Lovastatin exacerbates atypical absence seizures with only minimal effects on brain sterols

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Abstract AY-9944 (AY) exacerbates chronic recurrent seizures in rats that are analogous to atypical absence epilepsy in humans. The mechanism by which AY affects the slow spike-and-wave discharges associated with these seizures is not known, but is thought to involve inhibition of cholesterol synthesis. We tested the hypothesis that seizures seen with AY are due to significant reduction in brain cholesterol and/or elevated brain 7-dehydrocholesterol by assessing whether three other cholesterol synthesis inhibitors mimic AY seizures in rats. Effects of AY on brain sterols and spike-and-wave discharge duration were compared with those of two other late-stage cholesterol inhibitors [BM 15.766 (BM) and U18666A (UA)] and to an HMG-CoA reductase (early-stage cholesterol) inhibitor, lovastatin. With BM or UA, prolongation of seizure duration and brain sterol changes was similar to that caused by AY. AY effects on both brain sterols and seizure duration were dose-related. Lovastatin, with or without concurrent AY, mimicked AY seizures but reduced brain cholesterol by <10% and did not significantly change brain 7-dehydrocholesterol. Either lovastatin has a different mechanism of action than these late-stage cholesterol inhibitors or the brain sterol changes are not directly responsible for seizures in this model.—Serbanescu, I., M. A. Ryan, R. Shukla, M. A. Cortez, O. C. Snead III, and S. C. Cunnane. **Lovastatin exacerbates atypical absence seizures with only minimal effects on brain sterols.** ***J. Lipid Res.*** **2004.** 45: **2038–2043.**

Supplementary key words AY-9944 • BM 15.766 • brain cholesterol • cholesterol • epilepsy • seizures • spike-and-wave discharge • U18666A

Atypical absence seizures are chronic and frequent in generalized epilepsies such as Lennox-Gastaut syndrome. They are characterized by spontaneous, bilaterally synchronous, slow spike-and-wave discharges (SWD) and are usually associated with intermittent impairment of consciousness and the ability to move during seizures (1). Unlike typical absence seizures, behavioral and electrocorticographic (ECoG) correlates of atypical absence seizures are not concordant in seizure onset and offset (2).

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During postnatal brain development, administration of the cholesterol synthesis inhibitor AY-9944 (AY) leads to permanent and recurrent atypical absence seizures in adult rats (1). This effect is clearest in Long-Evans rats, which are already susceptible to mild atypical absence seizures, but in which AY augments the seizure duration by 10- to 20-fold. Hence, we refer to this markedly heightened seizure activity as the "AY model" or as "AY seizures." Seizure onset in the AY model occurs prepubertally (1). AY seizures in Long-Evans hooded rats and C3H mice (1–4) mimic the human condition in most respects, including developmental ECoG correlates, behavior, and pharmacological profiles (1, 5–7). Therefore, we have postulated that the AY model is a reproducible and valid model of atypical absence seizures.

Early studies with AY have shown that it decreases cholesterol levels in all tissues studied (4, 8–11). The decrease in cholesterol in the brains of AY-treated animals is associated with an elevation of the immediate cholesterol precursor, 7-dehydrocholesterol (2) (**Fig. 1**). We recently reported that decreased brain cholesterol is a transient effect of AY that reverses after AY injections are stopped, but that AY seizures are sustained long after the brain sterols return to normal (2).

We reasoned that if the AY-induced brain sterol changes (i.e., lowered cholesterol and elevated 7-dehydrocholesterol) are involved in the pathogenesis of AY seizures, other late-stage cholesterol synthesis inhibitors affecting 7-dehydrocholesterol conversion to cholesterol should induce seizures similar to those seen in the AY model (1). We also predicted that an early-stage cholesterol synthesis inhibitor that blocks synthesis of both cholesterol and its precursors (i.e., one that does not raise 7-dehydrocholes-

Abbreviations: AY, AY-9944; BM, BM 15.766; ECoG, electrocorticogram; LV, lovastatin; P, postnatal day; SWD, spike and wave discharge; UA, U18666A.

