Blocking Cholesterol Synthesis Impairs Acquisition of the Classically Conditioned Eyeblink Response

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Abstract—Smith-Lemli-Opitz (SLO) syndrome is a congenital disorder characterized by severe mental retardation. Patients with SLO lack 7–dehydrocholesterol (7 dH) reductase, which catalyzes the last step of cholesterol synthesis. Administration of an agent that blocks 7 dH cholesterol reductase, BM 15.966 (BM), leads to a biochemical profile which resembles that of SLO patients, i.e., lower plasma, liver, and brain cholesterol levels accompanied by the appearance of the precursors 7 dH and 8 dH cholesterol. In this article we address the functional consequences of chronic BM treatment on new motor learning by assessing acquisition of the classically conditioned eyeblink response. Just-weaned rats were fed BM by gavage for four months, with half of these rats given exogenous cholesterol during the last two months of BM treatment. Acquisition of the eyeblink response was impaired in BM-treated rats. Impaired acquisition of the eyeblink response was not accompanied by alterations in responsiveness to either the conditioned or unconditioned stimulus. Exogenous cholesterol, a clinically relevant countertreatment, failed to correct for the learning impairment produced by BM treatment. Chronic treatment with a cholesterol synthesis-blocking agent impaired associative learning in just-weaned rats.

CHOLESTEROL PLAYS a vital role in all animal cells. Cholesterol provides rigidity to cell membranes, is a major component of myelin, and serves as the precursor molecule for the synthesis of many steroid hormones. Physiological demand for cholesterol is greatest during development, and endogenous synthesis normally meets the demands both in the periphery and within the central nervous system (CNS). Disruption of endogenous synthesis during development can produce profound visceral and central abnormalities. An example is the Smith-Lemli-Opitz (SLO) syndrome (Smith et al., 1964). The SLO syndrome is a hereditary disorder characterized by very low levels of cholesterol and the appearance of its immediate precursors, 7-dehydrocholesterol (7dH cholesterol) and 8-dehydrocholesterol (8 dH cholesterol) (Irons et al., 1993; Tint et al., 1994). Cholesterol precursors accumulate because 7-dehydrocholesterol-delta-7-reductase, the enzyme that catalyzes the last step in the biosynthesis of cholesterol, is defective in SLO patients (Honda et al., 1995; Shefer et al., 1995).

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Integrative Physiological and Behavioral Science, April-June 2000, Vol. 35, No. 2, 120–131.

Clinical manifestations of SLO include functional and structural defects of every major organ system that contribute to the patients severe failure to thrive (Dellaire, 1969; Donnai et al., 1986; Thompson and Baraitser, 1986). The CNS is also severely affected. Neural abnormalities are numerous including irregular gyri, a small cerebellum and absence of cerebellar vermis, loss of nerve cells, gliosis, and hypomyelination (Cohen and Sulik, 1992). Functionally, SLO patients will often exhibit an abnormal electroencephalogram (Garcia et al., 1973). These structural and electrophysiological abnormalities contribute to the profound mental retardation associated with the syndrome (Smith et al., 1964; Blair and Martin, 1966)

Recently, a pharmacological model has been developed to investigate the biochemical changes that occur as a result of inhibiting 7dH cholesterol reductase. The drug BM 15.766 (BM), which specifically inhibits 7dH cholesterol reductase, was used to produce a sterol pattern in rats similar to that seen in SLO patients (Xu et al., 1995a; Xu et al., 1995b). In these studies, BM treatment began post-weanling—a time point that ensured the survival of the treated rats, but that also affected late-stage rapid myelination.

In a preliminary report, chronic treatment of just-weaned rats with BM impaired acquisition of the classically conditioned eyeblink response (Xu et al., 1998). We chose the eyeblink-conditioning paradigm to assess learning in BM-treated rats for several reasons. For one, performance factors may be explicitly measured in the eyeblink paradigm (Gormezano et al, 1985); a learning account is then supported through the elimination of performance accounts. For another, extensive research has detailed the neuroanatomical, neurophysiological, and neuropharmacological substrates of learning in the eyeblink conditioning paradigm (Thompson et al., 1997). Lastly, classical conditioning of the eyeblink response has been used successfully to illustrate developmental learning changes in rats affected by neurotoxic (Stanton et al., 1992; Stanton and Freemen, 1994) and neuropharmacological interventions (Stanton and Goodlett, 1998).

