Pharmacology Biochemistry and Behavior, Vol. 44, pp. 587-594, 1993
Printed in the U.S.A.

0091-3057/93 $6.00 + .00

Action of Chronic Choline Administration on Behavior and on Cholinergic and Noradrenergic Systems

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Received 6 March 1992

MOLINENGO, L., M. ORSETTI, P. GHI AND B. PASTORELLO. *Action of chronic choline administration on behavior and on cholinergic and noradrenergic systems.* PHARMACOL BIOCHEM BEHAV 44(3) 587-594, 1993.—Chronic administration of low doses (0.2–0.8 g/kg/day) of choline caused in the rat an increase of errors evaluated in the staircase maze after 20 days of interruption of daily training. An analogous pharmacological treatment caused no modification of the acetylcholine (ACh) and norepinephrine (NE) levels and no consistent modification of the density of muscarinic and α_1-adrenergic receptors. Only at higher doses (2.5 g/kg/day) did chronic administration (20 days) of choline cause in several sections of the CNS, an increase of ACh and NE levels and of the muscarinic receptor density. These observations indicate that only at high doses of choline are there consistent modifications of the central cholinergic systems, suggesting that the behavioral modifications observed at low doses of choline are not determined by an upregulation of the central cholinergic system.

Choline Chronic administration Staircase maze Cholinergic system Noradrenergic system

A number of attempts have been made to improve cognition in patients with suspected Alzheimer's disease by administration of choline or lecithin with inconclusive but in general negative results (18,37).

A strong rationale for testing choline in patients with memory deficits lays in the numerous pieces of evidence that suggest that cholinergic function is related to memory (1,4,12,36) and that administration of the acetylcholine (ACh) precursors (10,24) increases ACh concentration in the brain. In contrast, several reports (14,32,42,43) indicated that administration of choline does not alter the concentration of ACh in the brain.

However, choline does modify the responsiveness of central cholinergic neurons to pharmacological manipulations (38,40) and may be used for the synthesis of ACh under conditions of increased neural demand (44).

There is also evidence that the supposed cholinergic action caused by choline administration causes behavioral modifications in the animal (20). Fundar'o and Paschero (21) studied in adult and senescent rats the effect of chronic administration of choline on operant conditioning behavior. Bartus et al. (3) reported that the dramatic decrease in retention observed in senescent mice was completely missing in animals treated with choline, but choline administration does not improve memory in aged cebus monkeys (2) or passive avoidance behavior in old rats (4).

No modification of discriminating learning was observed by Beninger et al. (6) after 31 weeks of choline administration. Prado-Alcal'a et al. (34) reported improvement or deficits of a learned behavior according to the dose of choline applied directly into the caudate nucleus.

It may also be noted that central (tremors, hypokinesia, rigidity) or peripheral (salivation, defecation) cholinergic effects have never been reported after choline administration, and the hyperactivity observed by Wecker and Schmidt (41) after chronic administration of choline is in general associated with antimuscarinic agents.

There is also evidence that choline intake interferes with various neurochemical systems: Miller et al. (28) reported that choline modulates benzodiazepine receptor binding and GABA receptor activity. Moreover, there is evidence that muscarinic agonists evoke norepinephrine (NE) release in cortical slices (13,26) and a prejunctional inhibition of NE release at the periphery (19). These data suggest that the behavioral modifications caused by choline administration may be associated, through modifications of cholinergic systems, with various neurochemical effects.

In the present article, we examined whether the modifications of the rat behavior in the staircase maze test (8,9) caused by choline are associated to modifications of the ACh levels and muscarinic receptor density. The possible role played by catecholaminergic systems in determining the behavioral effects was also examined, evaluating the levels of NE and the

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density of α_1-adrenoceptors in different areas of the rat's CNS.

METHOD

Subjects

Male, albino rats (Morini, Wistar-derived strain) of 150–200 g were used. They were housed five per cage and fed ad lib with a standard rodent diet except when otherwise stated. They had free access to tapwater.