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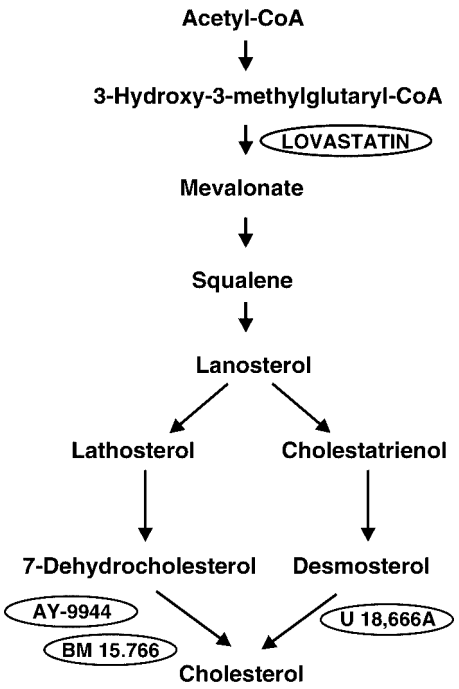

Fig. 1. Pathway of cholesterol synthesis indicating the known sites of action of the cholesterol synthesis inhibitors used in this study. AY-9944 and BM 15.766 inhibit 7-dehydrocholesterol-Δ^7 reductase, lovastatin inhibits HMG-CoA reductase, and U18666A inhibits desmosterol reductase.

terol) would not induce AY-like seizures. BM 15.766 (BM) and U18666A (UA) were chosen as examples of late-stage cholesterol synthesis inhibitors that would mimic AY; lovastatin (LV), an HMG-CoA reductase inhibitor, was chosen as the early-stage cholesterol synthesis inhibitor. Known sites of action of these drugs in the cholesterol synthesis pathway are shown in Fig. 1.

Three experiments are reported here. Experiment 1 was a comparison of the effects of our standard dose of AY to effects of BM or UA. Experiment 2 was an AY dose-response curve to determine whether brain sterols and SWD duration are equally affected at the same dose of AY. Experiment 3 was a comparison of our standard dose of AY to two different doses of LV or a combination of AY and LV.

MATERIALS AND METHODS

Animals

The Animal Care Committee of the Research Institute of the Hospital for Sick Children, Toronto, approved all experimental procedures. Untimed pregnant Long-Evans hooded rats were obtained from Charles River (St. Constant, Quebec, Canada) and housed in the animal facility at the Hospital for Sick Children. Long-Evans rats were used in these experiments because, unlike most other rat strains, they have a spontaneous tendency to produce SWD lasting ~25 s/h (1). All offspring were weaned at postnatal day 21 (P21), and then three animals of the same gender were housed per cage until the recording electrodes were implanted (2). All animals were housed in a controlled environment with a 12 h light/dark cycle, with lights on at 06:00 h. They were given ad libitum access to food and water. Body weight of test and control animals was monitored throughout the study.

Drugs

AY (*trans*-1,4-bis [2-chloro-benzylaminomethyl] cyclohexane dihydrochloride) was a gift from Wyeth-Ayerst (Philadelphia, PA). BM (4-[2-[4-(4-chlorocinnamyl) piperazine-1-yl]ethyl]benzoic acid, sulfate) was a gift from Dr. G. S. Tint, New Jersey Medical School (Newark, NJ). Both drugs inhibit conversion of 7-dehydrocholesterol to cholesterol at the 7-dehydrocholesterol Δ^7-reductase (Fig. 1). UA (3β-[2-diethylaminoethoxy]androst-5-en-17-one hydrochloride) was provided by Dr. R. J. Cenedella, Kirksville College of Osteopathic Medicine, Kirksville, MO. LV (1S-[1α(R*), 3α,7β,8β,(2S*,4S*),8a β]2-methylbutanoic acid-1,2,3,7,8a-hexahydro-3,7-dimethyl-8[2-(tetrahydro-4-hydroxy-6-oxo-2*H*-pyran-2-yl) ethyl]1-naphthalenyl ester) was purchased from Sigma-Aldrich (St. Louis, MO).

