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Global In Vivo Replacement of Choline by N-Aminodeanol. Testing a Hypothesis About Progressive Degenerative Dementia: I. Dynamics of Choline Replacement

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KNUSEL, B., D. J. JENDEN, S. D. LAURETZ, R. A. BOOTH, K. M. RICE, M. ROCH AND J. J. WAITE. *Global in vivo replacement of choline by N-aminodeanol. Testing a hypothesis about progressive degenerative dementia: I. Dynamics of choline replacement.* PHARMACOL BIOCHEM BEHAV **37**(4) 799–809, 1990.—Severe disruption of certain cholinergic pathways is a characteristic feature of Alzheimer's disease. Attempts to establish animal models by interfering with cholinergic function have not been very successful. We now present data which show a substantial and progressive replacement of free and phospholipid-bound choline by the novel choline isostere N-amino-N,N-dimethylaminoethanol during its dietary administration in place of choline. Free choline in blood fell to ~20% of controls after 10 to 30 days on diet. Phospholipid-bound choline in plasma was reduced to less than 15%, and in erythrocytes to about 22%. After 120 days of diet free and bound choline were reduced in most tissues to approximately 30% of controls. Only liver retained more than 80% of free choline. Acetylcholine was decreased to 33 to 50% of control. Total true and false transmitter in experimental animals was in all tissues less that acetylcholine in controls, suggesting that muscarinic transmission would be impaired. Moderate reduction of choline acetyltransferase activity was seen in striatum and myenteric plexus, and of QNB-binding in hippocampus, striatum and myenteric plexus.

Alzheimer's disease False cholinergic transmitter Choline deprivation HPLC of cholinergic compounds Phospholipids

DISRUPTION of central cholinergic neurotransmission is believed to be responsible for many of the behavioral features of Alzheimer's disease, including memory loss. There is a positive correlation between the loss of cortical choline acetyltransferase (ChAT) activity and the degree of dementia in patients with Alzheimer's disease (48). The reduction of cholinergic parameters is associated with a loss of cholinergic neurons projecting from the basal forebrain and medial septum to neocortex and hippocampus (49). It has been proposed that this specific vulnerability of cholinergic neurons derives from competition between the two functions of choline (Ch) in cholinergic neurons as a precursor of acetylcholine (ACh) and of the membrane constituent phosphatidylcholine (31,65). No entirely satisfactory experimental model of Alzheimer's disease is yet available. We have explored the approach of using an unnatural Ch isostere to exacerbate this competition and perhaps produce this selective vulnerability (32,42). N-Amino-N,N-dimethylaminoethanol (N-aminodeanol, NADe) has been shown in vitro and in vivo to be the precursor of a false cholinergic neurotransmitter (41,44). The potency of acetyl-N-aminodeanol (AcNADe) at muscarinic receptors is shown to be 4%, at nicotinic receptors 17% of that of ACh (45). However, the large store of endogenous Ch in the form of phosphatidylcholine in brain requires long-term substitution of NADe for dietary Ch to achieve a substantial replacement. We present here experiments in which the dynamics of such a replacement were studied in rats maintained on a Ch free, NADe-containing diet. The concentrations of free and phospholipid-bound Ch and NADe in plasma and erythrocytes were measured during 120 days on the Ch replacement diet. The results are compared with values from controls on a Ch-containing diet. After 120 days on the diets the levels of true and false precursors in free and bound form and of true and false transmitters have been measured in various tissues. An abstract of these results has been published (35). The physiological and behavioral consequences of this replacement are described in the following paper (53).

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METHOD

Animals and Diets

Pregnant rats were purchased from Bantin and Kingman (Pleasanton, CA). The pups were weaned at age 29 days and placed on experimental and control diets ad lib. The animals had at all times free access to a water supply. The basic diet consisted of dextrose (69%), casein (12%), soya oil (10%), mineral mix (5%), alphacel (ground cellulose, 2%), vitamin mix (1.5%) (10,52) and was tested to be Ch free (<14 $\mu mol \cdot kg^{-1}$). Either Ch chloride (control) or NADe chloride (experimental) was added at a concentration of 35.8 $mmol \cdot kg^{-1}$. NADe chloride was synthesized in our laboratory by a previously published method (43). Because of the possibility that NADe may be metabolized to a hydrazine derivative that depletes pyridoxine (13), the pyridoxine content of both diets was increased from the recommended level of 6 $mg \cdot kg^{-1}$ to 30 $mg \cdot kg^{-1}$. To distinguish the two diets, food dye FD&C No. 40 (red) was added to the experimental diet.

Samples

Blood samples were taken at intervals by carefully inserting a heparinized and subsequently dried hypodermic needle into a tail vein. A total of less than 150 μl blood was collected directly from the needle hub into two heparinized capillaries (Fisher, Microhematocrit Capillary Tubes), one for determination of free, one for phospholipid-bound Ch and NADe. The sealed capillaries (Fisher, Critoseal) were immediately cooled in ice and centrifuged at $6300 \times g$ for 5 minutes. The capillaries were broken at the interface between plasma and cells and the volume of each fraction established from the length of the corresponding segment. The plasma was blown into 1.5 ml 15% 1 N aqueous formic acid in acetone for the extraction of the free compounds or 4.0 ml chloroform/methanol [2:1, v/v, (18)] for the phospholipid-bound compounds. The cells were washed in 1.5 ml cold saline before adding the extraction medium. Tissue samples were dissected, weighed and immediately transferred to tubes with the same relative volumes of extraction medium as for the blood samples.

Extraction Procedure, Free Compounds

Plasma and blood cell samples in formic acid/acetone were allowed to extract on ice for 1 to 2 hours with occasional shaking. Ethylhomocholine was added as an internal standard and the samples were centrifuged at $12000 \times g$ for 20 minutes. Tissue samples were extracted in formic acid/acetone with internal standard at 4°C for 48 to 96 hours. The tissue was then removed. This procedure has been shown to extract Ch and ACh from tissue samples quantitatively (23). The tissue extracts or the supernatant of the blood samples were diluted with 300 μl distilled H_2O and extracted twice with 1.5 ml ether. Remaining traces of organic phase were removed by a stream of nitrogen directed over the aqueous phase. The mixture was made basic with 0.7 ml 2 M glycine buffer, pH 9.3, containing dipicrylamine (10 mM) (Pfaltz & Bauer) for ion-pair extraction of quaternary ammonium compounds into 3 ml dichloromethane (EM, Omni-Solv). The organic phase was transferred to a clean tube, evaporated to dryness under a stream of nitrogen and the capped tubes stored until measurement by HPLC.