Sections of the CNS Examined

The following sections were selected to evaluate ACh and NE levels and the density of muscarinic and α_1-adrenergic receptors: 1) the frontal and parietal cortex (cortex); 2) the olfactory bulbs with the cortex piriformis and tuberculum olfactorium (olfactory system); 3) the hippocampus; 4) the remaining brain (subcortex) after the cerebellum, pons, medulla oblongata, and residual parts of the cerebral cortex have been removed and discarded.

Staircase Maze

The staircase maze, described in previous research (8,9), was used.

A staircase of 13 steps with a corridor 17 cm long in the vertical wall was employed. Twenty rats were used. They were kept without food from 6:00 p.m.–12:00 a.m. and trained every morning to find food pellets (45 mg, Campden Instruments Ltd.) in the corridor corresponding to the 3rd, 6th, 9th, and 12th steps.

After 3 months of preliminary training, all rats ran rapidly onto the staircase and stopped only at the four reinforced steps. Once this training was completed, a trial without reinforcement was performed on the staircase.

In this trial (pretesting trial), the search for food on the 3rd, 6th, 9th, and 12th steps was considered a correct response and the search for food on any other steps an error. Then, daily training was interrupted for 20 days and a new trial with no reinforcement (testing trial) was performed. Therefore, the experimental scheme was: 1) 3 months of preliminary training; 2) pretesting trial; 3) no training and choline administration (20 days); 4) testing trial. Correct responses and errors were counted in the pretesting and testing trails. As the staircase was composed of 13 steps, the rat had the possibility in each trial of making four correct responses (the 3rd, 6th, 9th, and 12th steps) and nine errors.

The ratios (correct responses/total responses) × 100 were calculated from the experimental data obtained in the pretesting and testing trials.

Determination of ACh Levels

Forty rats of the same strain and weight as rats used in the behavioral test were used. They were killed by microwave irradiation of the head (2,450 mHz, 1.5 kw, 3.0 s). The skull was opened and the brain frozen (−30°C). The four sections of the CNS were collected and weighed.

The tissue was homogenized in a Polytron at 20,000 rpm for about 3 s in 2.0 ml boiling McIlwaine's citric disodium phosphate buffer (pH 4.0; 0.014 M). The tissue extracts after homogenization were kept for 30 s in boiling water, then transferred to ice-cold water, and diluted with equal volumes of frog-Ringer's solution containing eserine hemisulfate (20 μg/ml) and double-salt concentration to obtain an isotonic medium (5).

The extracts so obtained were centrifuged (1,000× *g* for 30 min). The supernatant was collected for the bioassay of ACh on the rectus abdominis muscle of the frog. The concentrations of ACh are given in μg/g fresh tissue.

Determination of NE Levels

Eighteen rats of the same strain and weight as rats used in the other experiments were killed by decapitation. The four brain areas were dissected. The tissue (80 mg tissue in 300 μl) was ruptured by sonication in ice-cold perchloric acid 0.1 M, containing EDTA 0.1% and sodium metabisulfite 0.05%. The samples were centrifuged at 50,000× *g* for 30 min at 4°C. The supernatant was purified on acid-washed alumina according to the method of Ehrenstrom and Johansson (17).

The NE concentration in the samples was evaluated according to the method of Keller et al. (25), which utilizes high-performance liquid chromatography (HPLC) with electrochemical detection.

The mobile-phase composition used was: 92 ml of a solution containing cytric acid 1.0 mM (pH 3.45), Na_2HPO_4 0.1 mM, EDTA 0.1 mM, heptansulfonic acid 1.0 mM (pH 3.45), and 8 ml acetonitrile.

The flow rate was 0.8 ml/min and the potential was +0.70 V. The obtained peaks were automatically integrated by the data module and the evaluation of the NE concentrations was made using external standards. The concentrations of NE (free base) are given in ng/g fresh tissue.

The instrumentation used was composed by a column μBondapak C18 (Waters Assoc., Italy), a pump (Waters 510), an electrochemical detector (Waters 460), and a data module (Waters 740).