In that preliminary report, rats treated with BM (n=3) for four months failed to acquire the eyeblink response after 300 training trials (Xu et al., 1998). However, rats given exogenous cholesterol during the last two months of BM treatment (n=3) acquired the eyeblink response normally. These data suggested that chronic BM-treatment in just-weaned rats impaired learning, an impairment that could be corrected through cholesterol supplementation. The purpose of the present study was to determine whether these preliminary findings were indeed reproducible, that is, whether chronic treatment with a 7 dH-delta-7-reductase inhibitor impairs acquisition of the classically conditioned eyeblink response in just-weaned rats. Further, we conducted a more detailed analysis on the effects of BM-treatment on brain cholesterol levels. As the preliminary report also evaluated the ability of exogenous cholesterol to mitigate against learning impairments, we evaluated that possibility here. As in our prior report (Xu et al., 1998), we expected BM-treated animals to not acquire the conditioned eyeblink response, and cholesterol supplementation to mitigate those effects.

Methods

Subjects

The committee on animal studies at the VA Medical Center, East Orange, NJ, approved all experimental protocols. Untimed pregnant female Sprague-Dawley rats, at approximately ten to fifteen days gestation, were received by the Neurobehavioral Unit Animal

Research Facility. The rats were maintained on a 12:12 light:dark schedule in 42 x 22 x 15 cm plastic tubs with free access to water and lab rodent chow (PMI Feeds Inc.). They were checked at least once a day for the presence of litters. After weaning, twenty-one days after birth, rats within the same treatment group were double housed with free access to water and rodent chow.

Drugs

BM 15.766, 4-(2-[l-(4-chlorocinnamyl)piperazin-4-yl]ethyl)-benzoic acid (BM) was a gift from Boehringer Mannheim GmbH, Mannheim, Germany. The BM was suspended in water (30 mg/ml) by sonication and administered daily by gastric gavage (30 mg/kg/day). The dosage was chosen based on previous work by Xu et al. (1995a).

Eyelink Surgery and Apparatus

The equipment and surgical procedures necessary to assess eyeblink conditioning in the freely moving rat have been elaborated previously (Servatius and Shors, 1994). Briefly, four electrodes were placed within the eyelid, two for measuring electromyographic (EMG) activity and two for stimulation. Conditioning was conducted in a modular test cage within an isolation chamber (Coulbourne Instruments, Allentown, PA). The EMG recording electrodes were connected to a differential AC amplifier with a 300–500 Hz band pass filter (A-M Systems model 1700, Everett, WA), and were amplified by 10K. A PC computer equipped with an A/D board (Kiethley-MetraByte DAS 1600, Taunton, MA) collected the EMG signals. The timing of stimulus presentation and EMG data collection was controlled using a program written in ViewDac (Keithley-Metrabyte).

The unconditioned stimulus (US) was a 10-ms square-wave stimulus of 10V (Bioelectric Stimulus Isolator, Coulbourne Instruments). This stimulus reliably elicited an eyeblink from all rats. The conditioned stimulus (CS) was a 500-ms white noise burst (82-dB, 10-ms rise/fall) (Coulbourne Instruments).

Procedures

Just weaned (40–50 g) rats were assigned to treatment groups with the provision that each treatment group had members from all litters. Rats were either given BM by gavage for four months (n = 18) or remained nontreated control rats (CON, n = 9). In addition, half of the BM-treated group were given free access to a 2 percent cholesterol diet in pre-formulated rat chow during the last two months of BM treatment (BM+CHOL, n = 9). After the four-month treatment period, all rats underwent the surgery for placement of subcutaneous eyelid EMG electrodes. One of the BM-treated rats died following this surgery, reducing the number of rats in this group to eight. Two days after surgery, rats were placed within the conditioning chambers for one hour to acclimate. During this period, EMG was sampled every 25–30 sec. These EMG samples were processed for eyeblink responses as a measure of spontaneous eyeblink activity. After this acclimation session all rats were returned to their home cages. The next day, rats were placed back into the conditioning chamber and exposed to the to-be-conditioned stimulus for 10 trials. The interstimulus interval for this test was 30–40 sec. Immediately following this test, eyeblink training commenced.

A delay-type conditioning paradigm was employed; that is, in paired trials the 500-ms

CS coterminated with the 10-ms US. Rats were trained for three daily sessions on consecutive days. Each session consisted of ten blocks of ten trials; each block began with a CS-alone trial, was followed by four paired (CS-US) trials, a single US-alone trial, and ended with four more paired trials. The intertrial interval was randomly determined to be between 20–30 sec.

