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BRIEF COMMUNICATION

Vitamin B_{12} Improves Cognitive Disturbance in Rodents Fed a Choline-Deficient Diet

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SASAKI, H., Y. MATSUZAKI, K. MEGURO, Y. IKARASHI, Y. MARUYAMA, S. YAMAGUCHI AND K. SEKIZAWA. *Vitamin B_{12} improves cognitive disturbance in rodents fed a choline-deficient diet.* PHARMACOL BIOCHEM BEHAV 43(2) 635-639, 1992.—The effect of vitamin B_{12} on learning disturbance was tested in rats. Rats were fed a choline-enriched, choline-deficient, and choline-deficient diet with vitamin B_{12}. Concentrations of acetylcholine in the brain were significantly lower in rats fed a choline-deficient diet than rats fed a choline-enriched diet. Passive avoidance learning shows that rats on a choline-deficient diet showed significantly impaired learning compared to rats on a choline-enriched diet. However, there was no significant difference of acetylcholine in the brain or in the passive avoidance learning between rats fed a choline-enriched and a choline-deficient with vitamin B_{12} diet. We, therefore, suggest that vitamin B_{12} potentiates learning in an acetylcholine-deprived brain.

Vitamin B_{12} Rat Acetylcholine Alzheimer's disease Passive avoidance learning

IT has been demonstrated that there is a specific deficiency in acetycholine (ACh), choline (Ch) acetyltransferase, and acetylcholinesterase (AChE) in autopsy material from patients with Alzheimer's disease (8). The severity of dementia is correlated with the neuropathologic indicators of cholinergic losses (24). The major anatomic lesion that correlates with Alzheimer's disease's cholinergic deficiency is the loss of a majority of the ACh-releasing neurons in the septal-diagonal band of the Broca-nucleus basalis system (7). Once these specific transmitter lesions were identified, the development of a treatment strategy for Alzheimer's disease became possible.

An animal model of a Ch-deficient brain has been established by feeding of a Ch-deficient diet (6). After mice had been fed either Ch-deficient or -enriched diets, the retention of learning from a single-trial, passive avoidance task was superior in Ch-enriched old mice and inferior in Ch-deficient old mice (2). As a coenzyme in the formation of S-adenosylmethionine, vitamin B_{12} is associated with the cholinergic metabolic pathways (26). Schrumpf and Bjelke (28) found that the levels of vitamin B_{12} in cerebrospinal fluid of patients with severe atrophy were slightly lower than those in a group of patients with moderate atrophy. This is in accordance with the results of Inada et al. (15), who found a decrease in vitamin B_{12} levels in demented brains.

Then, we speculated that administration of vitamin B_{12} to rats fed a Ch-deficient diet, resulting in decreased ACh in the brain, might enhance synthesis of ACh in the brain and improve cognitive disturbance. In the present study, we examined the hypothesis that vitamin B_{12} improves cognitive disturbance in rats with ACh-deprived brains, which encourages the treatment of ACh-deprived dementia with vitamin B_{12}.

METHOD

Animals

Studies were carried out using 4-week-old male Wistar rats kept in cages under a 12 L : 12 D condition. Rats were given free access to water, as well as to purified diets that were either Ch deficient or Ch enriched. ACh deprivation in rats on Ch-deficient diets has been reported previously (23). The Ch-enriched diet contains approximately 4 mg/g and the deficient diet 0 mg/g ChCl. Half the Ch-deficient diet included

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vitamin B_{12}, 10 mg/kg. All remaining ingredients were the same as standard rat chow, continuing approximately 1.6 mg/g Ch and from 30–50 μg/kg vitamin B_{12}. The Ch-enriched, Ch-deficient, and Ch-deficient diet with vitamin B_{12} were purchased from Japan Crea, Inc. (Tokyo, Japan). Rats were divided into three groups and were fed Ch-deficient (n = 8), Ch-deficient with vitamin B_{12} (n = 10), and Ch-enriched control (n = 10) diets. The diet with vitamin B_{12} was kept from exposure to light as much as possible to avoid resolution. To study Ch treatment on cognitive disturbance, an additional two groups of rats were fed either Ch-enriched, 4 mg/g Ch (n = 10), or standard rat chow, 1.6 mg/g Ch (n = 10). All remaining ingredients were the same as standard rat chow. When rats became 14 weeks old (after 10 weeks on the diet), they were trained and tested using the passive avoidance test for cognitive responses. All experiments were performed between 8:00 and 10:00 a.m.

Spontaneous Movement in Rats

Spontaneous movements of rats in the three groups were measured using an Animex counter (Animex III, Shimazu Co., Tokyo, Japan) for 60 min on a separate day from the passive avoidance learning day. In the additional two group of rats, spontaneous movements were also measured in the same way as in the three groups.