Treatments

In Experiment 1, AY (7.5 mg/kg; $AY_{7.5}$), UA (10 mg/kg; UA_{10}), or BM (20 mg/kg; BM_{20}) was injected subcutaneously at P2, P8, P14, and P20. AY was diluted in distilled water, and UA and BM were suspended in olive oil. In Experiment 2, suckling rats were injected subcutaneously with one of six different doses of AY (2.5, 5.0, 7.5, 10.0, 15.0, or 22.5 mg/kg; $AY_{2.5}$ to $AY_{22.5}$, respectively), at P2, P8, P14, and P20. In Experiment 3, the standard AY protocol was compared with either 50 mg/kg (LV_{50}) or 100 mg/kg (LV_{100}) gavaged on P2, P8, P14, and P20 or was compared with 7.5 mg/kg AY given subcutaneously plus 50 mg/kg LV (AY+LV) gavaged on P2, P8, P14, and P20. Control rats received an equivalent volume of distilled water or olive oil subcutaneously on the same postnatal day as the drug-treated litters. Seizure severity was assessed by measuring SWD duration in seconds for three consecutive 20 min intervals over each 1 h recording period, as described previously (1).

Electrocorticography

For implantation of skull seizure recording electrodes, rats were anesthetized with a single intraperitoneal injection of sodium pentobarbital (35 mg/kg) that lasted 3–4 h. Surgeries were performed in all animals at P55 and consisted of chronic implants containing two frontal and two parietal monopolar electrodes placed 1 mm deep and 2.2 mm anterior or posterior to the frontal parietal suture and 3 mm lateral to midline. Skull coordinates were measured relative to bregma at 0.0 (12). Electrodes were secured with dental cement and two screws attached to the parietal regions of the skull without penetrating the dura mater. All animals were then returned to the animal facility for a 4 day recovery period. ECoG recordings to assess baseline SWD duration in male and female rats from all treatment groups were performed at P59–P60. On seizure test days, the rats were placed freely moving in individual, warm Plexiglas chambers (Harvard Apparatus, Holliston, MA) and were connected to the recording equipment. There was an initial 20 min adaptation period to minimize movement artifact prior to ECoG recordings (1 h long), using a Grass Polysomnograph (Grass Instruments, Quincy, MA). All ECoG recordings were performed from 10:00 h to 14:00 h to minimize circadian variations (13).

Sterol analysis

Twenty-four hours after each injection (P3, P9, P15, or P21), two to eight rats from each group were anesthetized with sodium pentobarbital (35 mg/kg) and the brains were quickly removed. Brains were homogenized in 20 volumes of 2:1 chloroform/methanol to extract brain total lipids. 5-α-Cholestane was added as an internal standard for sterol quantification (2). Brain sterols were

obtained after saponification of the total lipid extracts in 1 N methanolic KOH. Sterols were derivatized using trimethylsilyl chloride and were analyzed by capillary gas chromatography as previously described (2, 14).

Statistical analysis

Data were expressed as the mean ± 1 SD for both the ECoG recording experiments and the brain sterol studies. Comparisons of group means were performed and analyzed by the two-tailed Student's *t*-test with a probability value of $P < 0.05$ chosen as an index of statistical significance. ANOVA for repeated measures was used to quantify the difference in SWD duration and brain sterols as a function of drug and dose.

RESULTS

Body weights

All rats were weighed immediately prior to each drug injection to determine the correct dose. At P20 in Experiment 1, body weights were similar in the control and BM_{20} groups (overall mean ± SD: 59 ± 4 g), but were lower than control values in the $AY_{7.5}$ and UA_{10} groups (52 ± 4 g; $P < 0.0001$). In Experiment 2, body weight at P20 was 22% lower in the AY_5, $AY_{7.5}$, AY_{10}, AY_{15}, and $AY_{22.5}$ groups compared with controls ($P < 0.05$). In Experiment 3, body weights in the different groups did not differ from control values throughout the experiment.