Muscarinic Receptor Binding

[^{3}H]Quinucliolinyl benzylare ([^{3}H]QNB) (39 Ci/mM, Amersham Corp., Arlington Heights, IL) binding to rat brain membranes was performed according to the method of Yamamura and Snyder (45). Twenty rats of the same strain and weight as rats used in the other experiments were used. Animals were killed by decapitation and brains quickly removed. The four brain areas were dissected out and homogenized in 10 ml ice-cold 0.32 M sucrose solution using a Potter-Elvehjem Teflon-glass homogenizer. The homogenates were then centrifuged at 1,000× *g* for 20 min and the resulting pellets discarded. Protein concentration in the supernatant fluid was determined according to the method described by Lowry et al. (27) using bovine serum albumin as a standard.

Aliquots of supernatant (25–50 μl; 0.2 mg protein) were incubated in triplicate with increasing concentrations of [^{3}H]QNB (0.05–2.0 nM) for 60 min at 25°C in Na-K phosphate buffer, pH 7.4. Incubations with atropine 1 μM were included to obtain unspecific binding.

The reaction was stopped by addition of 3 ml ice-cold phosphate buffer followed by filtration under reduced pressure over presoaked Whatman GF/B glass fiber filters. Filters were washed three times with 5 ml ice-cold buffer. Radioactivity was determined by allowing the dried filters to stand overnight in 5 ml Beckman Ready-Gel® scintillator (Beckman Instruments, Fullerton, CA) followed by liquid scintillation counting (Beckman LS-3801) at 40% efficiency. B_{max} and K_d values were estimated by Scatchard analysis and given in fmol/mg protein and pM, respectively.

α_1-Adrenoceptors Binding

[^{3}H]Prazosin (85 Ci/mM, Amersham) binding to rat brain membranes was performed according to the method of Glossman and Hornung (23).

Twenty rats of the same strain and weight as rats used in the other experiments were killed by decapitation. The different brain areas were quickly removed and homogenized with a Potter-Elvehjem Teflon-glass homogenizer in ice-cold 50 mM Tris-HCl and 1 mM EDTA, pH 7.4, buffer.

Homogenates were centrifuged at 4°C for 15 min at 48,000× *g*. The final pellet was resuspended in ice-cold Tris-HCl buffer (pH 7.4). Protein concentration of resuspended pellets was determined according to the method described by Lowry et al. (27) using bovine serum albumin as a standard.

Tubes containing [^{3}H]prazosin (0.5–2.5 nM) and an aliquot of resuspended tissue, corresponding to 1.5 mg protein, were incubated in triplicate (final volume 250 μl). After 15 min of incubation at 37°C, samples were diluted with 3 ml ice-cold Tris-HCl buffer and rapidly filtered through presoaked Whatman GF/B filters.

The filters were rinsed with three 5-ml washes of ice-cold Tris-HCl buffer. They were placed in vials containing 5 ml liquid of scintillation (Beckman Ready-Gel) and counted in a Beckman LS 3801 liquid scintillation counter (efficiency 40%). Specific binding was defined as the excess over blanks containing 1 μM unlabeled prazosin.

The value of B_{max} (fmol/mg protein) and K_d (nM) were estimated by Scatchard analysis.

Pharmacological Treatment

Rats of the control groups drank tapwater. Choline-enriched groups drank water in which choline chloride 1.5 and 5.5 g/l was dissolved.

Based upon the mean body weight (200–250 g) during the experiments and the mean fluid consumption (30 ml/day/rat), the doses of choline were evaluated. These values are given in Table 1 and in the figures. To avoid toxic peripheral effects that may interfere with the rat's performance, higher doses of choline were not used in the behavioral test. A dose of 2.5 g/kg/day of choline was given only to rats used for neurochemical measurements to confirm at a higher dosage neurochemical modifications of limited significance. Intragastric intubation was used to supply this dose of choline: Rats refused to drink water at a high choline concentration.

Administration of choline lasted 20 days.