Signal Processing

Eyelid EMG recordings were sampled at 1 kHz for 1 s. The EMG signal was low pass filtered using a locally weighted regression filter (Lowess, Stat-Sci, Tacoma, WA) with a time constant of 0.025 and a smoothing interval of 5. In this system, an eyeblink corresponds to activity greater than 0.4 (unitless). For the acclimation period, the entire 1 sec. record was processed for the appearance of eyeblinks. For the recordings in which a stimulus was delivered (the test of sensory reactivity and eyeblink conditioning), each 1 sec record was subdivided into a 200-ms prestimulus baseline. If an eyeblink occurred during the 200-ms prestimulus baseline, that trial was thrown out and a NA was recorded. For the test of sensory reactivity, an eyeblink must have occurred within 100 ms of stimulus offset to be scored. During conditioning, an eyeblink response was scored as an α-response (orienting response) if the onset occurred within 40 ms of the CS onset. An eyeblink was scored as a conditioned response (CR) if it occurred at least 100 ms after the CS onset and before the onset of the US. In addition to collecting the CR data, we also collected data on the unconditioned response (UR). The square-wave stimulation caused a significant stimulus artifact in the EMG record that dissipated by 40 ms after US offset. Therefore, UR magnitude was considered as the peak activity from 45–245 ms after US offset. Our unpublished data indicates that an eyeblink in this preparation is evident for 250 ms after US presentation.

Tissue Collection and Sacrifice

Once the behavioral tests were completed, the rats were deeply anesthetized with Nembutal (75 mg/kg body weight). About 3 ml of whole blood was collected from the inferior vena cava into tubes containing EDTA. The plasma was separated by centrifugation. The cerebellum and cerebrum were removed and placed on dry ice. The liver was quickly removed and dissected into small pieces. All samples were stored at –70° C until assayed for sterols.

Analytical Biochemistry

Gas chromatography mass spectroscopy was used for the determination of sterols. Neutral sterols were extracted with hexane from 0.1 ml plasma. For the formed tissues, approximately 1.0–1.5 g of liver, 0.5 g of cerebrum, and the entire cerebellum (~.45 g) were minced, and the sterols were extracted in methanol saturated chloroform (Shefer et al., 1988). 5-Cholesterolestane was added to the samples as an internal standard prior to extraction. All tissues were saponificated in 1N ethanolic NaOH at 130E C for one hour. 5-Coprastanol was added as an external standard to determine percent recovery. Trimethylsilyl ether derivatives of the sterols were prepared and quantitated by capillary gas-liquid chromatography on a Hewlett-Packard model 5890A (Hewlett-Packard, Palo Alto CA) equipped with HP ChemStation software and a 25-m high-polarity capillary

column (Chrompak CP-Wax 57CB, Raritan, NJ). The samples were injected at an initial temperature of 100° C at a flow rate of 1 ml/min. The major carrier was helium. A flame ionization detector was used, and the retention times and areas under the peaks were found using the HP ChemStation software. Concentrations of the sample sterols were determined by using a ratio method comparing the area under the peak and the known concentration of the internal standard to the area under the peak of the sterol of interest. The percent recovery of the external standard was never under 97 percent.

Statistical Analysis

The data involving only a single time point (spontaneous blink rate, test of sensory reactivity and biochemical data) were analyzed with one-way ANOVAs. For the test of sensory reactivity, data from 3 CON, 3 BM and 1 BM+CHOL rats were irretrievably lost; analyses therefore reflect these lower subject numbers.

Acquisition data were expressed as the percentage of CRs by blocks of trials; blocks only covered trials in which the CS was delivered. In addition, the acquisition data for the first session were expressed as the cumulative percent response over blocks of three trials. Statistical analyses of both forms of the acquisition data were performed with split-plot ANOVAs. Specific comparisons were performed with *post hoc* F-tests for simple effects. Additionally, trials to criterion were used to assess the speed of acquisition. Our criterion required greater than 75 percent CRs in twenty consecutive trials. If a rat did not acquire the response within 300 trials, then a value of 350 (the midpoint of the next session) was recorded for that rat. One-way ANOVAs were used to analyze the trials to criterion data. The average UR amplitude over blocks was also computed; blocks only included trials in which the US was delivered. For all analyses, specific comparisons were performed with Dunn's tests. The level of significance was set at $p < .05$. All data are presented as means ± standard errors of the means.

