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Global In Vivo Replacement of Choline by N-Aminodeanol. Testing a Hypothesis About Progressive Degenerative Dementia: II. Physiological and Behavioral Effects

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RUSSELL, R. W., D. J. JENDEN, R. A. BOOTH, S. D. LAURETZ, K. M. RICE AND M. ROCH. *Global in vivo replacement of choline by N-aminodeanol. Testing a hypothesis about progressive degenerative dementia: II. Physiological and behavioral effects.* PHARMACOL BIOCHEM BEHAV **37**(4) 811–820, 1990.—We have examined the progressive effects of replacement of dietary choline with NADe for a period of 120 days on a broad spectrum of behavioral and physiological functions known to involve the cholinergic system. The magnitudes of these effects tended to increase with time on the NADe diet, but those related to learning and memory were largely confined to the 60–120-day period. Neurochemical effects were concomitant with the replacement of Ch by NADe, being consistent with a hypocholinergic state as found in such progressive degenerative dementias as Alzheimer's disease. As cholinergic functioning was progressively impaired, basic physiological ("vegetative") processes appeared not to be affected. Apparently the rate at which the hypofunctional state developed was sufficiently slow for adjustments to occur, allowing the animal to adapt at a survival level to neurochemical changes. More complex behavioral functions were affected progressively, cognitive processes (e.g., learning and memory) being most sensitive and showing the least adaptability. We propose that the syndrome generated by NADe replacement of Ch represents an experimental model of progressive degenerative dementia.

False precursor (NADe) False transmitter Cholinergic function Muscarinic Developmental trends
Sensory, perceptual, and cognitive behaviors Progressive degenerative dementia Experimental model

THE present experiments were designed to test the hypothesis that a mechanism underlying dementing disease, e.g., Alzheimer's disease (SDAT), involves a competition for available choline (Ch) between biochemical pathways involved in synthesis of the neurotransmitter, acetylcholine (ACh) and in phospholipid metabolism (26,55). Such a competition could be precipitated by several inherited or acquired characteristics, could be age-related and could result in malfunctions of synaptic transmission, of the process by which cell membranes are renewed or both. Changes in synaptic transmission would have effects on behaviors that are cholinergically coded, making the hypothesis consistent with the fact that " . . . dementia is primarily a behavioral diagnosis" (36). The hypothesis also accounts for the relatively selective loss of cholinergic neurons observed in certain regions of the brains of SDAT patients. The hypothesis implies that both these types of effects are "progressive" in nature and suggests that the processes involved may proceed for some time, even years, before overt symptoms appear. In the present report we describe experiments focused on the early phases of the etiological chain of events. Other studies are underway designed to examine later phases that may lead to neuronal cell death.

The possibility that a false precursor leading to the synthesis of a false transmitter could serve as a pharmacological "tool" for examining neurotransmitter systems at a molecular level began to receive attention some half century ago. Early research examined choline (Ch) analogs that appeared to form false transmitters and studied their effects in vivo (8,35). Criteria that must be satisfied if a compound is to be accepted as a false transmitter were established (31). The first direct demonstration that a choline analog, triethylcholine, could be acetylated and released in a cholinergic

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synapse was reported by Ilson and Collier (25). Later it was appreciated that the synthesis of false transmitters might provide animal models for studying clinical states involving dysfunctions in the CNS. Experiments using such models have been carried out to measure effects on behavior, both normal (22,23), and abnormal (34). The results have been reviewed by Collier et al. (9), by Whittaker and Luqmani (54) and by Newton and Jenden (40).

A major difficulty in the earlier work was in demonstrating that administration of a Ch analog could result in its functionally significant replacement of Ch in vivo. The development of quantitative analytical techniques has now overcome this handicap. During the past five years N-amino-N,N-dimethylaminoethanol (N-aminodeanol, NADe), has been shown to satisfy fully the requirements of a false precursor, leading to the synthesis of a false cholinergic transmitter (37–39). It is also incorporated into phospholipids, replacing phosphatidylcholine in brain and other tissues (29,30). NADe is taken up by the Ch transport system in competition with Ch. It is acetylated by choline acetyltransferase (ChAT), stored as O-acetyl-N-aminodeanol (AcNADe) and released on stimulation. ACh stores are depleted as they are replaced by AcNADe. Upon release, AcNADe interacts with both muscarinic and nicotinic receptors and is hydrolyzed by acetylcholinesterase (AChE). Because the potency of AcNADe at these receptors is only 4% and 17% that of ACh, respectively, it results in a profound interference with cholinergic transmission, particularly at muscarinic sites. After replacing Ch in the diets of weanling rats for periods of 60–120 days, free NADe replaces 85–95% of the free Ch and up to 75% of lipid-bound Ch in plasma, brain and peripheral tissues as measured by HPLC (29,30). ChAT is reduced in the striatum, hippocampus, cortex and ileum, suggesting the possible loss of cholinergic neurones. Pilot studies have yielded evidence that these biochemical changes are reflected in concomitant changes in behavior (38).

The present experiments were designed to expand the earlier studies by examining a broader spectrum of behavioral and physiological functions and by exploring them in greater detail using an independent groups research design. Involvement of the cholinergic system in such functions has been extensively documented (45). For present purposes, functions selected for study were from among those known to involve the cholinergic system, i.e., to be "cholinergically coded." The general hypothesis tested was that the behavioral and physiological effects observed as a result of the replacement of Ch by NADe would be those expected of a hypocholinergic state, a major feature of such progressive degenerative dementias as Alzheimer's disease (26). Furthermore, it was predicted that the magnitudes of the effects would be determined by the extent of the replacement of free Ch, of phospholipid-bound Ch or both. The animals used in testing these hypotheses were among those participating in the experiments reported by Knusel et al. (29,30). Thus, it has been possible to relate the present results to concomitant neurochemical changes in the cholinergic system under the same experimental conditions.

METHOD

Animals

All animals in the present experiments were offspring of pregnant females purchased from Bantin and Kingman (Pleasanton, CA). They were housed in semibarrier cages in order to reduce the risk of infection from endemic disease pathogens frequently found in rat colonies (2, 11, 27). Filters were changed and cages washed (5% bleach solution and bactericidal soap) weekly. Litter in the base of each cage was changed twice weekly. All personnel were required to gown before entering the animal room. These precautions were considered essential in order to prevent viral and bacterial infections (2). The pups were weaned onto their respective diets at 29 days of age. At that time they were assigned randomly to one of two diets, i.e., experimental (NADe) or control (Ch).