Extraction Procedure, Phospholipid-Bound Compounds

All samples in chloroform/methanol were homogenized with a Polytron homogenizer (Brinkmann Instruments) and kept on ice for 1 to 2 hours with occasional shaking. Distilled water (400 μl) was added, the samples thoroughly mixed and centrifuged at $3000 \times g$ for 5 minutes. The interface between organic and inorganic phases was marked on the tube for later determination of the volume of the organic phase. An aliquot of the organic (lower) phase was evaporated to dryness under a stream of nitrogen. The phospholipids in the residue were hydrolyzed with 500 μl 6 N HCl at 100°C for 1 hour and the HCl partly neutralized with 305 μl 10 N NaOH. After adding internal standard and 2 ml formic acid/acetone the samples were processed as described above.

Chromatography

The dried samples from the dipicrylamine extraction were completely dissolved in 0.3–0.5 ml HCl (10 mM) and 1 ml dichloromethane and them extracted with 2 ml ether. The organic phase was aspirated and the residual traces of organic solvents evaporated under a stream of nitrogen. The sample was filtered through nylon filters (CAMEO, 0.45 μm pore size, Fisher Scientific) and the filtrate (10–100 μl) was injected into the HPLC apparatus. The chromatographic procedure was a modification of the method developed by Meek and co-workers (39). In order to separate Ch and NADe and their acetate esters as well as ethylhomocholine as internal standard, a C-8 reversed phase column (Econosphere, Alltech Associates) was used instead of the C-18 or resin based column of the original procedure. The HPLC apparatus was a Hewlett Packard HP 1090 Liquid Chromatograph with antoinjector and autosampler. An enzyme loaded anion exchange cartridge served as postcolumn reactor. The liberated H_2O_2 was detected amperometrically by a platinum electrode (Bioanalytical Systems) at +500 mV or +550 mV relative to a Ag/AgCl reference electrode (detector built in-house). A model 3392A signal integrator from Hewlett Packard measured peak sizes. The mobile phase consisted of Tris maleate buffer (30 mM: pH 7.0), octyl sodium sulfate (Kodak) (0.1–1.0 mM) as ion-pair reagent and triethylamine (Sigma) (0.2–20 mM) or tetramethylammonium (Sigma) (1.0–12 mM) as amine modifier. These concentrations were optimized for each individual column. The mobile phase was prepared daily and was filtered before use (Millipore filtration kit, 0.45 μm pore size). Flow rates of 1.5 or 2.0 ml/min were used with columns, detector cell and reference electrode at a constant temperature of 35°C. The enzyme column was prepared by a simple procedure. A new anion exchange cartridge (Brownlee Aquapore AX300, 3 cm × 2.1 mm) was flushed first with about 30 ml each of 80% acetonitrile in H_2O and subsequently with mobile phase. Ch oxidase (100 units, from alcaligenes sp., Sigma) in 0.5 ml 20 mM Tris maleate buffer (pH 7.0), followed by 300 units acetylcholinesterase (from electric eel, Sigma) in 1 ml H_2O were then loaded onto the cartridge with a syringe. Between adding the two enzymes the cartridge was again flushed with mobile phase.

Measurement of Choline Acetyltransferase

CAT activity was measured according to the method of Fonnum (19) with minor modifications. Tissues were homogenized in cold sodium phosphate buffer, pH 7.4; homogenates were kept at 0–4°C and assayed the same day. Triton X-100 (1%, v/v) was added to the homogenates to activate enzyme release. [1-^{14}C] acetylcoenzyme A (58 mCi/mmol, Amersham Corp., Arlington Heights, IL) was diluted with unlabelled acetylcoenzyme A (Sigma Chemical Co., St. Louis, MO) to a specific activity of 3.5 mCi/mmol and 115,000 dpm was added to each sample. The incubation mixture contained (mM final concentration in 56 μl total volume): acetyl-coenzyme A, 0.28; NaCl, 305.7; sodium phosphate buffer pH 7.4, 48; choline chloride, 8.8; EDTA pH

7.4, 1.0; and Tetram, 0.02. The reaction was initiated by adding acetylcoenzyme A to the sample which contained 10 μl of solubilized tissue homogenates, incubated at 37°C for 15 min and terminated by adding 1 ml of cold sodium phosphate buffer (10 mM, pH 7.4). Further processing of samples was as described (19).

Acetylcholinesterase (AChE) and Butyrylcholinesterase (BChE) Activity

Cholinesterase activity was assayed in cortex, hippocampus, striatum, heart, myenteric plexus, erythrocytes and plasma by the spectrophotometric method of Ellman et al. (17). Tissue was homogenized in ice-cold phosphate buffer (81 mM Na, 9.5 mM K; pH 7.4) and diluted to amounts appropriate for the assay: brain tissues, 0.8 mg original wet weight per tube; ileal longitudinal muscle, 4 mg/tube; ventricles 5 mg/tube; plasma, 50 μl/tube; packed erythrocytes, 2.5 μl/tube. Acetylthiocholine served as the substrate for AChE and butyrylthiocholine for BChE. Final substrate concentration was 0.5 mM. Incubation was in 3 ml phosphate buffer (pH 8) at 37°C for 5–10 min in presence of 0.3 mM 5,5-dithio-bis-2-nitrobenzoate and 10 μl of 0.1% quinidine sulfate. For each sample a tissue blank without substrate was processed identically.

QNB Binding

Muscle tissues were minced with scissors and all tissues were homogenized in 50 mM ice-cold phosphate buffer (81 mM Na, 9.5 mM K; pH 7.4) with a Polytron homogenizer, setting 6, for 20 s. Muscle homogenates were then filtered through a double layer of gauze. All preparations were diluted in phosphate buffer to amounts appropriate for initiation of the binding assay: ileal longitudinal muscle, 4 mg original wet weight per tube; ventricles, 5 mg/tube; brain tissues, 0.5 mg/tube. Muscarinic receptor number was estimated by binding of 0.4 nM [^{3}H] (−)-quinuclidinyl benzilate (QNB; specific activity 39.4 Ci/mmol; New England Nuclear) in a final volume of 2 ml by the filtration method of Yamamura and Snyder (66). Incubation was 1 h at 37°C. Nonspecific binding was determined by coincubation with 2 μM atropine sulfate and subtracted from total binding. All measurements were made in triplicate. Homogenate protein was quantitated using the Lowry assay (37) with bovine serum albumin as standard.