Results

Physiological

Chronic BM-treatment reduced plasma cholesterol levels, while administration of exogenous cholesterol raised plasma cholesterol levels above those of the CON rats $F(2,24) = 64.2$, $p < .001$ (See Table 1). Treatment with BM also caused the accumulation of 7 dH and 8 dH cholesterol in the plasma. Although exogenous cholesterol lowered 7 dH and 8 dH levels in BM-treated rats, these precursors remained evident in the plasma, $F(2,24) = 42.9$, and $F(2,24) = 8.6$, respectively, all $ps < .01$. The treatments also produced a similar pattern for cholesterol, $F(2,23) = 18.83$, 7 dH cholesterol, $F(2,23) = 60.3$, and 8dH cholesterol, $F(2,23) = 14.0$, in the liver, all $ps < .001$ (data not shown).

Similar to the peripheral tissues, BM treatment resulted in reduced cholesterol concentrations in the cerebrum (See Table 1); lower cholesterol levels were accompanied by an accumulation of 7 dH and 8 dH cholesterol. These impressions were supported by the analyses for cerebral cholesterol, $F(2,22) = 34.3$, 7 dH cholesterol, $F(2,22) = 120.5$, and 8 dH cholesterol, $F(2,22) = 82.8$, all $ps < .001$. In contrast to the peripheral tissues, administration of exogenous cholesterol to BM-treated rats did not alter cerebral cholesterol levels. Moreover, the countertreatment did not affect the BM-induced accumulations of either

TABLE 1
Effects of Chronic BM Treatment on Sterol Levels in the Plasma and Brain

		CON	*BM*	*BM+CHOL*
Plasma	Cholesterol	45.2±3.7	17.8±2.6*	82.9±5.0*+
	7 dH	ND	13.8±2.0*	3.9±.3*+
	8 dH	ND	2.0±7*	.3±.1*+
	Total Sterols	45.2±3.7	34.5±4.6*	77.6±8.9*+
Cerebrum	Cholesterol	15.1±1.3	5.7±4*	7.1±5*
	7 dH	ND	5.5±4*	5.9±3*
	8 dH	ND	.42±.02*	.51±04*
	Total Sterols	15.1±1.3	11.7±.7	13.6±8
Cerebellum	Cholesterol	21.1±.6	8.7±3*	8.5±.7*
	7 dH	ND	12.0±.8*	8.3±1.0*+
	8 dH	ND	1.2±09*	.9±.07*+
	Total Sterols	21.1±1.3	22.0±1.1	17.8±1.6

precursor in the cerebrum. Although sterol composition was altered by BM treatment, total cholesterol (the sum of the measured sterols) did not differ between the control and BM-treated groups, F (2,22) = 2.8, p = .09. However, the distribution of sterols, the proportions of precursors to the total sterols in the cerebrum were slightly, but significantly reduced in the BM+CHOL group compared to the BM group, F (1,14) = 4.8, $p < .05$.

The sterol pattern in the cerebellum resembled that of the cerebrum (See Table 1). Lower cerebellar cholesterol concentrations were evident in association with the accumulation of 7 dH cholesterol and 8 dH cholesterol concentrations. Although administration of exogenous cholesterol was unable to raise the cerebellar cholesterol level of BM-treated rats, the countertreatment was partially effective in lowering the BM-induced concentrations of 7dH cholesterol and 8dH cholesterol. These impressions were confirmed with the analyses of cerebellar cholesterol concentrations, F(2,23) = 169.9, as well as the concentrations of 7dH cholesterol, F(2,23) = 70.4, and 8 dH cholesterol, F(2,23) = 93.5, all $ps <$ .001. In contrast to the cerebrum, total sterols between the groups differed in cerebellum; total sterols were reduced in the BM+CHOL group compared to the BM and CON groups, F (2,22) = 3.7, $p < .05$. Like the cerebrum, the proportions of precursor to total sterols in the cerebellum were reduced in the BM+CHOL group compared to the BM-alone group, F (1,14) = 11.4, $p < .05$.