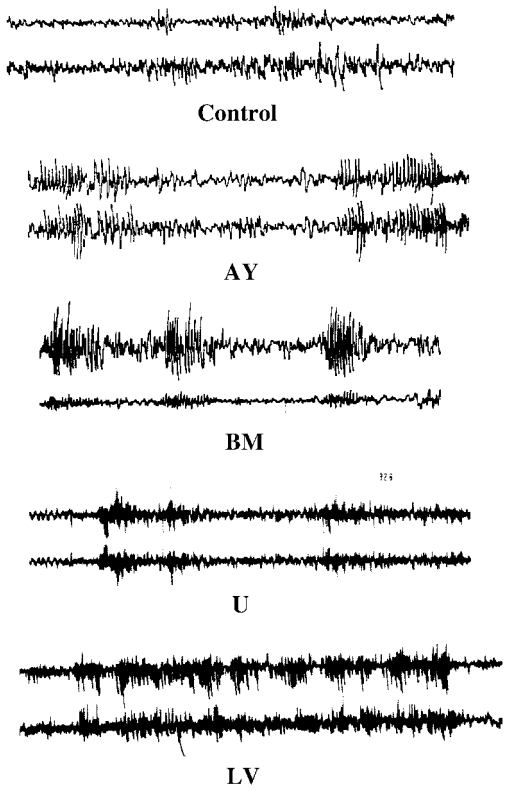

Fig. 2. Electrocorticograms (EcoGs) from electrodes implanted upon the frontoparietal cortex in Long-Evans hooded rats at P60. The baseline recordings in control (upper panel) showed one second spike and wave discharges (SWD) of moderate amplitude compared with those of AY (AY-9944), BM (BM 15.766), or UA (U18666A), which had higher amplitude SWD lasting 6 s for AY or BM and 4–5 s for UA. The AY, BM, and UA recordings showed recurrent and bilaterally synchronous SWD at ~5–6 Hz associated with intermittent behavioral arrest without a clear electrobehavioral concordance at the seizure onset and offset.