Dietary Treatment

The composition of the diets has been described in detail in earlier publications from our laboratory (29,30). Briefly, rats were fed, ad lib, a synthetic amino acid diet (ICN Nutritional Biochemicals, Cleveland, OH) in powdered or pelleted form. Administration continued to 120 days in groups of 10–15 rats.

Developmental Trends

The preweaning development of the animals was compared with the normal sequence of behavioral and physiological changes described by Baker et al. (2). The target behaviors ranged from mewling, huddling, suckling, righting and wriggling on the first postpartum day to play and sexual behavior appearing toward the 28th day of the observation period. Data were recorded for: (a) the day when the first animal in a litter showed the developmental sign and (b) the day when the sign was present in all members of the litter. These data were then compared to the established norms.

Body Weight

Body weights were recorded on a daily basis from the first day after birth until the animals were sacrificed at the end of the experiment, using a Sartorius High Capacity Balance Model 1404 MP8 and a Sartorius Model 7279 printer. The measures served as indices of possible differential effects of the diets on general health and were used in calculating the amounts of NADe and of Ch consumed per unit of body weight.

Food and Water Intake

The animals were given food and water ad lib. Intakes are expressed both as absolute values and as their ratios per kg body weight. Food bins and water bottles were weighed daily, intakes being calculated as the average differences in weights at the beginning and end of a three-day period. Amount of food eaten provided a measure of caloric intake. The amount of water consumed was used as an index of maintenance of body fluid balance.

Core Body Temperature

Core body temperature was measured using a YSI Series 500 probe and a YSI Model 49TA digital thermometer, with an accuracy of ± 0.05°C within the relevant temperature range. The probes were inserted into the rectum to a depth of 6 cm. Measures were taken immediately preceding testing at 15, 30, 60 and 120 days.

Nociception

Nociceptive thresholds were determined by the up and down procedure as developed in our laboratory (12). Measurement of footshock, i.e., flinch and jump, thresholds involved placing the animal in a test chamber, the floor consisting of stainless steel rods through which electric shocks of varying intensity could be delivered. Shock intensities were available in an exponential series from 0.05 to 4.0 mA in 20 steps. Use of the full range of in-

tensities was never necessary in determining thresholds. Each shock pulse (60 Hz) had a duration of 0.5 s and shocks were delivered at approximately 10 s intervals. Shock levels at the start of an up and down series were set at midpoints of the ranges within which preliminary experiments had shown the thresholds were likely to occur. The experimenter then adjusted the intensity in accordance with the response on each particular trial, i.e., raised 0.1 $\log_{10}$ unit when no response occurred and lowered 0.1 $\log_{10}$ unit when a response had been made. A "flinch" was defined as elevation of one paw from the grid floor and "jump" as rapid movement of three or more paws involving withdrawal from the floor. Thresholds were measured at 30 minutes after the recording of spontaneous activity at 15, 30, 60 and 120 days.

Spontaneous Activity

Spontaneous activity of the rats was measured in circular open-field chambers with a diameter of 60 cm. The apparatus and procedure have been described by Silverman et al. (52). Briefly, the interior walls of the chambers were fitted with two sets of infrared-sensitive photocells and infrared-emitting LEDs. One set, 4 cm above the floor, measured horizontal locomotor activity, while the second set, located 12 cm from the floor, concurrently measured vertical rearing activity. The chambers were interfaced with a TRS-80 Model III microcomputer which automatically recorded all light beam breaks and, at the end of each animal's daily 20-min session, printed the results in terms of horizontal and vertical activity during each 2-min interval. Activity was defined as the total beam breaks during a 20-min session and was measured at 15, 30, 60 and 120 days, immediately after temperature was taken in the morning.

Reactivity

Reactivity was defined as response to a sudden brief and intense change in the stimulus environment. An acoustic signal, the critical parameters of which have been described by Davis (14) and Pilz et al. (43), served as the stimulus. The apparatus and procedure were those described in detail by Silverman et al. (51). In essence, a microcomputer was used to trigger the acoustic signal, which was fed to a loudspeaker through an audio power amplifier. An animal's acoustic startle response was recorded using a moving-coil loudspeaker as a movement transducer. The transducer output was coupled to a peak-hold circuit that recorded the maximum voltage generated by the animal's response. After conversion to digital form, the data for each stimulus presentation were stored and then printed when all trials were completed.

Habituation

Habituation, defined as a "primitive form of learning," may be observed as decrements in behavioral responding " . . . when an animal is exposed repeatedly to a novel stimulus without an accompanying biologically relevant consequence such as food or shock" (24). In the present experiments there were two opportunities to observe the habituation process, i.e., during the assays for activity and reactivity. Counts of activity for 2-min periods during a 20-min assay session in the open field and the amplitude of responses to each of 20 presentations of the acoustic signal in the reactivity situation were recorded. These were plotted and examined by regression analysis for systematic trends indicative of intrasession habituation.

Inhibited (Passive) Avoidance

Inhibited (passive) avoidance was measured in a "step through" apparatus similar to that used by McGaugh and colleagues (33). The apparatus was composed of (a) a small compartment made of white plastic, (b) a larger, dark compartment of stainless steel and (c) a shock delivery unit adjustable for the intensity (mA) and duration (s) of the mild electric shock used as an aversive stimulus. The procedure involved two trials separated by a retention time of 48 hours. On trial 1 the animal was placed in the white compartment. Entry into the dark compartment led immediately to the closing of a guillotine door and administration of a 0.25-mA footshock for 0.5 s. Retention was tested after the 48-h delay, the measure being time taken to enter the dark after release from the white compartment. The times of animals not entering within 10 min were recorded as "600."

Conditioned Avoidance Response (CAR)

Effects of the dietary treatments on performance of a discrete trial, one-way CAR were observed using the apparatus and general procedure described by Russell and Macri (48). The animal was required to traverse an alley from a start to a goal compartment. The raising of a door to the start compartment activated a buzzer, the conditioned stimulus (CS), opened the alley for the animal's response and started a timer. If a response was not made within 10 s thereafter, an electric shock, the unconditioned stimulus (UCS), was automatically delivered. The shock was terminated and the timer stopped when the animal interrupted a light beam in the goal compartment. Thus, two responses could be studied, namely the times for an innate escape response stimulated by the UCS, R_e, and for a learned avoidance response to the CS, R_a. The standard shock intensity was fixed at 0.35 mA. Five trials were given on one day followed 48 h later by trials to the criterion of 7 avoidance responses in 10 consecutive trials or to a total of 25 trials, whichever came first.