Behavioral Data

During the adaptation session the spontaneous blink rates did not differ among the groups, F(2,24) = 0.10, p = 0.91. The overall rate of spontaneous blinks was 9.6± 1.1 blinks. Eyeblink responding to the future conditioned stimulus was assessed in terms of the proportion of eyeblink responses, response amplitude and response latency. Sensitivity to the to-be-conditioned stimulus did not differ between groups, F(2,16) = 0.55, p >.05. Moreover, the amplitudes, F(2,16) = 0.7, and latencies, F(2,16) = 1.3, of these eyeblink responses did not differ between groups, all $ps > .05$. Thus, chronic treatment with BM did not alter sensory reactivity to the CS prior to training.

Chronic treatment with BM impaired acquisition of the classically conditioned eyeblink response. This impression was confirmed with a 3 x 15 (Group x Trial Block) split-plot ANOVA comparing the acquisition of the eyeblink CR among groups. The analysis dem-

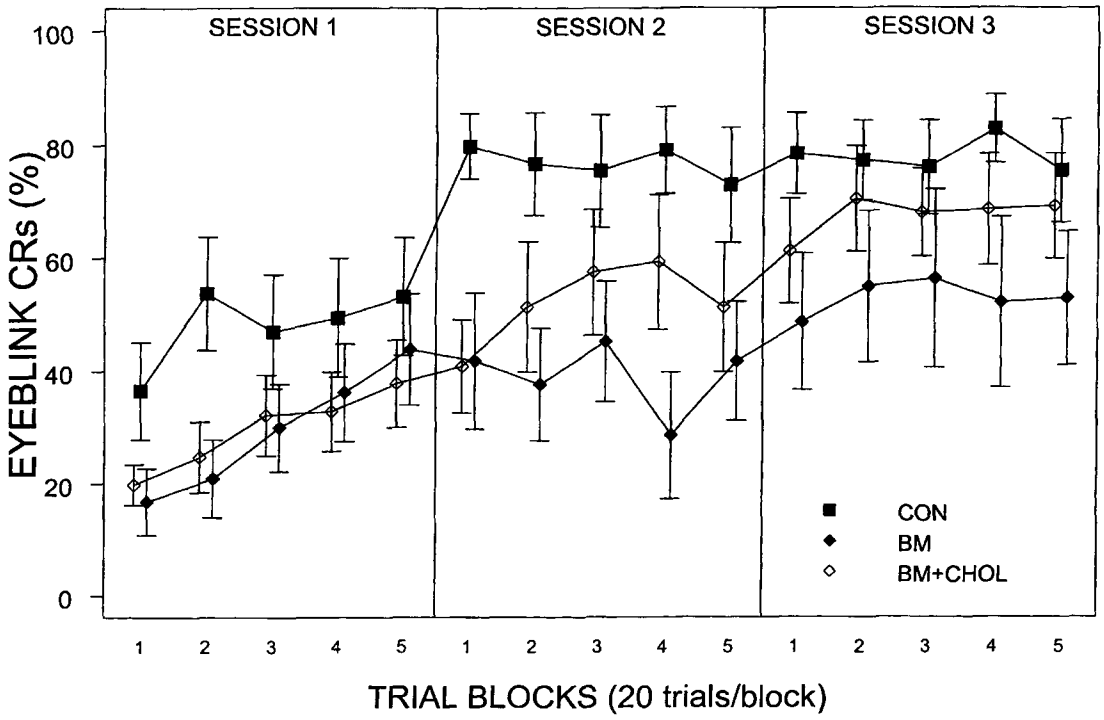

FIG. 1. Effects of Chronic BM Treatment on Acquisition of the Classically Conditioned Eyeblink Response. Acquisition of eyeblink CRs for the CON (n = 9), BM (n = 8) and BM+CHOL (n =9) groups was expressed as the proportion of eyeblink CRs by trial blocks.

onstrated only significant main effects of Group, $F(2,23) = 3.9$, and Trial Block, $F(14,322) = 12.2$, both $ps < .05$ (See Figure 1). From the initial block of trials, the percent of eyeblink CRs emitted by the CON rats were greater than that of the BM and BM+CHOL rats. To facilitate a comparison of the learning curves early in training, cumulative responses, over blocks of three trials, were compared for the first session of training. The analysis revealed significant main effects of Group, $F(2,23) = 3.7$, and Block, $F(28,667) = 344.0$, all $ps < .01$. These were qualified by the Group x Block interaction, $F(56,667) = 3.7$, $p < .05$ (See Figure 2, panel A). Rats treated with BM exhibited impaired acquisition of the eyeblink response; counter-treatment with exogenous cholesterol did not improve acquisition during the first session of training. Lastly, the degree of impaired learning was assessed using a measure of trials to a criterion of 75 percent CRs in twenty consecutive trials (data not shown). Both the BM (149±36) and BM+CHOL (156±28) groups required significantly more trials to reach the criterion than the CON (66±15) group, $F(2,23) = 3.5$, $p < .05$. All in all, the BM treated groups demonstrated slower acquisition of the eyeblink CR compared to the CON rats.