Passive Avoidance Learning in Rats

Passive avoidance learning was carried out according to the step-through procedure in the three groups and additional two groups of rats (16). The apparatus consisted of two compartments, one illuminated [300 × 250 mm; light (60 W) with a height of 200 mm to top of chamber], the other dark (200 × 150 mm with a height of 200 mm to top of chamber). The compartments were separated by a guillotine door (70 × 100 mm). A rat was placed into the illuminated safe compartment and then could enter the dark compartment through the door and stand on a grid floor. Once all four paws were on the grid, a foot-shock of a scrambled constant current (0.3 mA) and constant voltage (50 V, 50 Hz) was delivered to the floor grid for 3 s. The rat could escape from shock only by stepping back into the safe illuminated side. Then, the rat was returned to its home cage. Although rats quickly escaped the shock administered, we could not measure shock duration. Passive avoidance learning was repeated on the second, third, and fourth days in the same way as in the first trial, and the response latency in entering the dark compartment was measured. Results on the latency time of step-through were recorded for each experiment. The maximal latency time of those rats that did not move into the dark compartment during the observation period was calculated to be 300 s.

Determination of Brain ACh and Ch

Brain Ch and Ach were measured in Ch-enriched control (n = 10) and Ch-deficient rats (n = 8) and Ch-deficient with vitamin B_{12} (n = 10) and in the additional two groups of rats fed either Ch-enriched (n = 10) or standard rat chow (n = 10), which were used in the passive avoidance learning and spontaneous movement. One week after the last learning, rats, which were kept on the previous diets, were killed by microwave irradiation (microwave device NJE 2603 10 kW, New Japan Radio, Tokyo, Japan) at 9.0 kW for 0.75–1.15 s, which raised the brain temperature to 95.0 ± 1.7°C (12, 18). The brain was removed from the skull and dissected into seven regions according to the method of Glowinski and Iverson (9). The dissected tissue was homogenized with a mixture of 1 ml 0.05 M perchloric acid ($HCLO_4$) and 10 nmol/10 μl EHC using an ultrasonic cell disrupter (model US-300T, Nissei, Tokyo, Japan). The homogenate was centrifuged at 10,000 × g at 4°C for 15 min. The supernatant was filtered through a 0.45-μm millipore filter and then 5 μ supernatant was injected into a liquid chromatography with an electrochemical detection (LCEC) system for the determination of Ach and Ch (13,25). Tissue pellets obtained by centrifugation for the determination of protein were stored at −85°C until analysis. For assaying the protein concentration, a solution of 1 N NaOH was added to the pellets for the preparation of a final sample (10 ml) and homogenized. The homogenates obtained from tissues of the cerebellum, medulla-pons, hypothalamus, striatum, midbrain thalamus, hippocampus, and cortex were diluted with 1 N NaOH at the rate of 5, 3, 1, 2, 3, 2, and 10-fold, respectively. Using a Bio-Rad protein assay kit (Bio-Rad Labs., Richmond, CA), 0.1 ml of each of the above diluted homogenates was used for assaying protein concentration based upon the method of Bradford (4). Bovine serum albumine was used as the standard. The LCEC system consisted of an LC100P pump (Yokogawa Co., Ltd., Tokyo, Japan), an LC100S injector with 20-μl sample loop (Yokogawa), an LC-4A amperometric detector with platinum electrodes [Bioanalytical System (BAS), West Lafayette, IN], and an LC100W/F-PC work station (Yokogawa) for LC data processing. The analytical column was the BAS Acetylcholine Separation Column. A glassy carbon column was used as the precolumn, and an immobilized column containing AChE and Ch oxidase was used as the postcolumn. Analytical column temperature was set at 35°C (with a BAS Temperature Controller LC 22A). The mobile phase was 0.05 M phosphate buffer, pH 8.4, containing 1 nM $EDTD_2Na$ and 0.4 mM sodium 1-octanesulfonate (SOS). The flow rate was set at 0.8 ml/min. The electrode potential was set at +0.5 V against an Ag/AgCl reference electrode for the detection of hydrogen peroxide. The principle of the technique is based upon the separation of Ach and Ch in the separation column, followed by their enzymatic conversion through postcolumn reaction with AChE and Ch oxidase to hydrogen peroxide, which is detectable electrochemically by a platinum electrode.