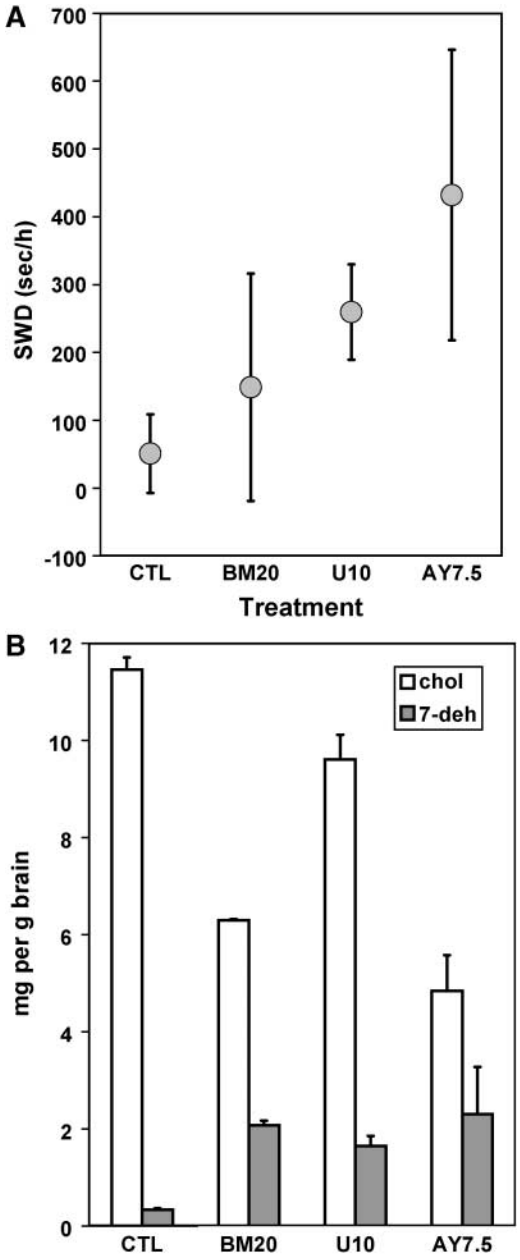

Fig. 3. Spike-and-wave discharge (SWD) duration at postnatal days 59–60 (A) and brain cholesterol (chol) and 7-dehydrocholesterol (7-deh) at postnatal day 21 (B) in controls (CTL) and rats injected with either 20 mg/kg BM 15.766 (BM20), 10 mg/kg U18.666A (U10), or 7.5 mg/kg AY-9944 (AY7.5) at P2, P8, P14, and P20 (n = 2–9/group). SWD duration was longer in each treatment group than in controls ($P < 0.05$) but did not differ significantly between drug treatments ($P > 0.10$; Student's *t*-test). Brain cholesterol was significantly reduced and brain 7-dehydrocholesterol was significantly increased in all three treatment groups.

Experiment 1: comparison of AY, UA, and BM

The ECoG recordings in all test rats showed similar slow, recurrent SWD occurring at 5–6 Hz and having am-

TABLE 1. Rat brain desmosterol on four postnatal days from 9 to 60

Treatment	Desmosterol, mg/g ± SD (*n*)			
	P9	P15	P21	P60
Control	0.05 ± 0.01 (2)	0.06 (1)	0.04 (6)	ND (20)
AY	0.49 ± 0.05[a] (8)	0.84 ± 0.26[a] (8)	0.80 ± 0.25[a] (8)	0.09 ± 0.02[a] (8)
BM	0.40 (1)	0.88 ± 0.09[a] (2)	1.11 ± 0.16[a] (2)	ND (5)
UA	0.03 ± 0.00 (2)	ND (3)	ND (3)	ND (5)

ND, not detected (<0.015 mg/g); SD, standard deviation. Brains were collected 24 h after dosing rats on postnatal days P2, P8, P14, or P20 with saline (control) or with one of three late-stage cholesterol inhibitors: AY, 7.5 mg/kg AY-9944; BM, 20 mg/kg BM 15.766; UA, 10 mg/kg U18666A.

[a] $P < 0.05$ versus control at P9.

plitude of 300–350 mV (**Fig. 2**). SWD duration was not statistically different in the $AY_{7.5}$, UA_{10}, or BM_{20} groups (**Fig. 3A**). However, the $AY_{7.5}$ and UA_{10} groups but not the BM_{20} group had longer SWD duration than did the controls ($P < 0.05$). In controls, brain cholesterol rose from ~3 mg/g at P3 to ~12 mg/g at P21 (data not shown). Brain cholesterol rose more slowly in the UA_{10}, BM_{20}, and $AY_{7.5}$ rats than in controls, with $AY_{7.5}$ causing the strongest inhibition at P21. 7-Dehydrocholesterol was present in controls at ~1 mg/g at P3 and decreased to ~0.3 mg/g by P21 (data not shown). At P21, brain cholesterol was lower and 7-dehydrocholesterol was 2- to 5-fold higher in the $AY_{7.5}$, BM_{20}, and UA_{10} groups than in the controls (Fig. 3B). At the time of seizure testing (i.e., ~40 days after stopping the drug treatments), brain cholesterol had returned to or above values in the control rats of the same age, and 7-dehydrocholesterol had declined to control values. In controls, very low levels of desmosterol were detected between P9 and P21 (**Table 1**). Desmosterol was not detected after P9 in the UA_{10} group, but was detected at P15 and P21 in the BM_{20} group and up to P60 in the $AY_{7.5}$ group (Table 1).

Experiment 2: AY dose-response study

SWD duration increased progressively with AY dose from ~300 s/h at $AY_{2.5}$ to ~700 s/h at AY_{15} and $AY_{22.5}$ (**Fig. 4A**). Desmosterol was detected in all rats in the AY dose-response study, even at P80 (60 days after stopping AY dosing; data not shown). Desmosterol rose with increasing AY dose, but leveled off at AY doses of 7.5 mg/kg and higher. Brain cholesterol declined to ~30% of control values as the AY dose rose from 2.5 to 7.5 mg/kg and did not decrease further at AY doses of 10 mg/kg and above (Fig. 4B). Brain 7-dehydrocholesterol showed the opposite trend to cholesterol, rising as the AY dose rose from 2.5 mg/kg to 5 mg/kg, remaining similar between 5 mg/kg and 15 mg/kg, and then rising again at 22.5 mg/kg (Fig. 4B).