Slower acquisition could be secondary to a deficit in responsiveness to the CS. The proportion of orienting responses were analyzed with a 3 x 15 (Group x Trial Block) mixed-ANOVA. The main effect of Group, $F(2,23) = 3.2$, $p = .058$, did not reach significance. The percentage of ORs by group were: CON 42±12%, BM 19±9% and BM+CHOL 23±6%. The latency of ORs was similarly analyzed; there were also no significant effects.

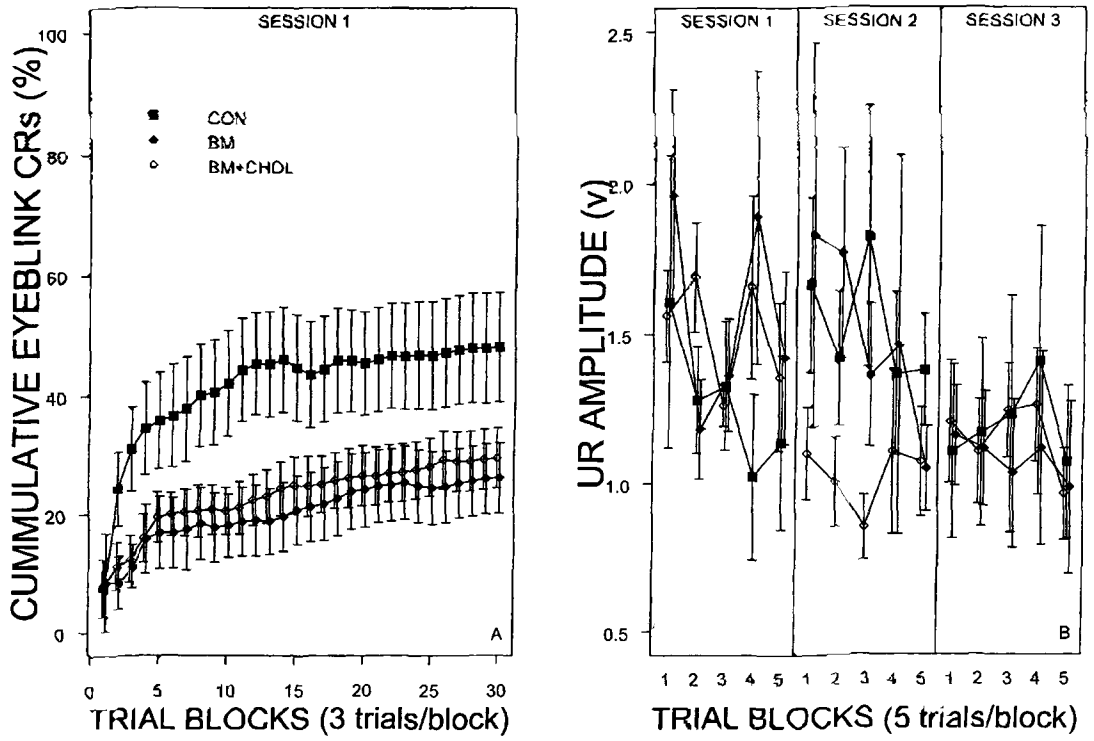

FIG. 2. Effects of Chronic BM Treatment on Learning Performance. Panel A on the left depicts the cumulative eyeblink CRs by blocks of three trials for the first training session. The legend in Panel A applies to both panels. Panel B on the right illustrates UR amplitudes in the US-alone trials.

