The Scopolamine-Induced Impairment of Spatial Cognition Parallels the Acetylcholine Release in the Ventral Hippocampus in Rats

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Received March 9, 2000 Accepted July 11, 2000

ABSTRACT—We investigated the relationship between the induction of spatial cognition impairment in the 8-arm radial maze task and regional changes (ventral hippocampus (VH), dorsal hippocampus, frontal cortex, and basolateral amygdala nucleus) in brain acetylcholine (ACh) release using microdialysis in rats treated with muscarinic (M) receptor antagonists. In a behavioral study, two M₁ antagonists, scopolamine (0.5 mg/kg, i.p. and 20 μ g, i.c.v.) and pirenzepine (80 μ g, i.c.v.), but not an M₂ antagonist, AF-DX116 (40–80 μ g, i.c.v.), disrupted spatial cognition in the 8-arm radial maze task. In brain microdialysis with Ringer's solution containing 0.1 mM eserine sulfate, scopolamine and AF-DX116, but not pirenzepine, increased ACh release in the VH. Moreover, in the bilateral injection of scopolamine (2 μ g/side), the VH and dorsomedial thalamus nucleus were important regions for scopolamine-induced impairment of spatial cognition. A simultaneous determination of the behavioral changes revealed that scopolamine (0.5 mg/kg, i.p.) markedly decreased the ACh contents and also increased the ACh release in all regions tested. Especially, the changes in the ACh release of the VH closely paralleled the induction of the scopolamine-induced impairment of spatial cognition. These results suggest that the blocking balance between M₁ and M₂ muscarinic receptor in the VH therefore plays a major role in the spatial cognition impairment induced by scopolamine in the 8-arm radial maze task.

Keywords: Spatial cognition, 8-Arm radial maze task, Acetylcholine release, Scopolamine, Ventral hippocampus

Cholinergic basal forebrain neurons that project to the cerebral cortex and hippocampus are well known to play a role in learning and memory in animals and humans (1, 2). In fact, many studies have shown a relationship between learning and memory functions and such changes in cholinergic markers as choline acetyltransferase (ChAT) (3), high affinity choline uptake (HACU) (4) and muscarinic receptor concentration (5) following both training in a water maze and passive avoidance. Recently, dorsal hippocampal acetylcholine (ACh) release has also been shown to increase during the performance of both a T-maze test (6) and a discrimination learning task (7). Thus, ACh is considered to be the most important neurotransmitter for learning and memory, although the role of other neurotransmitters in learning and memory has yet to be fully elucidated.

The muscarinic receptor antagonist scopolamine is well

known for inducing behavioral impairment regarding various different learning and memory tasks (1), and it has also been used as a pharmacological tool to evaluate the effects of cognitive enhancers on learning and memory deficits in animals and human (8, 9). We recently reported that scopolamine disrupted performance in both the 8-arm radial maze task and passive avoidance task, and cholinergic enhancers such as physostigmine and THA were found to reduce the impairment of such behavioral performance (10). Moreover, we also reported that the ACh contents in the frontal cortex (FC) significantly decreased in the scopolamine-treated behaviorally disrupted rats (11, 12). However, the detailed mechanism of scopolamine still remains unclear, since there have so far been no reports on the direct interaction between the behavioral changes and neurotransmitter changes by scopolamine in memory-impaired rats.

In the present study, to investigate the mechanism of scopolamine-induced impairment of spatial cognition in the 8-arm radial maze task, we observed the behavioral

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changes after the systemic and microinjection of M_1 and M_2 muscarinic receptor antagonists and then determined the regional contents and release of ACh using microdialysis in the ventral hippocampus (VH), dorsal hippocampus (DH), FC and basolateral amygdala nucleus (ABL).

MATERIALS AND METHODS

Animal

Male Wistar rats, weighing 200–250 g, were obtained from the Kyu Do Co., Ltd. (Saga); they were housed in groups of 4 to 5 per cage in a room with a controlled temperature ($23 \pm 2^\circ\text{C}$) and a relative humidity of $60 \pm 10\%$ with lights on from 7:00 to 19:00. The animals were placed under restricted food intake (10–12 g/day, CE-2; Clea Japan, Tokyo). Their body weights were maintained at approximately 80% of the free feeding level during the experimental period. The water was freely available in their home cages. All procedures regarding animal care and use were carried out based on the regulations dictated by the Experimental Animal Care and Use Committee at Facilities for Experimental Animals of Fukuoka University.