Experiment 3: AY compared with LV or AY+LV

SWD duration was increased by treatment with LV or AY (**Fig. 5A**). SWD were ~50% longer with LV_{100} than with LV_{50}, but these results did not differ significantly from $AY_{7.5}$ or AY+LV. Brain sterols did not change significantly in the LV50 group; cholesterol was slightly but significantly reduced in the LV100 group (Fig. 5B). Brain 7-dehydrocholesterol in the $AY_{7.5}$ group rose to ~6.5 mg/g; brain cholesterol was reduced by ~59%. Addition of 50 mg/kg LV to the standard $AY_{7.5}$ protocol did not significantly alter the brain sterol changes compared with $AY_{7.5}$ alone (Fig. 5B).

Relation between brain sterols and spike-and-wave discharge duration

As we reported previously (2), the changes in brain sterols reversed after stopping the drug treatments (data not shown). Because there was a delay between the last drug injection (P20) and seizure testing (P59–P60), we compared the SWD duration to the brain sterol levels on P21, which was 24 h after the drug injections were stopped. In the $AY_{7.5}$, BM_{20}, or UA_{10} rats in Experiment 1, SWD duration correlated positively with brain 7-dehydrocholesterol levels ($r = 0.925$, $P = 0.02$). In Experiment 2, SWD duration was positively correlated with AY dose ($r = 0.859$, $P = 0.03$) and negatively correlated with brain cholesterol ($r = -0.895$, $P = 0.02$). SWD duration rose continuously with AY dose, but did not increase at AY doses above 15 mg/kg (Fig. 4). There was no significant relationship between brain sterols and SWD duration in Experiment 3.

DISCUSSION

These results show that injection of either of the two late-stage cholesterol synthesis inhibitors (BM or UA) during the suckling period induces chronic, recurrent atypical absence seizures indistinguishable from those seen in AY-treated rats (1, 2). The conversion between 7-dehydrocholesterol to cholesterol is inhibited by AY and BM at the Δ-reductase level (Fig. 1). At 20 mg/kg, BM had potency similar to that of $AY_{7.5}$ in its effect on brain sterols, but increased SWD duration only by ~25% of that induced by $AY_{7.5}$ (Fig. 3). BM has not previously been shown to cause seizures, and UA had not previously been used in this seizure model, so the doses we selected were estimates based on seizure-inducing or brain sterol-altering effects seen demonstrated in other published research (8, 15).

UA is a known inhibitor of desmosterol conversion to cholesterol (15–17). However, four injections of UA at 10 mg/kg between P2 and P20 did not affect brain desmosterol but did raise brain 7-dehydrocholesterol (Fig. 3B). Thus, the effect of UA on brain sterol metabolism is not specific to the conversion of desmosterol to cholesterol but includes inhibition of 7-dehydrocholesterol conversions to cholesterol. In fact,