Statistical Analyses

The resulting data were analyzed for two general purposes. Significance of differences between treatment groups was tested using either parametric (ANOVA and Student's t) or distribution-free statistics (Mann-Whitney, Fisher Exact, Spearman rank-order correlation) depending upon the nature of the measuring instrument involved. Trends within the groups were studied by regression analysis that provided parameter estimates for slope and intercept constants (50). "Significance" is defined in terms of the 0.05 level of confidence and exact p values are given. Only when effects of treatment, time on diet and/or gender were statistically significant are they included in the text. Measures of central tendency are reported as mean $\pm$ SEM (n).

RESULTS

Analyses of physiological and behavioral differences between the two treatment groups have been carried out primarily on measures taken at one or several time points on the diet, i.e., 15, 30, 60 and 120 days. It is important for purposes of the analyses to recognize that measures at each of these time points involved independent groups of animals.

Developmental Trends

Application of the developmental sequence scale provided data for both NADe and Ch animals during their first 28 days postpartum that could be compared with standards established for normal animals (2). Spearman's rank order correlation coefficient was used to determine the magnitude of the relationship. In all comparisons the developmental sequences were very highly correlated

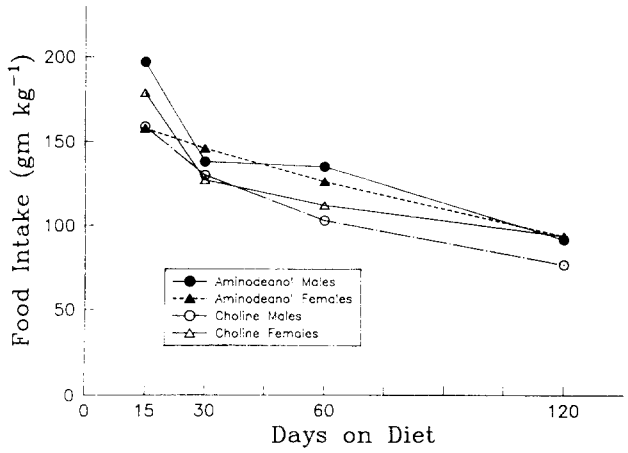

FIG. 1. Food consumed by female and male animals on the NADe and control (Ch) diets during the days of the experiments.

($r_s = .97$ or .98, $p < 10^{-10}$), indicating that there were no early developmental abnormalities.

Body Weight

Body weights for male and female animals were essentially the same when the control and experimental diets were introduced at 29 days postpartum. An immediate effect of the NADe diet was a decrement in weights of both sexes when compared with their controls [male, $t(9) = 3.37$, $p = 6.6 \times 10^{-3}$; female, $t(11) = 4.28$, $p = 1.1 \times 10^{-3}$]. Thereafter, regression analyses indicated that rates of increase in body weights on time within sexes and treatments were highly significant [ChCl: males, $F(1,2) = 65.168$, $p = 1.5 \times 10^{-2}$; females, $F(1,2) = 69.411$, $p = 1.4 \times 10^{-2}$; NADe: males, $F(1,2) = 531.352$, $p = 1.9 \times 10^{-3}$; females, $F(1,2) = 131.350$, $p = 7.5 \times 10^{-3}$] and that the slope constants were not significantly different.

Whole Brain Weight

Whole brain weights were recorded at sacrifice times: 15, 30, 60 and 120 days on the dietary regiment. The weights of female animals were consistently less at every time point ($p < 10^{-2}$, Student's t). Weights for the NADe animals were significantly less than those of the ChCl group (by 7–13%) at every time point ($p < 5 \times 10^{-2}$, Student's t), but increased with time, $F(3,61) = 15.76$, $p = 1.4 \times 10^{-7}$, at rates that were not significantly different. At the conclusion of the experiment, brain weights were 1.697 ± 0.25 g (12) and 1.840 ± 0.037 g (11) for NADe and control groups respectively. However, when expressed as a proportion of body weight, brain weights did not differ significantly between treatments for males, females or both combined.

Food and Water Consumption

Because the route of entry of NADe into the body was by food consumption, measures of intake were recorded at 15, 30, 60, and 120 days on the diets. Water consumption was also measured at these time points. Both water and food intake are reported in absolute terms and as intake per kilogram of body weight.

Three-way ANOVA showed a significant gender difference in absolute level of food consumption, $F(1,44) = 4.92$, $p = 3.2 \times 10^{-2}$, increases in intake over time on diet that approached significance, $F(3,44) = 2.55$, $p = 6.8 \times 10^{-2}$, and no significant treatment effects. Average consumption during the 120 days recorded was: females, NADe = 23.7 ± 2.70 g·day^{-1}, Ch = 23.7 ± 2.52 g·day^{-1}; males, NADe = 32.0 ± 1.65 g·day^{-1}, Ch = 26.2 ± 2.42 g·day^{-1}. As Fig. 1 shows, the ratio of food intake to body weight decreased steadily as time on diet increased, differences between measurement times being highly significant, $F(3,44) = 8.85$, $p = 1.1 \times 10^{-4}$. There were no significant gender or treatment differences.

Measures of absolute water consumption showed that female animals drank more water than males, $F(1,44) = 8.73$, $p = 5.0 \times 10^{-3}$. NADe animals drank more than their Ch controls, $F(1,44) = 9.1 \times 10^{-3}$, there being no significant trends over time. The ratios of water intake per kilogram of body weight also showed females to have higher values than males, $F(1,44) = 16.95$, $p = 2.0 \times 10^{-4}$. At all time points, the ratios for female and male animals in both the NADe and ChCl groups decreased with time on the diets, differences between measurement times being highly significant, $F(3,44) = 10.10$, $p = 3.5 \times 10^{-5}$. They were significantly higher for those on NADe, $F(1,44) = 7.63$, $p = 8.3 \times 10^{-3}$.

NADe Consumption

It was important to determine the amount of the false precursor consumed daily. Given that the diet contained 35.8 mmol·kg^{-1} of NADe and the average female ate 23.7 g·day^{-1}, the mean daily consumption for the 120 days of the experiment was 848 μmol of NADe per female rat. The corresponding intake for the average male rat was 1146 μmol·day^{-1}. When gender differences in body weights are taken into consideration, total daily consumptions are seen to be essentially the same: females, 4.8 mmol and males, 5.3 mmol·kg^{-1} of body weight·day^{-1}.

Core Body Temperature

ANOVA showed no significant differences between the Ch and NADe groups in core body temperature. A significant gender difference, $F(1,75) = 4.30$, $p = 4.2 \times 10^{-2}$, indicated that body temperature was higher in females than in males in both ChCl (0.24°C) and NADe (0.09°C) treatment groups. In both treatment groups, body temperature increased from 15 to 60 days and declined again at 120 days. Differences between times of measurement were highly significant, $F(3,75) = 15.92$, $p = 5.3 \times 10^{-8}$.