Reagents

ACh iodide and Ch iodide were purchased from the Sigma Chemical Co. (St. Louis, MO). Ethylhomocholine (EHC) iodide as an internal standard (IS) was synthesized from dimethyl-3-amino-1-propanol (Sigma) and iodoethane (Sigma) in the Department of Neuropsychopharmacology (Tsumura), Gunma University, School of Medicine. Vitamin B_{12} as methylcobalamin was donated by the Eisai Pharmaceutical Co. (Japan). Other reagents for extraction and chromatography were of the highest available purity and purchased from commercial sources.

Statistics

Mean values for latency time are reported as geometric means and geometric standard deviation of the mean (GSDM). All other values are reported as means ±SD. Statistical analysis was performed by one-way analysis of variance (ANOVA) and Kruskal–Wallis rank test. Significance was accepted at $p < 0.05$.

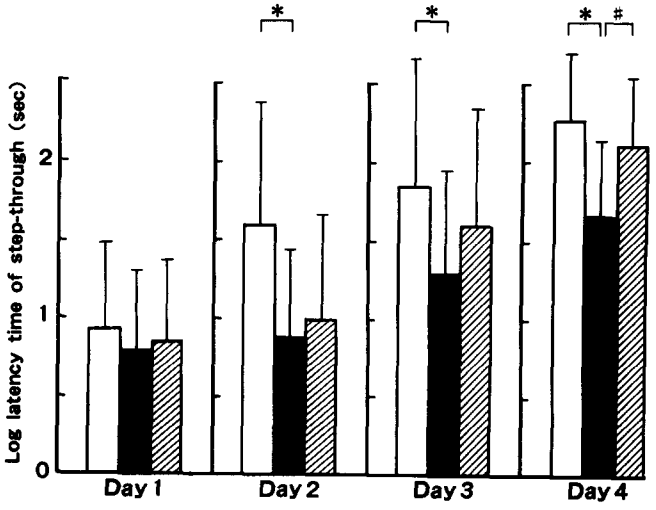

FIG. 1. Effect of vitamin B_{12} on passive avoidance learning (step-through) in rats fed a Ch-deficient diet. (□), control diet; (■), Ch-deficient diet; (▨), Ch-deficient with vitamin B_{12} diet. * and # show statistical significance ($p < 0.05$) between values in control and Ch-deficient diet and between values in Ch-deficient diet and Ch-deficient with vitamin B_{12} diet, respectively.

RESULTS

Spontaneous movements of rats in the Ch-enriched control, Ch-deficient diet, and Ch-deficient diet with vitamin B_{12} were 567 ± 184 (mean ± SD), 739 ± 218, and 662 ± 208, respectively. They were not significantly different.

On the first day, there was no significant difference in latency times among the three groups. The latency of rat entrance into the dark compartment increased on subsequent test days for all groups (Fig. 1). From the second to the fourth day, the latency times in control rats were higher than those of Ch-deficient rats ($p < 0.05$). In Ch-deficient with vitamin B_{12} rats, the latency times were significantly higher than those of Ch-deficient rats on the fourth day ($p < 0.05$). From the second to the fourth day, the latency times of control rats were not significantly different from the latency times of Ch-deficient with vitamin B_{12} rats. On the second, third, and fourth days, latency times over 300 s were observed in 2, 4, and 7 rats in the control group, respectively, and in 3 and 4 rats on the third and fourth days in the Ch-deficient with vitamin B_{12} group, respectively.

Tissue weights of rat brain regions are listed in Table 1. Tissue weights of rat brain in the Ch-deficient group were significantly lower than control rats for the cerebellum, midbrain thalamus, cortex, and whole-brain regions ($p < 0.05$). Vitamin B_{12} increased tissue weights of rat brain regions in the cerebellum, midbrain thalamus, cortex, and whole brain ($p < 0.05$). Concentrations of Ch and ACh in rat brain regions are shown in Tables 2 and 3, respectively. Ch levels in Ch-deficient rats were significantly lower than control rats for the cerebellum, cortex, and whole brain ($p < 0.05$) (Table 2). There were significant differences in ACH between control and Ch-deficient rats in the medulla pons, midbrain thalamus, cortex, and whole brain. Vitamin B_{12} recovered ACh in the medulla pons, midbrain thalamus, cortex, and whole brain.

Body weights of control, Ch-deficient, and Ch-deficients with vitamin B_{12} rats were 213 ± 33 g (mean ± SD), 130 ± 21, and 217 ± 24, respectively. Ch-deficient rats had a lower body weight than control rats ($p < 0.01$). Vitamin B_{12} recovered body weight significantly ($p < 0.01$).