RESULTS

Blood Levels

Rat pups were weaned on postnatal day 29 and levels of free and phospholipid Ch in plasma and red blood cells were determined. The animals were then placed on artificial diets, containing defined amounts of Ch of NADe as described in the Method section. Subsequent measurements of the levels of Ch and NADe in plasma and erythrocytes were made at various intervals up to 120 days on the diets (Figs. 1 and 2). For each measurement 30–150 μl blood was taken from the tail vein of randomly selected animals. Immediately after introducing the artificial diet, free Ch in the plasma of control animals rose from 20.1 ± 0.2 μM to a peak of 38.5 ± 1.9 μM on day 10 of the experiment (Fig. 1A). Subsequently, the level declined and final concentration on day 120 was 25.7 ± 1.0 μM. In the Ch-deprived and NADe-treated experimental animals, the level of free Ch fell sharply after start of the diet and reached 4.99 ± 0.19 μM after 120 days. Like free Ch in controls, total free precursor (combined free Ch and NADe) initially increased but fell again later. A maximum of 45.9 ± 3.1

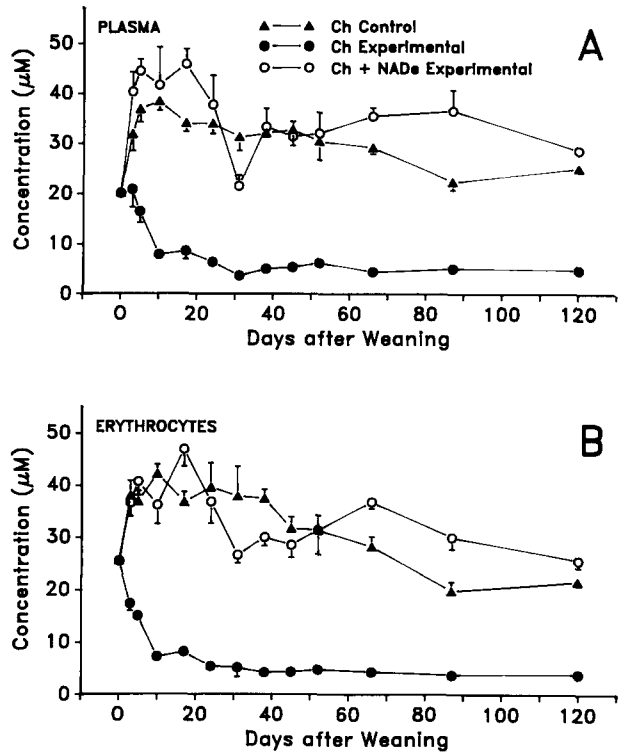

FIG. 1. Free choline (Ch) and N-aminodeanol (NADe) in plasma (A) and erythrocytes (B) during 120 days of dietary administration (35.8 mmol·kg^{-1}) of Ch chloride (control) or NADe chloride (experimental). Values are means; error bars give standard errors. N is between 4 and 10 animals per value. Late in the experiment (days 66, 87 and 120) the combined level of Ch and NADe in experimental animals significantly exceeded the level of Ch in controls ($p<0.05$, Student's t-tests), both in plasma and erythrocytes.

μM was reached at day 17 and the final level at 120 days was 29.4 ± 1.0 μM. A very similar pattern of changes was observed in packed erythrocytes (Fig. 1B). The initial level of free Ch was 25.6 ± 0.9 μM. In controls free Ch reached 42.2 ± 1.8 μM on day 10 and then fell slowly to 21.9 ± 0.9 μM on day 120. Free Ch in experimentals fell rapidly to a low level of less than 5 μM and was 4.68 ± 0.13 μM on day 120, while total free precursor in erythrocytes rose initially to 47.0 ± 3.4 μM on day 17 and then declined to a final concentration of 26.9 ± 1.3 μM. Molar ratios of NADe to Ch at 120 days were calculated to express a measure of the replacement of true by the false cholinergic precursor. This ratio was 4.91 ± 0.22 for plasma and 4.74 ± 0.20 for erythrocytes.

Changes in phospholipid-bound compounds followed a more steady pattern than the changes in free compounds. In plasma (Fig. 2A) initial bound Ch concentration was 2.37 ± 0.07 mM. A similar level was maintained in Ch-fed controls throughout the experiment and at day 120 this concentration was 1.88 ± 0.08 mM. Bound Ch in experimentals dropped sharply to 10–15% of control after only 10 days with a final level of 0.233 ± 0.016 mM on day 120. This drop was considerably faster than the decline in bound Ch in erythrocytes (Fig. 2B) and the time course seemed to parallel or even lead the change in free Ch (see Fig. 1). It should be noted that combined Ch and NADe in experimentals was much less than Ch in controls after only 3 and 10 days. Final combined concentration of the two compounds was 0.617 ± 0.030 mM. In erythrocytes (Fig. 2B), initial bound Ch

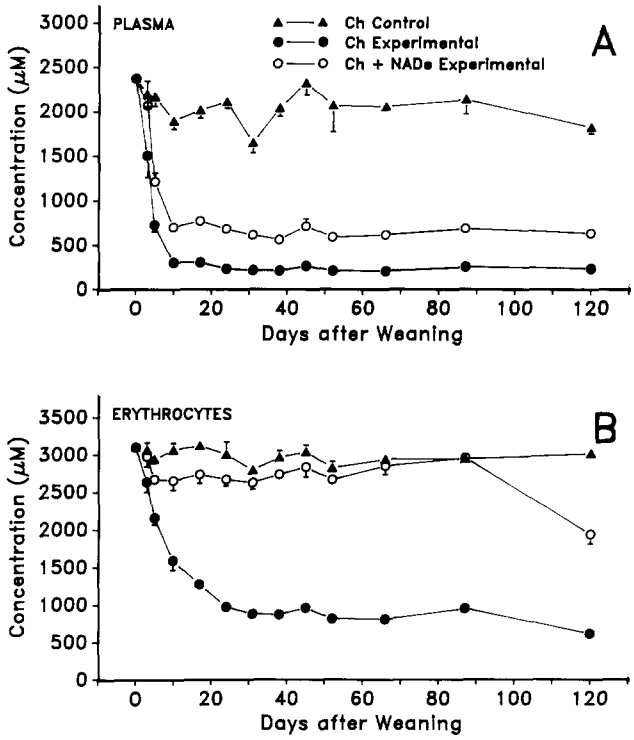

FIG. 2. Phospholipid-bound Ch and NADe in plasma (A) and erythrocytes (B) during 120 days of dietary administration (35.8 mmol·kg^{-1}) of Ch chloride (control) or NADe chloride (experimental). Values are means; error bars give standard errors. N is between 4 and 10 animals per value. For statistics see text.