Nociception (Algesia)

Results of analyses of nociceptive thresholds are summarized in Table 1. Sensory reflexive responding, as measured by flinch thresholds, showed systematic differences between the two dietary treatments. Young animals in both groups were so sensitive to the mild shock that their thresholds were below the sensitivity limit of our initial apparatus to measure. The sensitivity of the apparatus was then increased and a second series of measurements taken on new groups of animals, results of which are reported in Table 1. Overall, NADe animals were significantly hyperalgesic when compared with controls, $F(1,113) = 58.95$, $p < 10^{-10}$. Thresholds within each treatment group were not significantly different at 60 and 120 days. Female animals were more sensitive than males, $F(1,46) = 13.33$, $p = 7.0 \times 10^{-4}$. There was no significant interaction between gender and treatment.

Table 1 also presents analogous information about effects of the NADe diet on sensory perceptual processes as measured by jump thresholds. Animals in the Ch group became somewhat more hypoalgesic at 15 days, their thresholds increasing at 30 days and

TABLE 1

NOCICEPTIVE THRESHOLDS (mA r.m.s. × 0.5 sec), AS DETERMINED BY UP-AND-DOWN METHOD

	Flinch		Jump	
Days	NADe	ChCl	NADe	ChCl
15	0.065 ± 0.003 (24)*	0.091 ± 0.008 (12)*	0.085 ± 0.005 (10)	0.109 ± 0.007 (10)
30	0.074 ± 0.005 (23)*	0.121 ± 0.009 (12)*	0.097 ± 0.007 (10)	0.126 ± 0.011 (10)
60	0.054 ± 0.007 (10)	0.088 ± 0.005 (10)	0.094 ± 0.009 (10)	0.128 ± 0.007 (10)
120	0.050 ± 0.0052 (17)	0.085 ± 0.0054 (17)	0.066 ± 0.004 (17)	0.128 ± 0.007 (17)

*Thresholds were below the capability of the original apparatus to measure. Present data are from a replication of the experiment following alteration of the apparatus to lower the stimulus range.

Data are expressed as mean ± standard error (N).

remaining constant thereafter. Those on the experimental diet showed an opposite trend: thresholds did not change until 120 days, when the hyperalgesia that had characterized the responses at earlier times increased significantly. These effects are apparent in a significant treatment × day interaction, $F(3,75)=3.61$, $p=1.7\times10^{-2}$. There were no significant gender differences.

Spontaneous Activity

Measures of locomotor and rearing activity are summarized in Table 2. At none of the four assay times did the NADe and Ch groups differ significantly in the former. Their rearing behavior did differ, activity of the NADe animals being less than those on the Ch diet at 30, 60 and 120 days. The difference was significant overall, $F(4,77)=2.525$, $p=4.8\times10^{-2}$, and was not significantly different on different test days, $F(3,77)=1.89$, $p=0.14$. There was a gender difference in locomotion, females being significantly more active, $F(1,78)=30.64$, $p=4.0\times10^{-7}$, but not in rearing behavior. There was no significant gender × treatment interaction.

Reactivity

NADe animals were strikingly hyperreactive when compared with the Ch controls (Fig. 2). This difference was seen at 15 days, $t(19)=3.59$, $p=2.2\times10^{-3}$, and continued throughout the course of the experiment. Overall, the difference was highly significant, $F(3,45)=22.813$, $p=4.0\times10^{-9}$, and increased significantly with time [treatment × time interaction: $F(2,45)=8.123$, $p=9.7\times10^{-4}$]. Even at 30 days, a level was reached that sometimes led to clonic-tonic seizures.

Habituation

The design of the present experiments in principle provided data for the analysis of intrasession habituation in locomotor and rearing behaviors during 20-min exposures in the open-field assay and in the repeated trials of reactivity. Regression coefficients on intrasession time were calculated as estimators of habituation. There were no significant differences between Ch and NADe animals in slopes of their curves for habituation of locomotor behavior. However, as Table 3 summarizes, habituation of rearing behavior in the Ch group was significantly greater than in the NADe group at 15, 60 and 120 days. Overall, the difference was significant, $F(4,76)=2.956$, $p=2.5\times10^{-2}$, and did not change significantly with time, $F(3,76)=1.780$, $p=0.16$. No conclusions could be drawn in regard to habituation from the reactivity experiments because of missing data due to seizures.

Inhibited (Passive) Avoidance

The basic data for inhibited avoidance behavior consist of re-

TABLE 2

SPONTANEOUS ACTIVITY IN CIRCULAR OPEN FIELD (BEAM BREAKS PER MIN)

	A. Locomotion	
Days	Ch	NADe
15	63.8 ± 2.84 (10)	66.3 ± 2.54 (10)
30	65.8 ± 2.30 (10)	62.5 ± 2.37 (10)
60	60.4 ± 2.30 (10)	66.7 ± 2.68 (10)
120	55.7 ± 1.82 (17)	61.2 ± 1.57 (17)
	B. Rearing	
Days	NADe	Ch
15	11.7 ± 0.65 (10)	10.3 ± 0.57 (10)
30	13.6 ± 0.66 (10)	14.3 ± 0.63 (9)
60	11.9 ± 0.66 (10)	17.3 ± 0.78 (9)
120	12.0 ± 0.64 (17)	16.0 ± 0.64 (17)

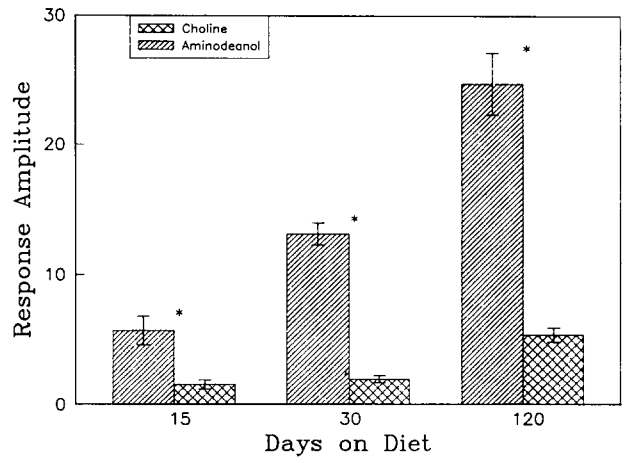

FIG. 2. Reactivity of animals in the NADe and control (Ch) groups, showing progressive hyperreactivity in the former as time on the diet increased (*$p<0.05$).