RESULTS

Staircase Maze

In Table 1, the means and SEs of the total number of responses (arrests on any step) and the percent of correct responses (arrest on the 3rd, 6th, 9th, and 12th steps) found in pretesting and after 20 days of interruption of the daily training (testing trial) are given for controls and rats treated with choline 0.22 and 0.82 g/kg/day for 20 days. Analysis of variance (ANOVA) indicates that choline administration caused no significant modification of the total number of responses given by the rat in the testing trial, $F(2, 17) = 0.93, p > 0.10$. ANOVA applied to the percent of correct responses given by the rat in the testing trial indicates that the modifications caused by the pharmacological treatment are significant, $F(2, 17) = 4.65, p < 0.5$–0.01.

Dunnett's test for the comparison with control indicates that the reduction of the percentages of correct responses after 20 days of interruption of the daily training and choline administration was significant at 0.82 g/kg/day choline ($p < 0.5$).

ACh Levels

The levels of ACh (μg/g fresh tissue) found in the sections of the CNS examined after 20 days of administration of 0.25, 0.80, and 2.5 g/kg/day choline are given in Fig. 1. The levels of ACh found in controls (cortex 3.25 ± 0.34 μg/g; hippocampus 4.3 ± 0.42 μg/g; olfactory system 4.55 ± 0.34 μg/g; subcortex 7.45 ± 0.54 μg/g) are in the range of the values given by other authors (11,31).

ANOVA indicates that the differences between the sections examined are significant, $F(3, 153) = 50.78, p < 0.01$. Choline administration caused a significant modification of ACh levels, $F(3, 153) = 8.60, p < 0.01$. Dunnett's test ($p < 0.5$) for comparison with a control indicates (Fig. 1) that only 2.5 g/kg/day choline caused an increase of ACh levels in the cortex, hippocampus, and subcortex.

TABLE 1

EFFECTS OF CHRONIC CHOLINE ADMINISTRATION (20 DAYS) ON THE STAIRCASE MAZE TEST

	Pretesting Trial		Testing Trial	
	Total Responses	% Correct Responses	Total Responses	% Correct Responses
Controls (7)	4 ± 0	100 ± 0	4.71 ± 0.36	80.28 ± 7.70
Choline 0.22 g/kg (7)	4 ± 0	100 ± 0	5.28 ± 0.64	54.75 ± 8.14
Choline 0.82 g/kg (6)	4 ± 0	100 ± 0	6.00 ± 0.93	43.33 ± 10.93*

The number of rats is reported in parentheses. Mean ± SEM of the total number of responses (arrests on any step) and of the percent of correct responses (arrests on 3rd, 6th, 9th, and 12th steps) found in pretesting trials and after 20 days of interruption of the daily training (testing trial) are given for controls and for rats treated with the given doses of choline.

*Probability of a casual result is less than 0.5 (Dunnett's test).