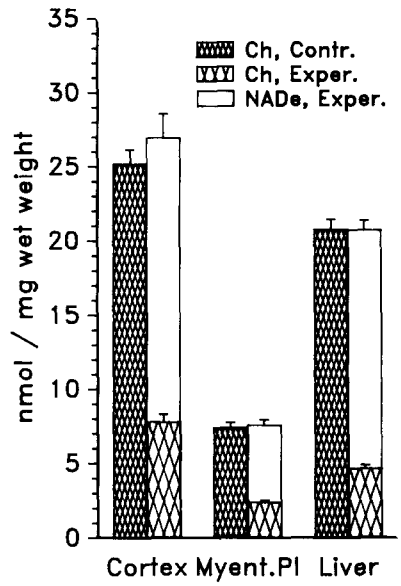

FIG. 3. Phospholipid-bound true (Ch) and false (NADe) precursor in rat tissues after 120 days of dietary administration (35.8 mmol·kg^{-1}) of Ch chloride (control) or NADe chloride (experimental). Data are presented as means and standard errors. Left bar: Ch in controls. Right lower bar: Ch in experimentals. Right upper bar: NADe in experimentals. Upper error bar for experimentals gives standard error of combined NADe+Ch. N = 15–17. Differences between treatment groups (Student's t-test): For Ch all differences were significant at $p<0.001$, for total precursor (Ch+NADe) there were no significant differences.

was 3.10 ± 0.06 mM and, as in plasma, was maintained in controls at a similar level during the entire experiment. In experimentals, bound Ch reached a low plateau after 30 days and the final level was 0.665 ± 0.035 mM. In contrast to plasma, the concentration of combined bound Ch and NADe in experimentals was at most times very similar to the level of bound Ch in controls. Only the measurement at 120 days showed a marked deviation from controls (1.97 ± 0.11 mM). The molar replacement ratio NADe/Ch at 120 days was 1.65 ± 0.08 for plasma and 1.97 ± 0.05 for erythrocytes.

Analysis of variance for levels of combined free precursors in plasma revealed a significant effect of time after onset of the diets, $F(12,158) = 6.14$, $p = 8.5 \times 10^{-9}$, between the levels in the two treatment groups, $F(1,158) = 10.60$, $p = 1.4 \times 10^{-3}$, and for differences in the pattern of the concentration changes [interaction of time and animal group, $F(12,158) = 2.04$, $p = 2.4 \times 10^{-2}$]. For the concentrations in erythrocytes, effects of time on diet, $F(12,159) = 8.21$, $p < 10^{-10}$, and interaction of time and group, $F(12,159) = 3.23$, $p = 3.6 \times 10^{-4}$, were statistically significant. No influence of gender on the levels of free compounds was detected in either plasma or erythrocytes. Analysis of variance of the data for the lipid-bound precursors in plasma, besides showing significant influences of time on diet and treatment group, $F(12,153) = 10.38$, $p < 10^{-10}$ and $F(1,153) = 710.81$, $p < 10^{-10}$, respectively, also revealed an effect of the gender of the animals. Further analysis showed the levels of bound precursors to be higher in females than in males. This was solely due to higher levels of bound NADe and not of bound Ch as might be expected from the reported higher methylation rates of phosphatidylethanolamine in females (5). On the average of days 3 to 120, females exhibited $20.0 \pm 5.6\%$ more bound NADe in plasma than males. No trend with time on diet was detectable.

Tissue Levels

After 120 days the animals were sacrificed. Free Ch and NADe, and where present, their acetate esters ACh and AcNADe were determined in cortex, hippocampus, striatum, heart, myenteric plexus, diaphragm and liver. The phospholipid-bound compounds were measured in cortex, myenteric plexus and liver. Animals to be used for measurement of brain Ch and ACh were sacrificed by microwave irradiation of the head. Others were sacrificed by decapitation in order to preserve enzymes and receptors which were measured as reported below. It is well known that the amount of free Ch in brain rapidly increases after decapitation, whereas the level of ACh decreases (8). The increase in Ch is probably due to phospholipid hydrolysis (33).

The levels of Ch and of ACh were greatly reduced in most tissues after 120 days of the NADe diet (Figs. 3, 4, 5). Compared to the Ch-fed controls the tissue content of phospholipid-bound Ch was reduced to 28.9% in cerebral cortex, 31.9% in myenteric plexus and 22.4% in liver (Fig. 3). Combined bound NADe and Ch in experimental animals matched the levels of bound Ch in control animals remarkably well. This corresponds to the results in erythrocytes for the most part of the experiment. Molar ratios of NADe to Ch as calculated for the precursors in blood were also determined for tissue. This ratio was 2.21 ± 0.13 for myenteric plexus, 2.49 ± 0.10 for cortex and 3.55 ± 0.17 for liver. Repeated measures analysis of variance with Bonferroni analysis ($p<0.05$)

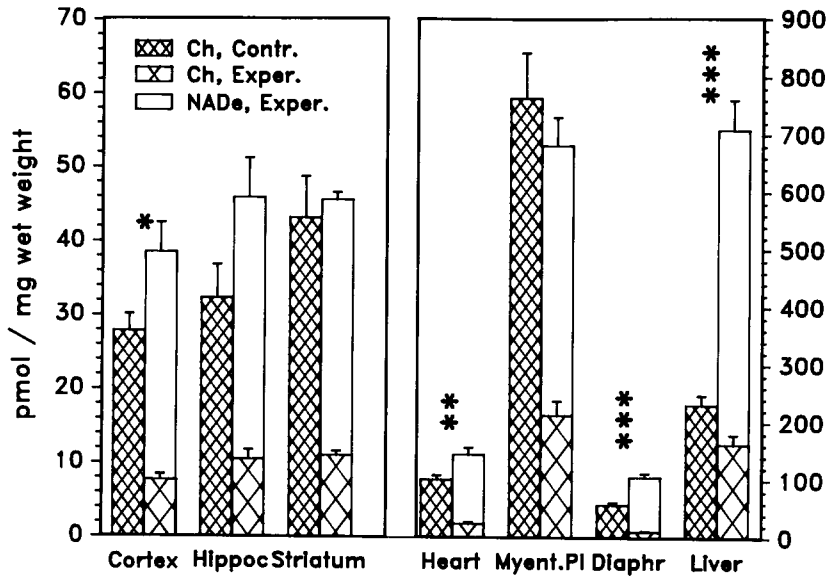

FIG. 4. Free true (choline, Ch) and false (N-aminodeanol, NADe) precursor in rat tissue after 120 days of dietary administration (35.8 mmol·kg^{-1}) of Ch chloride (control) or NADe chloride (experimental). Values are means and standard errors. Left bar: Ch in controls. Bars as in Fig. 3. N: brain areas = 4–5; other tissues = 15–17. Differences between treatment groups (Student's t-test): For Ch all differences were significant at $p<0.001$, for total precursor (Ch+NADe) differences were significant at $*p<0.05$, $**p<0.01$ or $***p<0.001$.