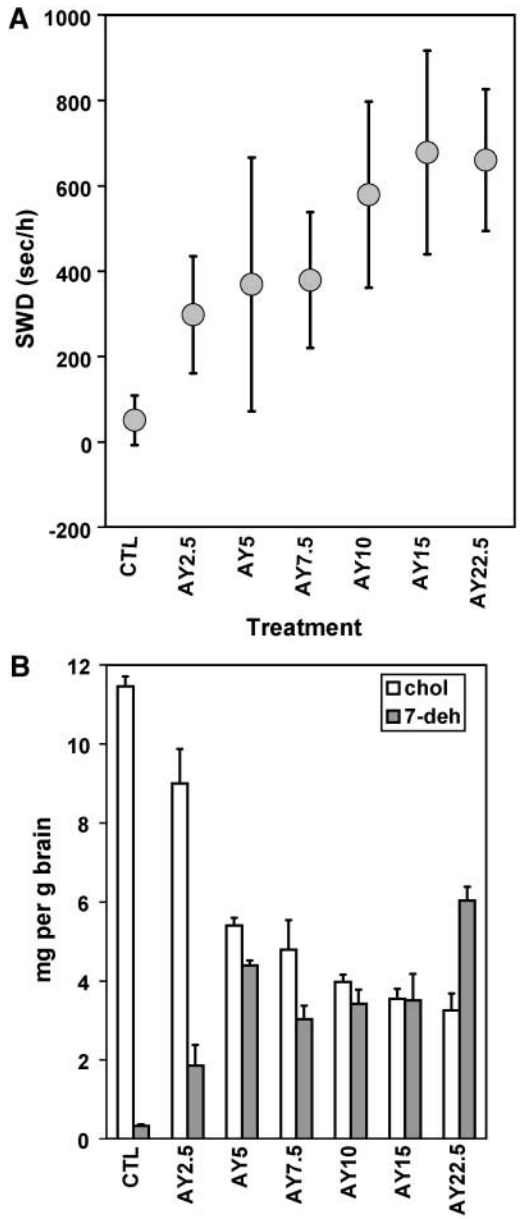

Fig. 4. Spike-and-wave discharge (SWD) duration at postnatal days 59–60 (A) and brain cholesterol (chol) and 7-dehydrocholesterol (7-deh) at postnatal day 21 (B) in controls (CTL) and rats treated with increasing doses of AY-9944 (AY; number shown with AY is the dose in mg/kg; n = 4–8/group). SWD duration increased with AY dose to 10 mg/kg but did not increase further above 10 mg/kg. The AY-induced reduction in brain cholesterol and the rise in brain 7-dehydrocholesterol were both dose-related.

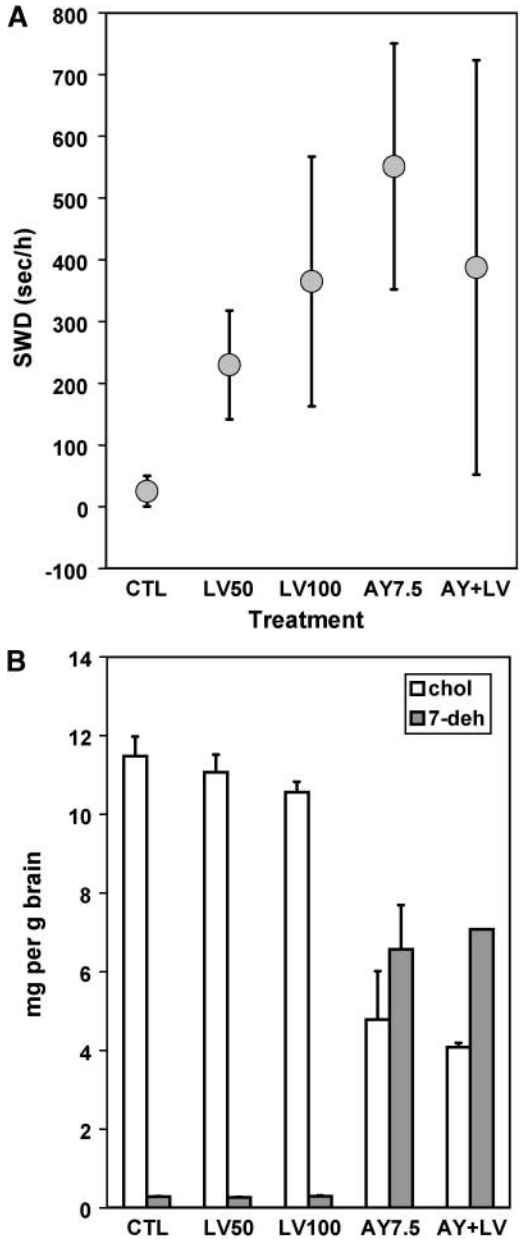

Fig. 5. Spike-and-wave discharge (SWD) duration at postnatal days 59–60 (A) and brain cholesterol (chol) and 7-dehydrocholesterol (7-deh) values at postnatal day 21 (B) in controls (CTL) and rats treated with lovastatin (LV; 50 mg/kg [LV50] or 100 mg/kg [LV100]), AY-9944 (AY; 7.5 mg/kg [AY7.5]), or a combination of 7.5 mg/kg AY and 50 mg/kg LV (AY+LV). Injections were done on P2, P8, P14, and P20 (n = 4–8/group). SWD duration was higher ($P < 0.05$) in the treatment groups than in the controls, but not different between treatments. Compared with controls, brain cholesterol was significantly reduced in the AY7.5 group, in the LV100 group, and in the AY+LV group, but not in the LV50 group ($P < 0.05$). 7-Dehydrocholesterol was elevated only in the AY7.5 and AY+LV groups.