TABLE 3

HABITUATION IN OPEN FIELD AS SHOWN BY REARING BEHAVIOR

Time on Diet	Slope Constants Ch	NADe	t	p*
15	-1.715 ± 0.370	-0.245 ± 0.498	$t(19)=2.37$	1.5×10^{-2}
30	-0.619 ± 0.476	-1.163 ± 0.501	$t(19)=0.79$	7.8×10^{-1}
60	-2.426 ± 0.952	-0.241 ± 0.378	$t(18)=2.14$	2.3×10^{-2}
120	-2.011 ± 0.664	-0.729 ± 0.357	$t(33)=1.70$	4.9×10^{-2}

Overall, $\beta=22.52$, $p=4.0\times10^{-3}$ [significance of aggregate probabilities (26)].
*One-tail test for NADe>Ch.
Data are regression coefficients on time.

sponse times on two trials, the first (training trial) resulting in punishment and the second (retention trial), taken as a measure of memory. In order to eliminate individual differences on the first trials, the ratio, retention time/training time, was used in the analysis of results. Because of the asymmetric spread of scores, this was converted to Log_{10} units as summarized in Fig. 3. The tests for statistical significance clearly show that, during the period on the diets, the retention times for NADe animals were significantly less than those for the Ch animals, $F(1,75)=18.60$, $p=5.4\times10^{-5}$, i.e., the shorter avoidance times of the NADe groups indicated poorer memory for the punishment received on the training trial. The difference increased with time on the diet, $F(3,75)=8.43$, $p=7.7\times10^{-5}$, with peak effects at 60 days.

Conditioned Avoidance Response (CAR)

The CAR assay provided four measures of behavior. Table 4 summarizes the analyses of these measures taken at 15, 30, and 60 days on the NADe and Ch diets. The escape response (R_e) is innate in origin, appearing as an unlearned reaction to a noxious stimulus. It is apparent that there was no systematic pattern of differences between effects of the two diets on this response over the period of 60 days.

Among the three parameters of the learned avoidance response the number of trials taken to reach the criterion increased as time on the NADe diet increased, $F(1,48)=7.46$, $p=8.8\times10^{-3}$, while there was no systematic trend in performance of the Ch groups (Fig. 4). The same was true of a second parameter, number of errors in trials to complete learning, $F(1,48)=6.17$, $p=1.7\times10^{-2}$. Thirdly, time of the avoidance response (R_a) became significantly longer at 60 days on the NADe diet [treatment × days, $F(2,48)=5.81$, $p=5.5\times10^{-3}$]. The results show that the group receiving NADe had higher scores than those on the Ch diet in 8 of the 9 (3 parameters × 3 time points) measures recorded. A binomial test indicates that this combination would not be expected by chance ($p=2.0\times10^{-2}$). In all instances higher scores mean less efficient learning, with its implications of poorer memory.

DISCUSSION

The basic rationale underlying our present experiments is: (a) that the false transmitter, by creating a hypocholinergic state, will be associated with changes in behavioral and physiological functions that are cholinergically coded and (b) that the extent of such changes will progress concomitantly with the extent to which NADe replaces Ch in the synthesis of the false transmitter and/or, in the longer range, produces effects on phospholipid metabo-

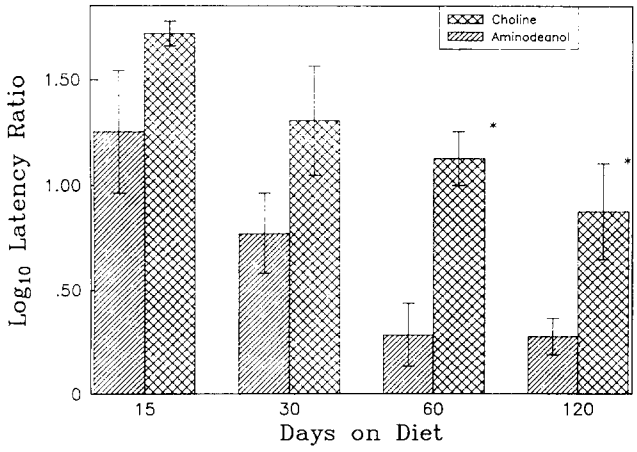

FIG. 3. Log_{10} latency ratio for retention/acquisition trials in the inhibited (passive) avoidance assay, showing progressively poorer memory in NADe than control (Ch) animals.

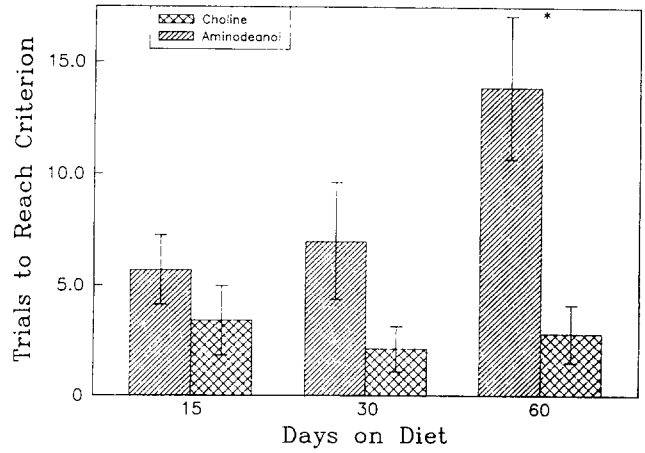

FIG. 4. Progressively poorer learning performance of NADe animals as evidenced by the number of trials required to reach criterion in the conditioned avoidance assay.

TABLE 4

CONDITIONED AVOIDANCE RESPONSE PARAMETERS*

	Trials		Errors		R_a (s)		R_e (s)	
Days	NADe	Ch	NADe	Ch	NADe	Ch	NADe	Ch
15	14.4 ± 1.70	11.6 ± 1.77	5.4 ± 1.07	4.1 ± 1.40	4.1 ± 0.38	3.7 ± 0.20	1.5 ± 0.15	2.3 ± 0.27
30	15.7 ± 2.69	12.6 ± 2.20	7.0 ± 1.84	5.6 ± 2.20	3.7 ± 0.29	4.6 ± 0.49	2.5 ± 0.41	2.5 ± 0.73
60	20.9 ± 2.59	12.4 ± 1.78	12.2 ± 2.35	4.2 ± 1.03	4.6 ± 0.43	3.2 ± 0.37	3.4 ± 0.72	1.7 ± 0.39

*n = 10 in all measures, except NADe = 9 for R_e.

lism that result in neuronal damage. After 60–120 days on the NADe diet, free Ch was 85–95% replaced with free NADe in plasma, brain and peripheral tissues and lipid-bound Ch was 65–75% replaced (29,30). Our present experiments were designed to measure several dependent variables periodically as these levels of replacement were progressively achieved. The magnitudes of these changes tended to increase with time in the NADe diet, but those related to learning and memory were largely confined to the 60–120-day period.