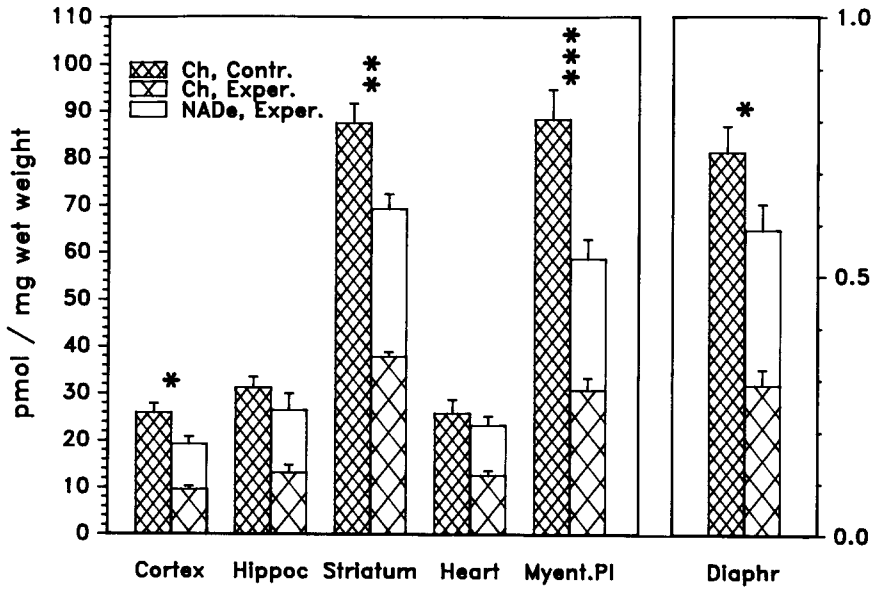

FIG. 5. True and false transmitters acetylcholine (ACh) and acetyl-N-aminodeanol (AcNADe) after 120 days of dietary administration (35.8 mmol·kg^{-1}) of Ch chloride (control) or NADe chloride (experimental). Data are presented as means and standard errors. Left bar: ACh in controls. Right lower bar: ACh in experimentals. Right upper bar: AcNADe in experimentals. Upper error bar for experimentals gives standard error of combined AcNADe+ACh. N: brain areas: N = 4–5; other tissues = 15–17. Numbers inside bars give number of animals. Differences between treatment groups (Student's t-test): For ACh all differences were significant at $p<0.001$, for total transmitter (ACh+AcNADe) differences were significant at $*p<0.05$, $**p<0.01$ or $***p<0.001$.

TABLE 1

MOLAR ACETYLATION RATIO (TRANSMITTER/PRECURSOR) OF CHOLINE (Ach/Ch) AND N-AMINODEANOL (AcNADe/NADe) IN RAT TISSUES AFTER DIETARY ADMINISTRATION OF Ch CHLORIDE (CONTROL) OR NADe CHLORIDE (EXPERIMENTAL) FOR 120 DAYS

	Control		Experimental		
Area	N	Ch/ACh	N	Ch/ACh	NADe/AcNADe
Hippocampus	(4)	1.03 ± 0.15	(4)	1.36 ± 0.11	0.369 ± 0.030
Cortex	(5)	0.90 ± 0.10	(4)	1.23 ± 0.19	0.289 ± 0.020
Striatum	(5)	2.08 ± 0.26	(4)	3.40 ± 0.18	0.860 ± 0.048
Heart	(16)	0.26 ± 0.03	(15)	0.44 ± 0.04	0.099 ± 0.010
Myent. Plexus	(16)	0.13 ± 0.01	(15)	0.15 ± 0.01	0.062 ± 0.004
Diaphragm	(17)	0.014 ± 0.001	(15)	0.018 ± 0.002	0.0036 ± 0.0003

Data are expressed as mean ± standard error.

showed the ratio in liver to be significantly higher than in cortex and myenteric plexus.

Free Ch was reduced in most areas to approximately 30% of controls (Fig. 4). Only the liver with 82.4% of control still exhibited a level of free Ch which was comparable to the Ch-fed rats, but the difference from controls was statistically significant ($p<0.01$; Student's t-test). The total amount of free precursors in experimental animals numerically exceeded the corresponding level of Ch in controls in all but one tissue (myenteric plexus). The difference was statistically significant for cortex, heart, diaphragm and liver. In the liver of NADe-fed animals total free precursor exceeded the level of Ch in controls by more than 200% (Fig. 4). This finding confirms an earlier similar observation with deuterium labeled NADe (44). The molar replacement ratio NADe/Ch was also calculated. Separate analysis of variance with Bonferroni analysis ($p<0.05$) for the brain areas and the other areas proved the ratio to be higher in cortex (4.02 ± 0.17) than in hippocampus (3.40 ± 0.21) and striatum (3.12 ± 0.21) and higher in diaphragm (5.17 ± 0.30) and lower in myenteric plexus (2.35 ± 0.19) than in liver (3.39 ± 0.20) and heart (3.82 ± 0.20).

The concentration of ACh in experimentals in all examined cholinergic tissues was reduced to about 40% of controls (Fig. 5). The degree of this reduction was always less than for the precursor in the same tissues (see Fig. 4). However, the total amount of true and false transmitter in experimental animals was always less than the amount of ACh in Ch-fed controls (Fig. 4). The molar ratio of AcNADe to ACh was in all tissues close to 1. Separate statistical analysis for brain and other areas showed significant differences between tissues only for the areas outside the brain, where the ratio in heart (0.85 ± 0.06) was significantly lower than in diaphragm (1.10 ± 0.09).

Molar ratios between cholinergic transmitter and precursor levels were calculated in Table 1 to express the extent of acetylation of the precursor. The results show that in the NADe-fed animals the degree of acetylation of Ch is about four times higher in most tissues than the degree of acetylation of NADe. The mean of all tissues is 3.82 ± 0.30. With the possible exception of diaphragm, acetylation of Ch is higher in NADe-treated animals than in the Ch-fed controls. It is also apparent from Table 1 that the proportion of Ch that is acetylated is very different in the various tissues. The ratio in striatum is about twice the ratio in the other brain areas. Heart and myenteric plexus have lower ratios, and diaphragm is by far the lowest.