the increased SWD duration caused by UA seems more related to its inhibition of 7-dehydrocholesterol than to inhibition of desmosterol conversion to cholesterol. In the rats treated with AY, UA, or BM, a positive relationship was seen among lower brain cholesterol, elevated brain 7-dehydrocholesterol, and increased SWD duration (as detailed in Results). These findings support our previous impression that the seizure morphology and duration induced by AY depends in some way on these changes in brain sterols (2).

Despite the consistency between altered brain sterols and prolonged SWD duration with AY, BM, and UA (Experiment 1), and a positive relation between AY dose, brain sterol changes, and SWD duration (Experiment 2), our LV results raise questions about the interpretation of these results. LV blocks conversion of mevalonate to subsequent intermediates in cholesterol synthesis by inhibit-

ing HMG-CoA reductase (Fig. 1). It has other effects on LDL receptor expression that may actually be more important for the plasma cholesterol-lowering effect than inhibition of HMG-CoA reductase. In fact, LV increases HMG-CoA reductase expression while decreasing its activity, so its plasma cholesterol-lowering effect is not exclusively related to inhibition of HMG-CoA reductase. In any event, the doses of LV we tested had only minor effects on brain sterols but significantly increased SWD duration, analogous to the effects of the lower doses of AY (Figs. 4, 5).

In a pilot study, we found that the increase in SWD duration did not differ whether LV (100 mg/kg) was injected every day or every 4 days during P2–20 (data not shown). Hence, the lowest injection frequency was chosen. Four injections of LV at 100 mg/kg did not increase SWD duration significantly more than four injections at 50 mg/kg and did not alter the effects of 7.5 mg/kg AY (Fig. 5). Hence, LV did not have a clear dose-response effect on SWD duration. If elevated brain 7-dehydrocholesterol is responsible for the increased SWD duration caused by AY (by blocking the increase in brain 7-dehydrocholesterol), LV might be expected to have inhibited the effects of AY. If reduced brain cholesterol were responsible for the increased SWD caused by AY, we would have predicted that LV would exacerbate AY-induced seizures. Our LV data show that neither effect occurred.

These results leave open at least two possibilities. First, our experimental design involves sterol measurement on the whole brain 24 h after each drug has been dosed. Although sufficient to see clear brain sterol changes after AY, UA, or BM, this 24 h delay and/or sterol measurement in the whole brain may not permit detection of a rapid, transient, or highly localized reduction in brain cholesterol occurring after LV injection, if during this time the seizure-inducing effect of lower cholesterol in a critical area in the brain has occurred. A corollary of this interpretation is that the effect of AY on brain sterols is much more severe than is needed to induce seizures. Indeed, our dose-response data suggest that the lowest dose of AY we used (2.5 mg/kg) increased seizure duration by more than 10-fold while reducing brain cholesterol by only 23% (Fig. 3). Thus, changes in brain sterols observed 24 h later or in the whole brain may be a relatively insensitive marker of the process by which these seizures are initiated.

Second, effects of cholesterol synthesis inhibitors (early or late stage) on SWD duration in this model may occur downstream of cholesterol itself; that is, these inhibitors may induce seizures predominantly because they inhibit or otherwise disrupt synthesis or action of neurosteroids derived from cholesterol. Neurosteroids have previously been implicated in seizures, some inhibiting and others exacerbating seizures (18, 19). The seizure-enhancing effects of cholesterol synthesis inhibitors could therefore arise via changes in neurosteroids, regardless of whether brain levels of cholesterol itself or cholesterol intermediates are affected.

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