Homeostatic Functions

Introduction of the concept of "homeostasis" (4,6) brought into perspective the "self-correcting" ("vegetative") processes essential to normal functioning of living organisms. The question has been raised as to whether dysfunction of one or more of these processes might underlie such disorders as SDAT. In our present experiment we chose to observe four of them. The NADe diet had no significant effects on total caloric intake or core body temperature. NADe animals consistently drank more water than the controls. Body weight showed a brief initial decrement that disappeared by 70 days, the rate of growth being similar to that of the ChCl control animals from Day 15. Such results strongly suggest that the behavioral effects of NADe are unlikely to be attributable to imbalances in basic homeostatic mechanisms.

Behavioral Effects

Interactions between behavioral variables and NADe replacement of Ch may be evidenced in (a) differences between NADe and ChCl controls animals and (b) progressive trends in the magnitudes of behavioral changes as the replacement (time on the NADe diet) increased. Both are considered in the paragraphs to follow. The various behaviors will be discussed within the three basic categories: sensory-reflexive (innate) behaviors, sensory-perceptual processes and cognitive processes (learning and memory).

Sensory-Reflexive Processes

Among the behaviors measured were two that have been established as primarily sensory-reflexive (innate) in nature, i.e., appear without the necessity for learning. One of these is the flinch response in our assay for nociception. Although voluntary behaviors, e.g., preening, may follow, the initial reaction directly linked to the shock stimulus is a stereotyped withdrawal reflex. The second measure of innate behavior is reactivity to the auditory stimulus, which has received much attention as the "acoustic startle reflex"(14). It is mediated in the rat by a short neural pathway with only a few synapses contained within the brain stem and the spinal cord (21) and has a response latency of 8 ms as recorded electromyographically (16). It can be modulated by higher brain systems (53), but the basic response is innate. The fact that, beginning at 30 days, the acoustic startle stimulus produced convulsive seizures in some NADe animals illustrates the way in which the basic startle reflex may become coupled to more complex responses. In the present case, this coupling requires participation of motor areas in the cerebral cortex and occurs with so short a latency as to make differentiation from the startle reflex difficult.

Both these behaviors were significantly affected by the NADe diet. Flinch thresholds were reduced, resulting in a marked hypersensitivity (hyperalgesia); changes in the acoustic startle reflex beginning at 15 days clearly showed the existence of a state of hyperreactivity. These changes in sensory-reflexive behavior have a resemblance to a syndrome that is referred to as "psychomotor agitation" in SDAT and in some affective disorders.

Sensory-Perceptual Processes

Clearly apparent in SDAT and other degenerative disorders of aging are signs of a sensory-perceptual nature, e.g., failure to "understand" events in the physical and psychological environments, spatial disorientation, and eventually a general loss of response to most stimuli. All of these go well beyond reflexive behavior involving responses coordinated at higher levels in the CNS. Four of the behavioral measures in the present experiments provided information about effects of NADe on such processes. Locomotor and rearing activities in the open-field situation were two of them. These exploratory behaviors are characterized by the fact that they make new information available to the individual (17). There has been empirical evidence for at least four decades that the search for novel stimulus conditions can be a significant determinant of exploratory behavior [see (3,17)]. Whether or not particular stimuli are "novel" requires not only the sensory processes involved in registering them, but also the more complex processes of perceiving them in relation to past experience. Results of the present experiments show that, even when 85–90% of free Ch was replaced by NADe after 60 days on the NADe diet, sensory-perceptual processes involved in locomotor activity in the novel open-field situation were not significantly affected. However, rearing behavior was significantly suppressed after both 60 and 120 days of treatment.

Two other behavioral measures involved the use of electric shock, but also incorporated responses other than reflexive. The jump reaction in our assays required the participation of both sensory and perceptual processes (7). Similar sensory-motor coordination was involved in the escape of the CAR assay. Both behaviors were initiated by reflexive responses to the shock. The escape response involved a learning process that resulted eventually in the acquired avoidance response, R_a. Furthermore, measures of behavior in the nociceptive assay (jump) indicated that NADe ani-

mals were significantly hyperalgesic throughout the four test periods, whereas there was no significant trend in escape response times in the CAR assay as NADe replacement of Ch progressively increased. The hyperalgesic state is characteristic of decreased cholinergic activity.

Cognitive Processes

The fact that impairment in learning, memory and other cognitive processes characterizes SDAT from its early stages led us to include a broad range of measures of learning and memory in our present experiments. "Learning" and "memory" are theoretical constructs defined empirically by the operations involved in studying deductions from them. The two constructs are intimately related in that systematic changes in behavior during repeated exposures to the same stimulus conditions (learning) require the retention (memory) of information about responses made to earlier exposures. From this perspective our present experiments provided nine measures of memory, ranging from what has been termed a "primitive form" (habituation) to the de novo acquisition of a CAR dependent upon differential reinforcement.

The present data on habituation are similar to some earlier findings following manipulation of the cholinergic system, but are different in other regards. Administration of organophosphorus anticholinesterases leading to elevated ACh in brain has been shown to result in decreased levels of locomotion and of rearing without affecting habituation (47). There is evidence that intrahippocampal injections of the cholinergic neurotoxin, AF64A, increase open-field activity, but, again, do not affect habituation (1). In terms of current theoretical models of habituation (42) these results could be interpreted as meaning that the cholinergic neurotransmitter system is involved in modulating response levels, but not directly in the neurochemical events basic to eliciting the response, i.e., in "extrinsic," but not "intrinsic" habituation (15). In our present experiments, replacement of ACh by the false transmitter, AcNADe, produced significant effects on habituation in rearing behavior: animals on the control diet showed typical intrasession habituation; animals fed the NADe diet did not habituate within the 20-min assay period.

Inhibited (passive) avoidance has been used more widely than other behavioral assays for studying effects of drugs and other treatments on memory per se. In our present experiments the replacement of Ach by the false transmitter occurred progressively throughout the experimental period, and it is not possible to differentiate among effects on learning, memory and retrieval from memory. However, all of these involve processes in which memory is the common element. Our present results have three features of special interest. First, statistically significant differences in measures of memory between the NADe and control animals occurred only at 60 and 120 days, although the former had consistently poorer measures of memory throughout the experimental period. Secondly, the control group showed a consistent trend toward decreasing performance at the four assay times, a trend that might be related to increasing chronological age. Thirdly, animals on the NADe diet also decreased systematically in their performance, but at a much faster rate than those on the control diet, reaching an asymptote by the 60-day assay.