Slower acquisition could be secondary to a deficit in responsiveness to the US. The UR amplitudes were analyzed with a 3 x 5 x 3 (Group x Trial block x Daily Session) split-plot ANOVA. The main effect of Daily Session, $F(2,308) = 6.4$, was significant, $p<.001$. Moreover, the Trial Block x Daily Session interaction, $F(4,308) = 4.3$, $p < .001$, was significant. Regardless of group assignment, the amplitude of the UR decreased with training (See Figure 2, Panel B). For the first two daily sessions, UR amplitudes in the initial trial blocks were greater than those in the latter blocks. In contrast, the amplitude of UR responding during the initial trial block of the last training session was less than the initial blocks for the two preceding sessions; the lower amplitude responses in the third daily session were maintained over the entire session. In addition, we analyzed the UR in US-alone trials. The URs in US-alone trials were analyzed with a 3 x 5 x 3 (Group x Trial Block x Daily Session) mixed-ANOVA. The pattern of results for this analysis mirrored that for the UR amplitudes described above (data not shown).

Discussion

Consistent with our earlier work (Xu et al., 1998), chronic treatment with BM produced a sterol pattern that is similar to that observed in patients with SLO, i.e., lower levels of

cholesterol accompanied by an accumulation of the precursor 7 dH cholesterol in plasma and liver. Moreover, 8 dH cholesterol also appeared in the plasma and liver of BM-treated rats. In previous work, 8 dH cholesterol did not appear when BM treatment began at a similar developmental stage, with similar doses and for a similar time period (Xu et al., 1995a; Xu et al., 1995b). The accumulation of such high tissue levels of 7dH cholesterol likely promoted the conversion of some of the 7dH cholesterol to 8dH cholesterol by an isomerase; a conversion that also occurs in SLO patients (Batta et al., 1995). In this regard, the sterol profile in just-weaned rats chronically treated with BM more closely resembled the sterol profile of SLO patients.

Brain sterols were likewise affected by BM treatment. The sterol patterns were similar in the cerebrum and cerebellum, i.e., reduced cholesterol levels and accumulation of its immediate precursors, including 8 dH cholesterol. This altered sterol pattern in the CNS is consistent with earlier work with rats treated AY 9944 (trans) (AY), another 7dH cholesterol-delta-7–reductase inhibitor (Dvornick and Pill, 1968). Since the turnover of brain cholesterol is much slower than in peripheral tissues, predominately reflecting the sterol content of cellular structures and myelin, inhibition of brain cholesterol suggests that sterol compositions were persistently affected by chronic BM treatment.

The primary purpose of the present study was to determine the functional significance of persistently altered brain sterols on learning and memory. The model system employed was the classically conditioned eyeblink response. Learning of the eyeblink response depends on cerebellar input. If impaired cholestereol synthesis altered myelin composition in the cerebellum, hence neurotransmission, then acquisition of tasks dependent on the cerebellum should be impaired. Indeed—consistent with our preliminary report—chronic treatment with BM impaired acquisition of the eyeblink CR. The BM-treated group emitted fewer eyeblink CRs and required more trials to reach criterion than the nontreated rats. Compared to the preliminary report, however, the BM-treated rats in the present study performed better. In our preliminary report, not one of the three BM-treated rats acquired the eyeblink response in 300 trials. In contrast, most of the BM-treated rats (6/8) acquired the eyeblink CR during the 300 trials in the present study. The performance of the BM-treated rats in the present study appears to be a better representation of the effects of chronic BM treatment on acquisition of the eyeblink response. In addition, there were a number of procedural differences that may account for the improved performance of the BM-treated rats. In the preliminary study, rats were trained in a single session of 300 trials, whereas in the present study training took place over three daily sessions of 100 trials each. The poorer learning performance of the BM-treated rats in the preliminary report may have reflected fatigue in addition to BM treatment.

Children with SLO have profound gross motor abnormalities (Smith et al., 1964). Although our pharmacological model does not affect the rat to the degree of the genetic abnormality, there was still a concern that chronic BM treatment would affect sensory-motor performance. An advantage of the eyeblink-conditioning paradigm is that nonassociative and performance factors related to the acquisition of the eyeblink CR can be explicitly measured. An associative account is supported after the elimination of performance accounts. With regard to sensory reactivity to the CS, we evaluated acoustic responsiveness both prior to and during training. In both, reactivity to the CS did not differ between BM-treated and CON rats. However, the analysis of orienting responses (ORs) did indicate a trend toward fewer responses in the BM-treated rats, suggesting that attention to the CS or cue-saliency may be reduced in BM-treated rats. A future study examining CS intensity may bring out a subtle deficit in cue saliency in BM-treated rats. We also

evaluated sensory reactivity to the US. As expected, the magnitude of the UR generally decreased with continued training. We found no difference among the groups in the magnitude of the UR. Thus, the aversiveness of the US appeared to be similar between nontreated and BM-treated rats; differences in US sensitivity did not contribute to the observed learning deficit.