In the additional two groups of rats, all parameters were also measured in the same way as those in the three groups. There was no significant difference of spontaneous movements between the two groups: 598 ± 230 (mean ± SD) and 647 ± 242 in rats fed Ch-enriched and standard rat chow, respectively. Latency time of step-through was not significantly different between them; on the first, second, third, and fourth days, log latency times of step-through were 0.9 ± 0.6 s (geometric mean ± GSDM) vs. 0.8 ± 0.5, 1.6 ± 0.8 vs. 1.4 ± 0.9, 1.9 ± 0.8 vs. 1.8 ± 0.9, and 2.3 ± 0.7 vs. 2.2 ± 0.8 in Ch-enriched vs. standard rat chow, respectively. Tissue weights of rat whole brain was 1,681 ± 71mg (mean ± SD) vs. 1,639 ± 64 in Ch-enriched vs. standard rat chow, respectively. They were not significantly different. Tissue weights in rat brain regions were also not significantly different between them. Whole-brain choline levels were 182 ± 45 pmol/mg protein (mean ± SD) vs. 161 ± 52 in Ch-enriched vs. standard rat chow, respectively (not significantly different). Ch levels in rat brain regions were also not significantly different between groups. Whole-brain ACh levels were 339 ± 54 pmol/mg protein (mean ± SD) vs. 296 ± 48 in Ch-enriched vs. standard rat chow, respectively. They were not significantly different. ACh levels in rat brain regions were also not significantly different between groups.

DISCUSSION

We confirmed previous reports that a Ch-deficient diet reduces ACh in the brain, which causes cognitive disturbance in rats (2,27). Vitamin B_{12} at about a 200 times higher dose than standard rat chow reproduced brain ACh up to control values in rats fed a choline-deficient diet and improved cognitive disturbance in rats. Deprivation of brain ACh is one of the

TABLE 1

TISSUE WEIGHTS OF RAT BRAIN REGIONS

Group	Tissue Weight (mg. mean ± SD) in Rat Brain Regions							
	Cerebellum	Medulla Pons	Hypothalamus	Striatum	Midbrain Thalamus	Hippocampus	Cortex	Whole Brain
Control (n = 8)	237 ± 12	211 ± 21	108 ± 11	130 ± 9	193 ± 17	111 ± 6	620 ± 33	1,610 ± 58
Ch free (n = 10)	215 ± 15*	198 ± 34	105 ± 9	128 ± 25	157 ± 13*	115 ± 8	555 ± 27*	1,472 ± 76*
Ch free + B_{12} (n = 10)	239 ± 8†	215 ± 20	112 ± 6	139 ± 12	189 ± 15†	115 ± 9	620 ± 51†	1,665 ± 48†

*Significantly different from control group, $p < 0.05$ (Student's t-test).
†Significantly different from Ch-free group, $p < 0.05$ (Student's t-test).

TABLE 2

EFFECT OF VITAMIN B_{12} ON CEREBROREGIONAL Ch LEVELS IN RATS FED A Ch-DEFICIENT DIET

Group	Ch Levels (pmol/mg protein, mean ± SD) in Rat Brain Regions							
	Cerebellum	Medulla Pons	Hypothalamus	Striatum	Midbrain Thalamus	Hippocampus	Cortex	Whole Brain
Control (n = 8)	159 ± 55	184 ± 38	149 ± 45	172 ± 30	198 ± 66	189 ± 77	172 ± 69	173 ± 55
Ch free (n = 10)	94 ± 32*	138 ± 64	110 ± 56	116 ± 54	205 ± 48	159 ± 42	98 ± 32*	119 ± 31*
Ch free + B_{12} (n = 10)	141 ± 71	169 ± 116	141 ± 91	181 ± 110	182 ± 74	237 ± 110	137 ± 49	157 ± 63

*Significantly different from control group, $p < 0.05$ (Student's t-test).

major pharmacological abnormalities of Alzheimer's-type dementia. Because a great deal of vitamin B_{12} enhanced ACh synthesis in the present animals, vitamin B_{12} may be one of the pharmacological treatments for patients with Alzheimer's-type dementia.

In the previous study, we observed that the passive avoidance learning shows that rats on a Ch-deficient diet showed significantly impaired learning compared to rats on a Ch-enriched diet and that nicotine administered intraperitoneally significantly potentiated learning in rats on a Ch-deficient diet, as well as in rats on a Ch-enriched diet (27). Passive avoidance has been used more than any other behavioral procedure for the evaluation and screening of drugs for effects on learning and memory. However, Heise (11) pointed out that any drug administered prior to learning trials will probably affect sensory processes, motor systems, motivational arousal, or other nonlearning aspects of performance to some extent. If a drug is given before the learning trial, the warning stimulus may become averse by drug influence on the visual or auditory input (20). Kameyama et al. (17) reported that in pretraining treatment with drugs that have an analgesic action the effect of foot-shock as a negative reinforcement is weakened and the degree of retention may become poor as with amnesia. In the present study, we did not administer any drug just before the trials. However, the present passive avoidance procedure is characterized as a broad test for learning, memory, performance-enhancing, cognition-activating, or psychostimulant activity (5).