Parameters of CAR performance provide evidence of impaired memory during the process of acquiring the avoidance response. The latter involves what is often referred to as "reference" memory (44). The animal had to master the general rule that CS was a signal to move to the goal end of the straightaway; it was not required to remember what responses it had made on earlier trials ("working memory"). In all three measures of the CAR, animals on the NADe diet were inferior in performance to the control subjects, the trends being apparent at all three assay times and statistically significant at 60 days. NADe animals took more trials to learn the CAR, made more errors and were slower in response times. Such results are consistent with earlier reports summarizing preclinical and clinical research (41).

Analogies With PDD

A published report of the U.S. National Institute of Health Consensus Development Conference on Differential Diagnosis of Dementing Diseases (36) contains the statement that " . . . dementia is primarily a behavioral diagnosis." It is characterized by a decline in " . . . memory, other cognitive capacities, and adaptive behavior . . . In some demented patients, changes in motor, sensory, and visual systems appear . . . the most frequent of the dementing diseases are progressive in nature." In the present experiments, we have tested a hypothesis about the etiology of an impaired cholinergic system; produced a progressive impairment of function; and measured behavior effects as observed in sensory, motor and cognitive functions. Our results have shown that, as cholinergic function was progressively impaired, basic physiological ("vegetative") processes appeared not to be affected. Apparently the rate at which the impairment developed was sufficiently slow to allow the organism to adapt at a survival level to changes in the environment. However, complex behavior functions were affected progressively as time on NADe diet increased. Cognitive functions were most sensitive to change and showed the least adaptability. Differential sensitivities and failure to adapt may reflect different threshold requirements in cholinergic function for normal behavior of different kinds (47). It is important to recognize that our experiments have been limited to one neurotransmitter system, whereas clinical evidence indicates that other such systems are also involved. However, the central involvement of the cholinergic system in PDD has been pointed out by many investigators, e.g., " . . . although 11 major transmitter systems have been implicated in AD, the most dramatic and consistent findings are related to deficits in the cholinergic system (13)."

Further studies are in progress to determine whether the syndrome produced by NADe continues to progress beyond 120 days of treatment (10), whether it is reversible when a normal diet is restored, whether it is associated with histological changes, and whether it responds to pharmacological intervention. We believe that these studies will establish whether the behavioral deficit is due to functional changes, neuronal loss or both, and will help to define the investigational utility of NADe syndrome as an experimental model of progressive degenerative dementia that meets the basic criteria for the validity of animal models (49).

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REFERENCES

1. Bailey, E. L.; Overstreet, D. H.; Crocker, A. D. Effects of intrahippocampal injections of the cholinergic neurotoxin AF64A on open-field activity and avoidance learning in the rat. Behav. Neural Biol. 45:263–274; 1988.

2. Baker, H. J.; Lindsey, J. R.; Weisbroth, S. H. Housing to control research variables. In: Baker, H. J.; Lindsey, J. R.; Weisbroth, S. H., eds. The laboratory rat. vol. I: Biology and diseases. New York: Academic Press; 1979:169–192.