ChAT, ChE and Muscarinic Receptor Density

The activities of the cholinergic marker enzymes ChAT and AChE were determined after 120 days on the experimental and control diets in cortex, hippocampus, striatum, heart and myenteric plexus. QNB binding was measured in the same areas in order to assess muscarinic receptor density. The results are given in Figs. 6 and 7. Although the levels of ChAT activity and QNB binding were generally somewhat lower in experimentals than controls, they were surprisingly similar in the two animal groups, despite the prolonged Ch deprivation of the experimental animals. A decrease of ChAT activity to 81.4% of control was observed in striatum, $t(16) = 3.625$, $p = 2.3 \times 10^{-3}$, and to 80.1% in myenteric plexus, $t(31) = 2.134$, $p = 4.1 \times 10^{-2}$. No significant changes were seen in cortex, hippocampus, or heart. QNB binding was significantly reduced in hippocampus, striatum and myenteric plexus ($p<0.05$; Student's t-test), but not in cortex and heart. The largest relative decrease was measured in myenteric plexus with 73.1% of control receptor density remaining in the experimental group.

There were no significant differences in whole brain AChE levels at any of the times measured. The only significant regional difference was found in the striatum, AChE activity in NADe animals being less than in the Ch group [87.7 ± 5.4 percent, $t(17) = 2.28$, $p = 3.6 \times 10^{-2}$]. Ileal smooth muscle activity levels were significantly higher in the NADe animals [140 ± 17 percent, $t(17) = 2.34$, $p = 3.2 \times 10^{-2}$], but cardiac AChE in the NADe group was lower than in the control [73.0 ± 5.1 percent; $t(32) = 5.20$, $p = 1.1 \times 10^{-5}$]. AChE activity, measured in erythrocytes at four time points, showed a significant treatment difference, $F(1,72) = 4.61$, $p = 3.6 \times 10^{-2}$]. This result appears to be due to a higher level of activity in the NADe animals at 30 days, the only significant difference at any of the time points. Plasma BChE activity showed significant differences in all three basic parameters: treatment, $F(1,59) = 8.42$, $p = 5.6 \times 10^{-3}$; time on diet, $F(3,59) = 30.12$, $p = 1.0 \times 10^{-9}$; and gender, $F(1,59) = 48.62$, $p = 3.0 \times 10^{-9}$. In both treatment groups, plasma BChE activity levels were always higher in females than in male animals, the difference increasing with time on the diets [gender × time, $F(3,59) = 11.31$, $p = 7.3 \times 10^{-6}$]. Differences between treatment groups also increased with time on the diets [treatment × time, $F(3,59) = 4.99$, $p = 3.8 \times 10^{-3}$].

DISCUSSION

Our results show that replacing Ch in the diet of young rats by the synthetic Ch isostere NADe leads to a significant reduction of free Ch, phospholipid-bound Ch and ACh in tissues and blood and to their replacement, in large part, by NADe, phospholipid-bound NADe and AcNADe. The degree of replacement, ex-

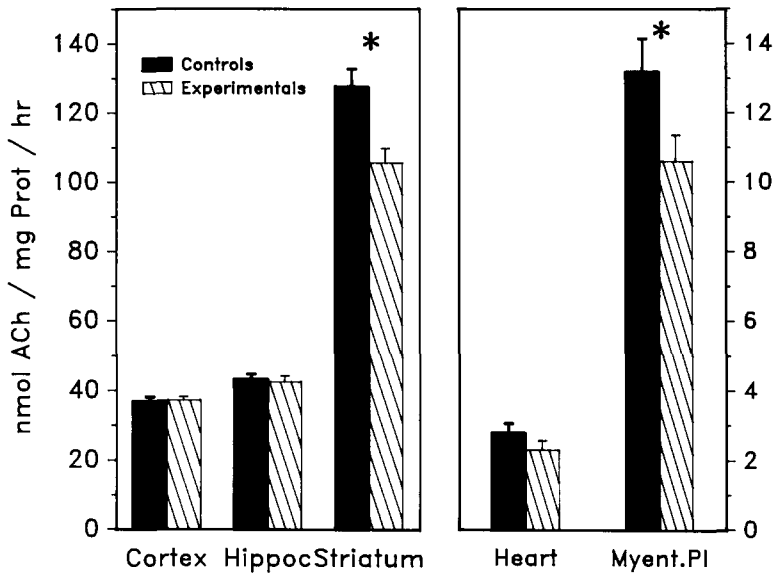

FIG. 6. Activity of choline acetyltransferase (ChAT) in rat tissues after 120 days of dietary administration (35.8 mmol·kg^{-1}) of Ch chloride (control: left bars) or NADe chloride (experimental: right bars). Data are presented as means and standard errors. N: brain areas = 8; other tissues = 15. Differences between treatment groups (Student's *t*-test) $*p<0.05$, $**p<0.01$.

pressed as the ratio of NADe to Ch, varied in different compartments of the body. In particular, it decreased from free precursor in blood to precursor and transmitter in tissues. The levels of total free precursor (combined free NADe and Ch in experimental animals, free Ch in control animals) in plasma and erythrocytes followed a distinct pattern and did not appear to have reached stable levels even after 120 days on the diets. In both animal groups, experimentals and controls, an initial sharp increase in concentration was observed. Zeisel and Wurtman (69) reported stable levels of plasma Ch of about 10 μM Ch in rat pups at 20 days of age. However, it is well known that the amount of Ch ingested bears a direct relationship to its plasma concentration in man and animals (26–28). The sharp increase of free precursor in blood after weaning observed in the present study could be due