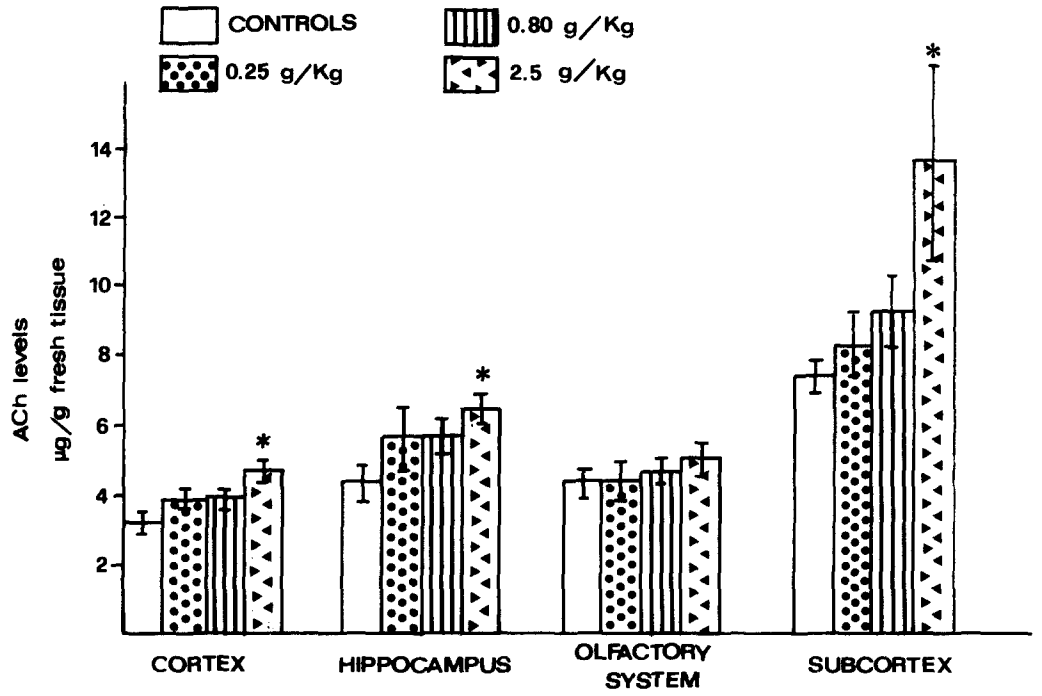

FIG. 1. Levels of acetylcholine (ACh) (μg/g fresh tissue) found in sections of the CNS after chronic administration of choline 0.25, 0.8, and 2.5 g/kg day. Shown are mean values ± SEM of at least nine rats per point. *The probability of a difference to controls (Dunnett's test) is <0.5.

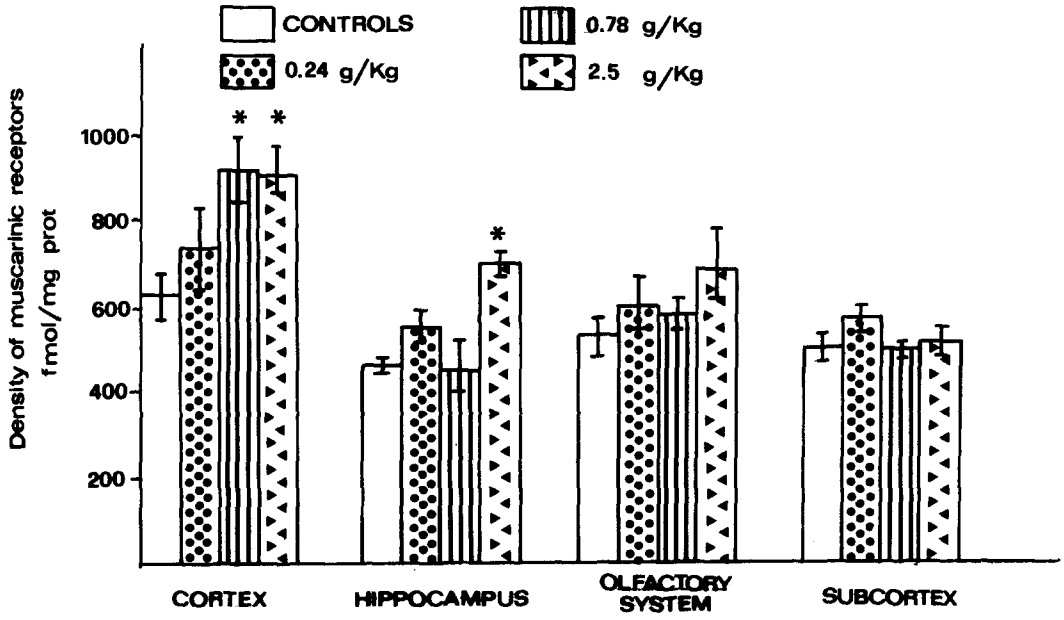

FIG. 2. Density of muscarinic receptors (B_{max} in fmol/mg protein) found in sections of the CNS after chronic administration of choline 0.24, 0.78, and 2.5 g/kg/day. Shown are mean values ± SEM of at least four rats. *The probability of a difference to controls (Dunnett's test) is <0.5.