3. Berlyne, D. E. Conflict, arousal and curiosity. New York: McGraw-Hill; 1960.
4. Bernard, C. Lecon Sur Les Proprietes Physiologiques et Les Alterations Pathologiques des Liquides de L'Organisme. vols. I and II. Paris: Balliere; 1859.
5. Bremer, J.; Greenberg, D. M. Biosynthesis of choline in vitro. Biochim. Biophys. Acta 37:173–179; 1960.
6. Cannon, W. B. The wisdom of the body. Rev. Ed. London: Kegan, Paul, Trench, Trubner; 1939.
7. Casey, K. L.; Melzack, R. Neural mechanisms of pain: a conceptual model. In: Way, E. L., ed. New concepts of pain and its clinical management. Philadelphia: Davis; 1967:13–31.
8. Channon, H. J.; Smith, J. A. B. The dietary prevention of fatty livers, triethyl-β-hydroxyethyl ammonium hydroxide. Biochem. J. 30: 115–120; 1936.
9. Collier, B.; Boksa, P.; Lovat, S. False cholinergic transmitters. Prog. Brain Res. 49:107–125; 1979.
10. Coleman, P. D. Editorial: How old is old? Neurobiol. Aging 10:115; 1989.
11. Cooper, J. F.; Needham, J. R.; Hetherington, C. H. The use of a simple barrier system to exclude murine pathogens. Lab. Anim. 11: 47–48; 1977.
12. Crocker, A. D.; Russell, R. W. The up-and-down method for the determination of nociceptive thresholds in rats. Pharmacol. Biochem. Behav. 21:133–136; 1984.
13. Cutler, N. R.; Narang, P. K. Cognitive enchancers in Alzheimer's disease. In: Cutler, N. R.; Narang, P. K., eds. Drug studies in the elderly. New York: Plenum Medical Book Co.; 1986:313–332.
14. Davis, M. Neurochemical modulation of sensory-motor reactivity: acoustic and tactile startle reflexes. Neurosci. Biobehav. Rev. 4:241–263; 1980.
15. Davis, M.; File, S. E. Intrinsic and extrinsic mechanisms of habituation and sensitization: implications for the design and analysis of experiments. In: Peeke, H. V. S.; Petrinovich, L., eds. New York: Academic Press; 1984:287–323.
16. Davis, M.; Gendelman, D. S.; Tischler, M. D.; Gendelman, P. M. A primary acoustic startle circuit: lesion and stimulation studies. J. Neurosci. 2:791–805; 1982.
17. Eaton, R. C. Neural mechanisms of startle behavior. New York: Plenum Press; 1984.
18. Ellman, G. L.; Courtney, K. D.; Andres, V.; Featherstone, R. M. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7:88–95; 1961.
19. Fisher, R. A. Statistical methods for research workers. New York: Hafner Publishing Co.; 1950:99–101.
20. Fonnum, F. Recent developments in biochemical investigations of cholinergic transmission. Brain Res. 62:497–507; 1973.
21. Fox, J. E. Habituation and prestimulus inhibition of the auditory startle reflex in decerebrate rats. Physiol. Behav. 23:291–297; 1979.
22. Glick, S. D.; Crane, A. M.; Barker, L. A.; Mittag, T. W. Effects of N-hydroxyethylpyrrolidinium methiodide, a choline analogue, on passive avoidance behavior in mice. Neuropharmacology 14:561–564; 1975.
23. Glick, S. D.; Mittag, T. W.; Green, J. P. Central cholinergic correlates of impaired learning. Neuropharmacology 12:291–296; 1973.
24. Heise, G. A. Behavioral methods for measuring effects of drugs on learning and memory in animals. Med. Res. Rev. 4:535–558; 1984.
25. Ilson, D.; Collier, R. B. Triethylcholine as a precursor to a cholinergic false transmitter. Nature 254:618–619; 1975.
26. Jenden, D. J. The pharmacology of cholinergic mechanisms and senile brain disease. In: Scheibel, A.; Wechsler, A. P., eds. The biological substrates of Alzheimer's disease. New York: Academic Press; 1986:205–215.
27. Jonas, A. M. The research animal and the significance of a health monitoring program. Lab. Anim. Sci. 26:339–344; 1976.
28. Jones, L. V.; Fiske, D. W. Models for testing the significance of combined results. Psychol. Bull. 50:375–382; 1953.
29. Knusel, B.; Jenden, D. J.; Lauretz, S. D.; Booth, R. A. Rice, K. M.; Roch, M.; Waite, J. J. Global in vivo replacement of choline by N-aminodeanol: Testing a hypothesis about progressive degenerative dementia: I. The dynamics of choline replacement. Pharmacol. Biochem. Behav. 37(4): in press; 1990.
30. Knusel, B.; Lauretz, S. D.; Booth, R. A.; Jenden, D. J. Dietary replacement of choline by N-aminodeanol in rats, measured by HPLC. Soc. Neurosci. Abstr. 13:1196; 1987.
31. Kopin, I. J. False adrenergic transmitters. Annu. Rev. Pharmacol. 8:377–394; 1965.
32. Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265–275; 1951.
33. McGaugh, J. L. The search for the memory trace. Ann. NY Acad. Sci. 193:112–123; 1973.
34. Matthews, R. T.; Chiou, C. Y. Effects of diethylcholine in two animal models of parkinsonism tremors. Eur. J. Pharmacol. 56:159–162; 1979.
35. Maw, G. A.; du Vigneaud, V. An investigation of the biological behavior of the sulfur analogue of choline. J. Biol. Chem. 176:1029–1036; 1948.
36. National Institutes of Health (USA). Differential diagnoses of dementing diseases. National Institutes of Health, Consensus Development Conference Statement. Alzheimer's Dis. Assoc. Disord. 2:4–15; 1987.
37. Newton, M. W.; Crosland, R. D.; Jenden, D. J. In vivo metabolism of a cholinergic false precursor after dietary administration to rats. J. Pharmacol. Exp. Ther. 235:157–161; 1985.
38. Newton, M. W.; Crosland, R. D.; Jenden, D. J. Effects of chronic dietary administration of the cholinergic false precursor N-amino-N, N-dimethylaminoethanol on behavior and cholinergic parameters in rats. Brain Res. 373:197–204; 1986.
39. Newton, M. W.; Jenden, D. J. Metabolism and subcellular distribution of N-amino-N, N-dimethyl aminoethanol (N-aminodeanol) in rat striatal synaptosomes. J. Pharmacol. Exop. Ther. 235:135–146; 1985.
40. Newton, M. W.; Jenden, D. J. False transmitters as presynaptic probes for cholinergic mechanisms and functions. Trends Pharmacol. Sci. 7:316–320; 1986.
41. Olton, D. S.; Gamzu, E.; Corkin, S. Memory dysfunction: an integration of animal and human research from preclinical and clinical perspectives. Ann. NY Acad. Sci. V444; 1985.
42. Peeke, H. V. S.; Petrinovich, L. Habituation, sensitization and behavior. New York: Academic Press; 1984.
43. Pilz, P. K. D.; Schnitzler, H.-U.; Menne, U. Acoustic startle threshold of the albino rat (Rattus norvegicus). J. Comp. Psychol. 101:67–72; 1987.
44. Rawlins, J. N. P.; Olton, D. S. The septo-hippocampal system and cognitive mapping. Behav. Brain Res. 5:331–358; 1982.
45. Russell, R. W. The cholinergic system in behavior: the search for mechanisms of action. Annu. Rev. Pharmacol. Toxicol. 22:435–463; 1982.
46. Russell, R. W.; Booth, R. A.; Lauretz, S. D.; Smith, C. A.; Jenden, D. J. Behavioral, neurochemical and physiological effects of repeated exposures to subsymptomatic levels of the anticholinesterase, soman. Neurobehav. Toxicol. Teratol. 8:675–685; 1986.
47. Russell, R. W.; Booth, R. A.; Smith, C. A.; Jenden, D. J.; Roch, M.; Rice, K. M.; Lauretz, S. D. Roles of neurotransmitter receptors in behavior: recovery of function following decreases in muscarinic receptor sensitivity induced by cholesterase inhibition. Behav. Neurosci. 103:881–892; 1989.
48. Russell, R. W.; Macri, J. Some behavioral effects of suppressing cholinergic transport by cerebroventricular injection of hemicholinium-3. Pharmacol. Biochem. Behav. 8:399–403; 1978.
49. Russell, R. W.; Overstreet, D. H. Animal models in neurobehavioral toxicology. In: Bond, N. W., ed. Animal models in psychopathology. Sydney: Academic Press; 1984:23–57.
50. SAS user's guide: Statistics, Edition 5. Cary, NC: SAS Institute; 1985: 656–661.
51. Silverman, R. W.; Chang, A. S.; Russell, R. W. A microcomputer-controlled system for measuring reactivity in small animals. Behav. Res. Methods Instrum. Comput. 20:495–498; 1988.
52. Silverman, R. W.; Chang, A. S.; Russell, R. W. Measurement of activity in small animals using a microcomputer-controlled system. Behav. Res. Methods Instrum. Comput. 20:537–540; 1988.
53. Tischler, M. D.; Davis, M. A visual pathway that mediates fear-conditioned enhancement of acoustic startle. Brain Res. 276:55–71; 1983.
54. Whittaker, V. P.; Luqmani, Y. A. False transmitters in the cholinergic system: implications for the vesicle theory of transmitter stor-

age and release. Gen. Pharmacol. 11:7–14; 1980.
55. Wurtman, R. J.; Blusztajn, J. K.; Maire, J. C. "Autocannibalism" of choline-containing membrane phospholipids in the pathogenesis of Alzheimer's disease—A hypothesis. Neurochem. Int. 7:369–372; 1985.
56. Yamamura, H. I.; Snyder, S. H. Muscarinic cholinergic binding in rat brains. Proc. Natl. Acad. Sci. USA 71:1725–1729; 1974.