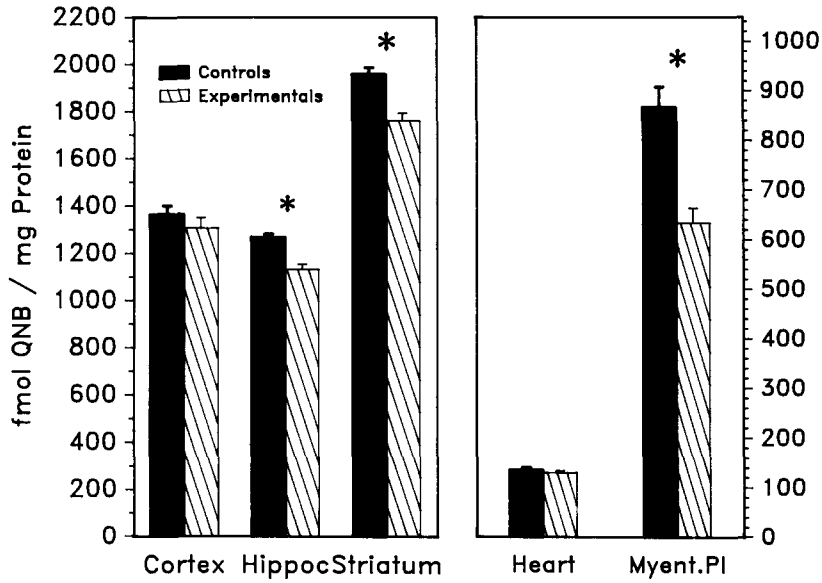

FIG. 7. QNB binding in rat tissues after 120 days of dietary administration (35.8 mmol·kg^{-1}) of Ch chloride (control: left bars) or NADe chloride (experimental: right bars). Values are means and standard errors. N: brain areas = 8; other areas = 15. Differences between treatment groups (Student's *t*-test): $***p<0.001$.

to the relatively high dietary content of 35.8 mmol·kg^{-1} of Ch or NADe. The Ch concentration in normal lab chow is about 6 mmol·kg^{-1} and Zeisel (68) and Wurtman (64) give values for human and cow's milk of less than 0.6 mmol·kg^{-1} of total (free and bound) Ch. It has been shown recently (54) that the uptake kinetics of intestinal tissue slices for free Ch are about the same in neonatal and adult rats. The amount of food consumed relative to body weight decreased with age, which could account for a gradual decrease in blood Ch levels, as seen in control animals after 10 days on diet. In the experimental animals such a simple relationship could hardly explain the rate of the decrease after the initial peak and is not consistent with the subsequent concentration pattern. It must be concluded that in these animals adaptive changes in the Ch metabolism occur still later than 1 month after start of the experimental diet.

The levels of total phospholipid-bound precursors in erythrocytes were similar in both animal groups and remained relatively stable. In controls bound Ch was also remarkably constant in plasma. In the experimental group however the plasma level of total bound precursor (Ch+NADe) fell very quickly after the diet was begun. This suggests that the hepatic secretion of bound precursors into the bile is drastically reduced in the NADe group. Secretion of phospholipid-bound Ch by the liver into the bile and subsequent transport to the intestines, where it is reabsorbed (11,20) is the most likely source of phospholipid-bound Ch in the bloodstream of our animals since their food (control and experimental) was completely devoid of bound Ch.

De Novo Synthesis of Ch?

It is of interest to consider the origin of the Ch remaining in the animals after living on a virtually Ch free diet (less than 14 μmol·kg^{-1} diet) for 120 days. Two sources have to be considered: Ch already present in the body at the time of weaning, and de novo synthesis of Ch by rat tissues or intestinal microflora. Ch can be synthesized in animal tissues by sequential methylation of phosphatidylethanolamine to phosphatidylcholine [see (47) for review]. This methylation activity is present in most animal tissues, but is highest in liver (47,69). Yost et al. (67) showed that in the lung of Ch-deficient rats this methylation pathway is stimulated. There can be no doubt that some Ch synthesis took place in our animals, although the low methionine content of the diet minimized this route. It has been shown that in rats deuterium-labeled methyl groups of dietary NADe appear to a minor extent in Ch (44). It is not known whether NADe is deaminated and methylated after incorporation into a phosphatide. In any event, no major synthesis of Ch has to be postulated to explain the level of Ch remaining in the experimental animals after 120 days on a Ch-free diet. The amount of phospholipid-bound Ch, even in cholinergic tissues, is many times higher than free Ch and ACh combined (Figs. 3 to 5). Free Ch therefore can be neglected for a rough estimate of total Ch in the organism. In an earlier study in our laboratory (44) animals were sacrificed after 33 days on a similar diet. At the time of sacrifice the molar Ch fraction of total bound precursor (Ch+NADe) was 0.433 ± 0.006 in cortex and 0.162 ± 0.006 in liver. In our study it was after 120 days 0.289 ± 0.007 in cortex and 0.224 ± 0.008 in liver. Unfortunately, myenteric plexus was not measured in the earlier study, but after 120 days the Ch fraction was 0.319 ± 0.012, similar to the Ch fraction in cortex. The values for cortex (and myenteric plexus) are therefore assumed to be representative of the majority of the tissues. The liver, as evident from our results, is likely to be an exception among the tissues with regard to its Ch metabolism. The data from erythrocytes (Fig. 2) suggest that the tissue levels of combined bound Ch and NADe do not change substantially during prolonged NADe feeding. Using the choline fraction in cortex and taking into account the mean body weights of 80, 155 and 315 grams at days 0, 33 and 120, respectively (data not shown), and setting the amount of Ch at day 0 to 100% it can be calculated that the amount of Ch at day 33 was approximately 84% and at day 120 114% of the original amount on day 0. This means that the Ch which was present at weaning could largely account for the Ch which was still measured after 120 days of the Ch-free, NADe-substituted diet, despite a four-fold increase in body weight. The finding that the ratio between Ch dehydrogenase and Ch kinase activity in liver cells can be shifted several-fold under certain experimental conditions (60) and that Ch deficiency reduces the hepatic concentration of betaine by 98% after only 2 days (59) make it seem likely that Ch is well conserved when it is in short supply. The amount of Ch excreted by the animals on the NADe diet is likely to be minimal. In urine after 120 days on the NADe diet it was approximately 40 nmol·day^{-1} (data not shown). It seems possible that more prolonged replacement of dietary Ch with NADe will lead to further replacement of Ch in various functional compartments including phospholipids and ACh.

Source of Ch and NADe in the Brain

It is generally believed that the brain derives a substantial part of its Ch from phospholipid-bound Ch, taken up from the blood (2,58). Lysophosphatidylcholine has been shown in squirrel monkey to cross the blood-brain barrier as an intact molecule (29). Enzyme systems which are capable of liberating Ch from its bound form are present in the brain (25, 30, 57, 68) and seem to be influenced by muscarinic stimulation (7). Jenden and co-workers (9) calculated that in the rat, sources within the brain account for up to 93% of free Ch in brain. However, there is also a saturable carrier system that transports free Ch from blood into the brain (12). We have shown that the molar ratio of free NADe to Ch in brain lies between the molar ratio of the free compounds in blood and the ratio of the bound compounds in both blood and brain. This suggests that both free and bound compounds in plasma contribute to the pool of free compounds in brain. It is also consistent with the assumption that bound Ch serves as the major source for the free Ch in brain. There is a net loss of Ch from the brain in the intact animal as demonstrated for several species, including rat and man (2). It is likely that relatively more Ch than NADe is lost from brain to blood because of more effective enzymatic release of Ch or its more efficient transport into blood. The high molar ratio of NADe to Ch in blood is easily explained by a preferential removal of Ch from the blood, mainly by the liver (6,70) and kidney (1). Thus, the high levels of residual Ch and of NADe in liver after 120 days of Ch-free diet may be due to increased uptake into the liver in the NADe-fed animals. NADe has been shown to be a comparable, but quantitatively poorer substrate than Ch for various metabolic steps (41,44).