In the present study, we measured ACh in seven regions of the brain. ACh was significantly reduced in the medulla pons, midbrain thalamus, and cortex in rats fed a Ch-deficient diet. The rest of the brain also showed a tendency to decreased ACh. Deprived ACh in the brain has been reported in rodents fed dietary Ch deficiency (6,23). In the present experiment, a Ch-enriched diet did not further improve both latency time and ACh concentration in the brain compared to rats fed standard rat chow. Some reports describe negative results after prolonged treatment with a Ch-enriched diet compared with standard rat chow (21). Choline at 4 mg/g might not be high enough to further increase brain ACh as compared with that in rats fed standard rat chow. We adopted rats fed a Ch-enriched diet as controls. Whitehouse et al. (29) observed that neurons of the nucleus basalis of Meynert undergo a profound and selective degeneration in patients with Alzheimer's-type dementia and provide a pathologic substrate of the cholinergic deficiency in their brains. Because the nucleus basalis of Meynert is a major source of cholinergic innervation, ACh depression in the brain in the present model is along the lines of an Alzheimer's-type dementia.

As a coenzyme in the formation of S-adenosylmethionine, vitamin B_{12} is associated with the cholinergic metabolic pathways (26). Nadeau and Roberge (22) reported increased specific Ch acetyltransferase activity measured in several structures of cat brain after daily supplementation with vitamin B_{12}. The present results are the first proving that a great deal of vitamin B_{12} increased ACh synthesis in ACh-deficient brain tissues. Ikeda et al. (14) reported significantly lower levels of methyl-B_{12} in cerebrospinal fluid of patients with Alzheimer's dementia. Agamanolis et al. (1) suggested that experimental deprivation of vitamin B_{12} in rhesus monkeys makes a demyelinative condition, with degeneration, loss of axons, and marked gliosis occurring at a later stage. Hector and Burton (10) summarized the literature by stating that a vitamin B_{12} deficiency causes several types of psychiatric disorders: delirium with slow mentation, fear, memory change, and/or delusions, depressive illness, acute psychosis, usually with para-

TABLE 3

EFFECT OF VITAMIN B_{12} ON CEREBROREGIONAL ACh LEVELS IN RATS FED A Ch-DEFICIENT DIET

Group	ACh Levels (pmol/mg protein, mean ± SD) in Rat Brain Regions							
	Cerebellum	Medulla Pons	Hypothalamus	Striatum	Midbrain Thalamus	Hippocampus	Cortex	Whole Brain
Control (n = 8)	80 ± 21	432 ± 59	411 ± 70	815 ± 92	497 ± 65	282 ± 32	301 ± 46	350 ± 41
Ch free (n = 10)	67 ± 19*	340 ± 66*	341 ± 80	627 ± 69	422 ± 51*	248 ± 49	203 ± 80†	269 ± 38†
Ch free + B_{12} (n = 10)	84 ± 17	435 ± 116‡	432 ± 96‡	800 ± 142	485 ± 88‡	273 ± 32	296 ± 45§	346 ± 41§

*Significantly different from control group, $p < 0.05$ (Student's t-test).
†Significantly different from control group, $p < 0.01$ (Student's t-test).
‡Significantly different from Ch-free group, $p < 0.05$ (Student's t-test).
§Significantly different from Ch-free group, $p < 0.01$ (Student's t-test).

noid flavorings, and, in rare cases, secondary mania. On the other hand, they found no convincing evidence that a vitamin B_{12} deficiency causes dementia. Mitsuyama and Kogoh (19) studied 14 patients with various types of dementia. Serum B_{12} concentrations were found to be within the normal range in all patients. However, after administration of daily intramuscular injections of vitamin B_{12} for more than 60 days patients with a high elevation of cerebrospinal fluid vitamin B_{12} levels showed reduced anxiety, improved mood, increased communication, and increased interaction with other people and the environment, and in self-ratings the scores on their own competence were enhanced. So far, vitamin B_{12} is used for the treatment of peripheral neuropathy in such cases as diabetes mellitus (3). Although it has not proved that vitamin B_{12} also enhances ACh synthesis in the brain of Alzheimer's dementia, the present results suggest the possibility that vitamin B_{12} is a candidate in the treatment of Alzheimer's-type dementia.

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