Apparatus

As previously reported (13), behavioral testing was conducted on an 8-arm radial maze (Neuroscience Co., Tokyo), a modified one originally developed by Olton and Samuelson (14). It was elevated 50 cm from the floor. The maze consisted of a central platform, 24 cm in diameter, with 8 arms extending radially. Each arm measured 50 cm in length, 10 cm in width, and 50 cm in height, with transparent plastic side walls. Food cups for the reinforcers were located near the end of each arm. The maze was located in a room containing many extra-maze visual cues. For the behavioral analysis, an image motion analyzer, AXIS-30 (Neuroscience Co.), was used to quantify the task performance of rats in the 8-arm radial maze. The high speed analyzer had an automatic tracking system which was able to track each rat's movement in the maze with a CCD camera equipped with a personal computer, which then analyzed the movement in real time and assessed the length of the route, frequency of arm visits, velocity of walking and time required to accomplish the task.

Behavioral procedure for an 8-arm radial maze task

The test animals were trained in a group to be habituated to the apparatus and food pellet for 3 days before each test animal's actual training began. This 10-min period of habituation was repeated 3 times a day. The habituation interval was more than 1 h. For each training session, the test animal was placed in a circular plastic wall in a platform at the middle of the 8-arm radial maze. Then, after 1 min, the ring was lifted and the test animal was allowed to

move freely in the maze. The trial continued until the test animal had either entered all 8 arms or 10 min had elapsed. If the test animals proceed in the 8-arm radial maze task by using sequential routines that consist of repeating a given angular direction (e.g. 45° or 135°) by repeated training, these animals were excluded in the present experiment. The performance of the test animal in each trial was assessed by three parameters; the number of correct choices in the initial 8 chosen arms, the number of errors which was defined as choosing arms that had already been visited, and the time that elapsed before the animal ate all 8 pellets. If the test animals made 7 or 8 correct choices and less than one error in three successive sessions, they were then used for the drug evaluation the next day.

Surgical operation for intraventricular injection and microinjection

The test animals that had acquired spatial cognition according to the above criteria were first anesthetized with sodium pentobarbital (50 mg/kg, i.p.; Tokyo Kasei, Tokyo) and placed into a stereotaxic apparatus (Type SR-6; Narishige Scientific Instrument Lab., Tokyo). The skull was exposed and drilled through to the duramater at the coordinate of the ventricle (0.8 mm anterior to the bregma, 1.3 mm lateral to the midline and 3.8 mm ventral from the skull surface with the incisor bar set at -3.3 mm below the interaural line). Each coordinate of brain regions for the microinjection of scopolamine was determined according to Table 1. The coordinates were selected using the brain atlas of Paxinos and Watson (15). A 21-gauge stainless steel guide cannula was implanted at the outer edge of the duramater through the drilled opening, lowered perpendicularly to each depth and then was permanently secured with dental cement and bone screws. A recovery period of approximately 3 to 7 days was allowed and the test animals were confirmed not to demonstrate any abnormal behavioral performance in the 8-arm radial maze task before the muscarinic (M) antagonists were administered.

Intraventricular injection and microinjection

A 27-gauge stainless steel injection cannula was used to infuse the drug. The injection cannula was connected to a 1 ml Hamilton syringe (Hamilton Co., Reno, NV, USA) via a polyethylene tubing (type PE10, i.d. of 0.28 mm, o.d. of 0.61 mm; Becton Dickinson, Parsippany, NJ, USA). Five μl of M antagonists or 2 μl of scopolamine was injected bilaterally into each lateral ventricle or each brain region, respectively, through the injection cannula that extended 0.5-mm beyond the tip of the guide cannula, using a Microinjection Pump (CMA/100; Carnegie Medicine Co., Stockholm, Sweden). The rate of injection was $1.0 \mu\text{l}/\text{min}$, and the injection cannula was left in place for a further 3 min to allow the drug to diffuse away from the tip.