Muscarinic Receptors

The B_{max} s (fmol/mg protein) found in the various experimental situations are given in Fig. 2. The values found in controls, ranging from 625 ± 47 fmol/mg protein (in the cortex) to 458 ± 14 fmol/mg protein (in the hippocampus), are in the range of the values given by Yamamura and Snyder (45).

ANOVA indicates that the differences between sections, $F(3, 73) = 17.20, p < 0.01$, and between controls and treated groups, $F(3, 73) = 8.47, p < 0.01$, are significant. Dunnett's test for the comparison with a control (see Fig. 2) indicates that the increase of B_{max} caused by choline is significant ($p < 0.5$) in the cortex at 0.78 and 2.5 g/kg/day and in the hippocampus at 2.5 g/kg/day only. No variation of the affinity constant (K_d) for the radioligand was observed: The maximal variation observed was from 112 ± 16 to 95 ± 9 pM. These values are in the range of values given by Yamamura and Snyder (45).

NE Levels

The levels of NE in the sections of the CNS examined after chronic administration of the different doses of choline are given in Fig. 3. The values found in controls (cortex 332 ± 26 ng/g; hippocampus 523 ± 25 ng/g; olfactory system 329 ± 19 ng/g; and subcortex 851 ± 72 ng/g fresh tissue) are in the range of values given by Ehrenstrom and Johansson (17).

ANOVA indicates that the differences between controls and treated groups, $F(3, 65) = 33.75, p < 0.01$, and between sections of the CNS, $F(3, 65) = 23.65, p < 0.01$, are significant.

The significance of the differences compared to controls (Dunnett's test, $p < 0.5$) are given in Fig. 3. The results reveal that the increase of levels of NE caused by choline in the hippocampus and olfactory system is significant only at 2.5 g/kg/day choline.

α_1-Adrenergic Receptors

The B_{max} s (fmol/mg protein) found in the various experimental situations are given in Fig. 4. The values found in controls (from 38.3 ± 4.1 fmol/mg protein in the olfactory system to 26.7 ± 2.3 fmol/mg protein in the subcortex) are in the range of values given by Perry et al. (33) for Buffalo strain rats.

ANOVA indicates that choline administration caused no significant modification in the B_{max}. The differences between sections are significant at the level of 1.0–0.01, $F(3, 73) = 5.70$.

No variation of the affinity constant K_d for the radioligand [^{3}H]prazosin was observed. The values found varied from 0.3