Levels of Precursors and Transmitters in Tissues

It seems likely that the generally higher tissue levels of total free precursors in experimentals than in controls could be explained by the higher plasma levels observed after prolonged NADe substitution. In animals on the NADe replacement diet for 120 days, free Ch in brain areas was reduced to about 30% of controls, while ACh was reduced only to about 40% (Figs. 4 and 5). Ch in the brain of the experimental animals therefore is acetylated more effectively than in controls. This is also evident in the ratios of transmitter to precursor in experimentals and controls (Table 1). ChAT activity was not substantially different in the ex-

perimental group, and ChAT is in any case not believed to be a rate limiting factor for ACh synthesis. Other explanations for increased acetylation of the available Ch in NADe-fed and Ch-deficient animals are possible. Most of the Ch that is acetylated has entered cholinergic terminals through the high-affinity transport system. It is possible that this system is upregulated in a prolonged state of cholinergic insufficiency. Alternatively, an increased acetylation ratio could mean that the mole fraction of free Ch which is available for acetylation is higher than the mole fraction that is measured in total brain areas. The molar ratios between cholinergic transmitter and precursor levels (Table 1) show that in the NADe-fed animals the degree of acetylation of Ch is about four times higher in most tissues than the degree of acetylation of NADe. The mean ratio of all tissues is 3.82 ± 0.30. This agrees well with the calculated ratio of 4.33 for the availability of the two precursors for acetylation as determined from their respective high-affinity transport kinetics in synaptosomes (41). However, the increased acetylation ratio for Ch and the higher level of total precursors were not sufficient to compensate for the poor acetylation ratio for NADe in the experimental group, so that the level of total transmitter was always lower in experimental animals than in controls.

Functional Replacement of Ch by NADe

At muscarinic receptors, AcNADe is 4% as effective as ACh (45). Since the total level of true and false transmitter is less after NADe substitution, a significant muscarinic hypofunction can be expected. Behavioral effects of the Ch-free, NADe-substituted diet have indeed been found (32,46) and are detailed further in the following paper (53). Further studies are required to determine whether the NADe regimen leads to other changes in central cholinergic neurons than simply a disruption of cholinergic transmission due to a reduction of active transmitter. We will report later a study of the reversibility of the observed neurochemical and behavioral deficits after reintroducing Ch in the diet of Ch-deprived animals.

Our measurements of the activity of the cholinergic marker enzyme ChAT in the brain of experimental animals showed a moderate but statistically significant decrease in striatum but not in cortex and hippocampus. In Alzheimer's disease a loss of ChAT-activity in cortex and hippocampus has consistently been reported [for reviews see (3); see also (24)]. A profound reduction of ChAT in cortex and hippocampus has also been observed after experimental lesion of the basal forebrain cholinergic system [for review see (56)]. Our finding is difficult to reconcile with an assumption that our experimental regimen led to a major loss of cholinergic neurons. The striatum contains cholinergic local interneurons and therefore cholinergic cell bodies and terminals. In contrast, cortex and hippocampus are cholinergically innervated by distant cells located in the basal forebrain. However, there is no obvious reason why ChAT should be more affected in cell bodies than in terminals. In myenteric plexus a significant decrease of ChAT activity was detected, but a smaller decrease was found in heart. This finding confounds the picture, given the presence in both tissues of cholinergic parasympathetic cell bodies and terminals.

QNB binding under conditions which were used in our experiments reflects the number of muscarinic receptors. We found a decrease in binding in hippocampus, striatum and myenteric plexus. Levels in cortex and heart were not significantly changed. Changes in muscarinic receptors in Alzheimer's disease are less well established than changes in enzyme activities. Mild decreases or no changes are reported (34, 36, 50, 51). A recent study found only modest decreases in M_1 and M_2 muscarinic receptor sites despite substantial decreases in the activities of ChAT and AChE (55). In rat experiments lesions of the nucleus basalis magnocellularis led to a marked reduction in the levels of cholinergic enzyme markers by 42% in neocortex but only minor decreases of 13% and 14% in total and M_1 muscarinic receptors (62). In animals it has also been shown that the number of muscarinic receptors can be altered by pharmacological intervention. Chronic administration of cholinesterase inhibitors leads to a decrease in muscarinic receptors (14, 16, 22) and application of muscarinic agents also results in adaptive changes in receptor number (4, 40, 63). Results reported for ibotenic acid or electrolytic lesion of the nucleus basalis are controversial. McKinney and Coyle (38) reported an acute decrease in muscarinic receptor number after the lesion but a later return to control levels. De Belleroche et al. (15) found a moderate decrease in receptor number in cortex, while Westlind et al. (63) observed an increase in muscarinic receptor number in hippocampus. Our finding of a moderate decrease of receptor number after NADe treatment is difficult to interpret. If NADe treatment leads to a loss of cholinergic neurons in the brain, a decrease in presynaptic muscarinic receptors would be expected, but even a complete loss of cholinergic neurons would probably not result in a loss of more than 25% of the receptors (49). Based on results with pharmacological manipulation of the cholinergic system (see above) an increase in the number of postsynaptic muscarinic receptors in our study could be expected because of the limited availability of ACh. Interestingly, however, chronic scopolamine treatment leads to muscarinic receptor upregulation only in intact but not in nucleus basalis lesioned rats (61). Alternatively, although AcNADe is a very weak partial agonist, it could be speculated that it is able to prevent postsynaptic upregulation of receptor number. Further studies are required to resolve these questions.

We conclude from our results that prolonged Ch deprivation and NADe administration in rats leads to a significant hypofunction of muscarinic cholinergic neurotransmission. The following paper (53) shows that the observed biochemical cholinergic changes are accompanied by physiological and behavioral changes known to be associated with cholinergic hypofunction. However, while the reduction of Ch in most compartments of the organism was severe, our treatment most likely did not result in a major loss of central cholinergic neurons. The levels of cholinergic precursor after 120 days on the diet were still declining, and it is possible that longer treatment of the animals will lead to further cholinergic impairment. More prolonged studies are under way.

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