We also assessed the ability of exogenous cholesterol—a clinically relevant countermeasure prescribed for SLO patients—to affect the biochemical and behavioral abnormalities produced by chronic BM treatment. Treatment with a high cholesterol diet resulted in very high levels of cholesterol in the periphery. In addition, the levels of 7dH and 8dH cholesterol in the periphery of the BM-cholesterol group were lower than that of the BM group. These data demonstrate that the high cholesterol diet was helpful in lowering the concentration of the precursors in the periphery. High plasma cholesterol concentrations exert a negative feedback inhibition on the rate limiting enzyme of cholesterol synthesis, HMG-CoA reductase, thus reducing the levels the precursor in plasma and liver (Goldstein and Brown, 1984). In contrast to the peripheral tissues, the high cholesterol diet for the most part was not able to counteract the effects of BM treatment on cholesterol and precursor concentrations in the CNS. However, the countertreatment was partially effective in the cerebellum. Exogenous cholesterol reduced the 7 dH and 8 dH cholesterol levels in the cerebellum compared to those in the BM group, this lowering of precursors was also reflected in the lowering of the proportion of precursors to total sterols. The mechanism for the reduction of precursors in the cerebellum is likely independent of cholesterol levels, inasmuch as the exogenous cholesterol did not raise cerebellar cholesterol levels.

Our preliminary data suggested that exogenous cholesterol may be effective in counteracting the learning impairment that resulted from chronic BM treatment. The BM+CHOL group (n = 8) in the present study performed similarly to the BM+CHOL group in the preliminary report (n = 3), that is, acquisition was slower than CON rats, but was generally complete within 300 trials. Yet, the high cholesterol diet was not effective in counteracting the learning impairment that resulted from chronic BM treatment. As noted above, the BM-alone group performed better in the present study compared to a similar group in the preliminary study. Whatever accounted for the improved performance of the BM-alone group in the current study is not immediately apparent. Nonetheless, exogenous cholesterol failed to improve the associative deficit produced by chronic BM treatment. Together, the biochemical and behavioral data suggest that exogenous cholesterol treatment may have limited utility in counteracting the effects of cholesterol-synthesis inhibitors.

These data clearly demonstrate that chronic treatment of cholesterol synthesis inhibitors to just-weaned rats impairs associative learning. The mechanisms by which impaired cholesterol synthesis affected eyeblink conditioning remain unknown. Several tentative hypotheses may be offered. First, cholesterol is necessary for myelination. To ensure the survivability of the rat pups, the inhibitor was fed to just-weaned rats, late in the period of rapid myelination (Gottlieb et al., 1977). However, gross microscopic inspection of the brain of BM-treated rats did not reveal any overt structural abnormalities (unpublished observations). Moreover, we did not observe gross motor impairments that would accompany myelin abnormalities or alterations in response latencies that may be expected from neural transmission problems. Second, cholesterol is a substrate for the synthesis of neurosteroids. Neurosteroids function as neuromodulators, some of which reported to have the ability to improve learning and memory performance (Robel et al., 1995; Baulieu, 1997). However, neurosteroids require very small amounts of cholesterol and therefore were not likely to be affected by BM treatment. Third, low cholesterol levels may increase

cellular membrane fluidity altering the function of membrane-associated proteins (i.e., receptors and ion channels), thus interfering with neural functions. Lastly, the observed learning impairment may have been the result of an accumulation of 7 dH and 8 dH cholesterol. These precursors may affect learning directly by being toxic to normal cellular transmission, or indirectly through their incorporation into myelin (Smith and Hasinoff, 1970). However, further research will be necessary to dissociate the effect of lower cholesterol levels from that attributable to increased precursor levels.

The results of this study underscore the importance of cholesterol synthesis in maintaining normal function. Blocking the synthesis of cholesterol impaired acquisition of the eyeblink conditioned response. The impairment was not accompanied by deficits in sensory reactivity to the CS or US. The clinically relevant countertreatment of exogenous cholesterol was not effective in ameliorating the learning deficit. A practical deterrent for assessing the true nature of learning deficits from chronic BM treatment is the length of the treatment protocol (4 months). Future research should be aimed toward determining the minimum treatment period required to affect associative learning.

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