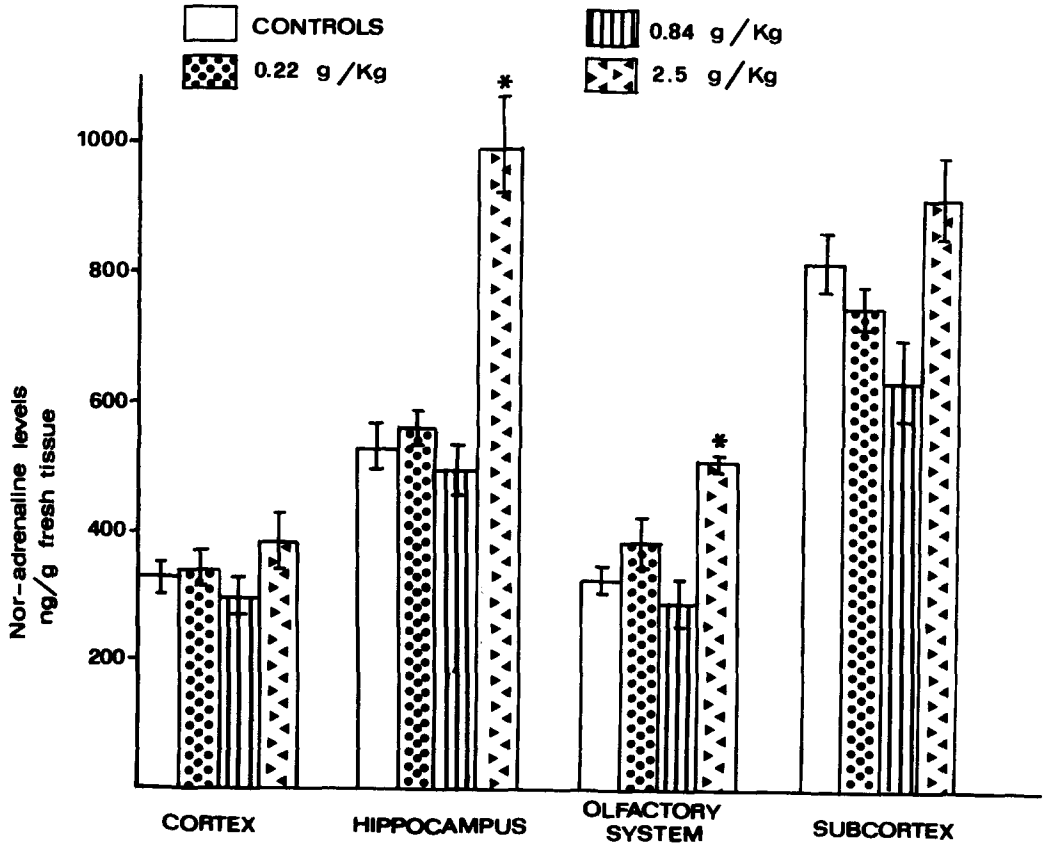

FIG. 3. Norepinephrine (NE) levels (ng/g fresh tissue) found in sections of the CNS after chronic administration of choline 0.22, 0.84, and 2.5 g/kg/day. Shown are mean values ± SEM of at least four rats per point. *The probability of a difference to controls (Dunnett's test) is <0.5.

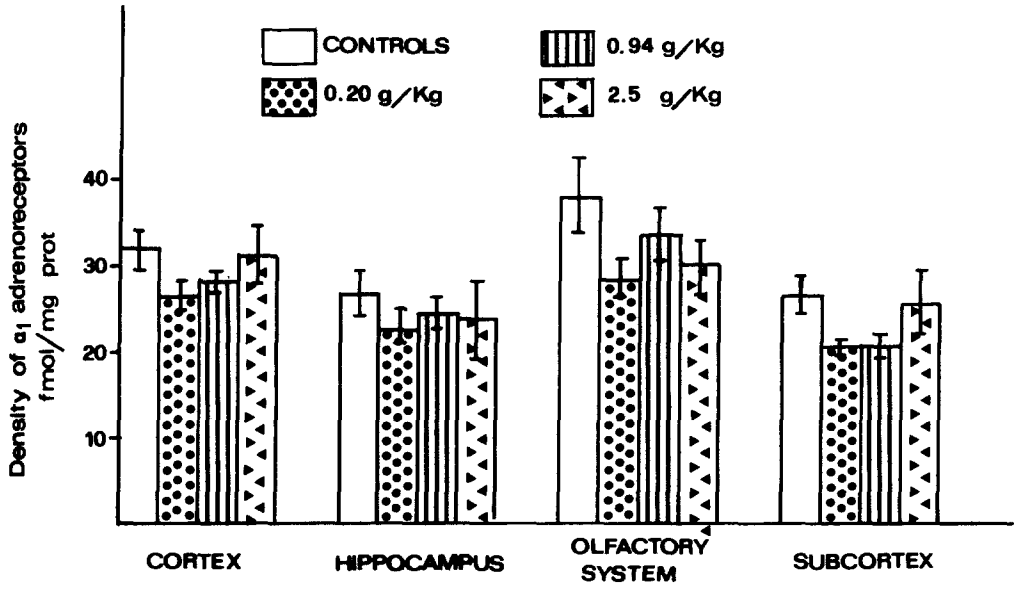

FIG. 4. Density of α_1-adrenoceptors (B_{max} in fmol/mg protein) found in sections of the CNS after chronic administration of choline 0.2, 0.94, and 2.5 g/kg/day. Shown are mean values ± SEM of at least four rats per point.

± 0.07 to 0.29 ± 0.08 nM and are in the range of values given by Perry et al. (33).

DISCUSSION

Cohen and Wurtman (11) observed an increase of ACh levels at doses of choline over 1 g/kg/day. At lower doses of choline (0.1–0.4 g/kg), Wecker and Schmidt (41,43) and Pedata et al. (32) reported no modification of ACh levels in the rat brain. We obtained an increase of ACh concentrations in the cortex, hippocampus, and subcortex only after 2.5 g/kg/day choline.

These observations indicate that an increase of levels of ACh is observed only at doses of choline over 1 mg/kg. At lower doses, choline displays no effect on ACh levels.

Choline 2.5 mg/kg/day also caused an increase of the density of muscarinic receptors in the cortex, hippocampus, and olfactory system. The decrease in the density of muscarinic receptors observed after chronic administration of diisopropylfluorophosphate (16,22,35,39) was attributed to the enhanced synaptic ACh levels.

However, our results indicate that chronic administration of 2.5 g/kg/day not only causes an increase of ACh but also an increase of the density of muscarinic receptors. It may be noted that we offered evidence that chronic administration of disulfiram causes, in certain CNS structures, an increase of ACh levels and of the density of muscarinic receptors (30), while after chronic administration of alfamethylparatyrosine there is a reduction of ACh levels and an increase of the density of muscarinic receptors (29). These observations indicate that modifications of ACh levels and of the density of muscarinic receptors are often not directly correlated.

In some ways, the results we obtained concerning the noradrenergic system support this conclusion. Choline (2.5 g/kg/day) increased the levels of NE in the hippocampus and olfactory system but no modification of the density of α_1-adrenoceptors was observed.

These modifications of NE concentration in the brain suggest that at high doses of choline, which causes evident modification of the central cholinergic system, there is also an interference with the catecholaminergic components.

Interactions between ACh and NE have been identified in brain structures. Muscarinic agonists evoke NE release in cortical slices (13,26) while cholinergic stimulation of hippocampal synaptosomes inhibits NE synthesis and release (7). ACh activates neurons in the locus coeruleus, the source of NE input to the forebrain, through actions at nicotinic and muscarinic receptors (15).

These observations suggest that interaction between cholinergic and adrenergic systems may differ according to the structures of the CNS examined. This conclusion is in agreement with our results that indicate that at high doses of choline there is a modification of NE levels in the hippocampus and olfactory system but not in the cortex and subcortex.

Further work will be necessary to characterize the relative importance of these interactions and identify mechanisms and sites of action.

At lower doses of choline, there is no modification of the total number of responses but there is a reduction of correct responses in the staircase maze; no modification of ACh and NE levels or of the density of muscarinic and α_1-adrenoceptors was observed at these doses of choline.

Therefore, the neurochemical data we obtained do not support the hypothesis that the behavioral effects of chronic administration of choline are the consequence of modification of central cholinergic or noradrenergic systems.

It may be further noted that in our experiments chronic administration of low doses of choline caused a reduction of correct responses, which may suggest that choline administration displayed an amnestic effect characteristic of antimusca-

rinic agents (e.g., scopolamine). An analogous observation was reported by Wecker and Schmidt (41): Chronic choline administration caused a hyperactivity in general associated with antimuscarinic agents.

In conclusion, the results of the present experiments indicate that only at high doses of choline are there clear modifications of the central cholinergic system, but the neurochemical and behavioral effects observed at lower doses of choline do not indicate that choline administration caused an upregulation of the cholinergic system.

The necessity to explore the possibility that choline administration causes modification of various neurochemical systems is emphasized by the observations of Miller et al. (28), indicating that dietary intake of choline modulates the behavioral response to benzodiazepines and the GABA–benzodiazepine–chloride channel complex through induced alteration in membrane phospholipid metabolism.

ACKNOWLEDGEMENT

This work was supported by Ministero della MURST (40% 1989).

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