Table 1. The coordinates of each brain region for the microinjection of scopolamine

Brain region	Abbreviations	A (mm)	L (mm)	H (mm)	Angle
Frontal cortex	FC	2.0	± 2.0	2.8	—
Parietal cortex	FPC	1.2	± 5.0	3.5	—
Dorsal hippocampus	DH	−3.8	± 2.0	3.5	—
Ventral hippocampus	VH	−4.8	± 5.0	8.0	—
Dorsomedial thalamus nucleus	DMT	−2.3	± 0.7	5.5	—
Central amygdaloid nucleus	ACE	−2.3	± 4.2	8.0	—
Basolateral amygdaloid nucleus	ABL	−2.3	± 4.8	8.7	—
Medial caudate putamen	CPM	0.2	± 2.5	6.0	—
Lateral hypothalamus	LH	−2.3	± 1.7	8.5	—
Mammillary body	MB	−4.8	1.6	9.5	10°
Basal forebrain	BF	−0.8	± 2.8	7.5	—
Medial septal nucleus	SEP	0.7	1.1	6.7	10°
Dorsal raphe nucleus	DR	−7.8	2.4	7.0	20°
Median raphe nucleus	MR	−7.8	2.9	8.5	20°

A, anterior to the bregma; L, lateral to the midline; H, ventral to the surface of the skull measured at the bregma.

Scopolamine and AF-DX116 were injected i.c.v. 10 min prior to the test, and pirenzepine was given i.c.v. 30 min prior to the test. In the microinjection of scopolamine, the test was carried out 10 and 30 min after the microinjection of scopolamine into each brain region. The performance in the radial maze task during non-injected trials was not affected by repeated intraventricular injections. When microinjections were made repeatedly into the same rat, a minimum of three days was allowed between microinjections.

Determination of the brain ACh contents

Following the observation of 8-arm maze performance, the rats showing a scopolamine (0.5 mg/kg, i.p.)-induced impairment of spatial cognition were sacrificed by microwave irradiation (5.0 kW, 1.5 s, Microwave Applicator; Muromachi Kikai Co., Tokyo) to prevent degradation of ACh content by cholinesterase and then underwent an ACh assay as previously described (13). The brain was immediately removed and cut into 1-mm coronal cryosections and then punched out. Each regional sample was added to ethylhomocholine (EHC, 2 nmol) as an internal standard and homogenized with 500 μ l of formic acid/acetone (15/85). Following centrifugation, the supernatant was extracted with diethylether. The HPLC-ECD system (Waters Assoc., Milford, MA, USA), utilizing an EicomPak AC column and enzyme column (EICOMPAK AC-GEL; Eicom, Kyoto), was used for the ACh determination. The brain ACh contents were quantified by calculating the area under the curves using an integrator (Waters Model 730; Waters Assoc.) and their contents were then determined using an internal standard method.

Brain microdialysis procedure for ACh release

A brain microdialysis was performed as previously

described (13). Briefly, the only test animals that reached the criterion of the 8-arm radial maze task were stereotaxically implanted with a guide cannula (AG-8, Eicom) under pentobarbital anesthesia (50 mg/kg, i.p.) as well as the above operation of microinjection. The guide cannula was placed in the site according to the atlas of Paxinos and Watson (15), and the coordinates were measured from the bregma: VH A: −4.8, L: 5.0, V: 6.0; DH A: −4.0, L: 3.3, V: 1.8; FC A: 2.8, L: 2.0, V: 2.0; ABL A: −2.8, L: 4.8, V: 7.5. A microdialysis probe (A-I-8-02: 2 mm dialysis membrane for DH, FC and ABL; A-I-8-03: 3 mm for VH; Eicom) was inserted into the guide cannula of rats placed in plastic cages (30 × 30 × 35 cm) 2 days following scopolamine treatment for the 8-arm radial maze task (4–5 days following the surgical days); and the animals were perfused with Ringer's solution containing 0.1 mM eserine sulfate (Sigma) at a flow rate of 2 μ l/min by means of a syringe pump (CMA/100). The syringe was connected to the probe inlet by polyethylene tubing; the probe outlet was connected to the sample loop (300 μ l) of the analytical system by polyethylene tubing. The sample (20 μ l) was collected by a refrigerated collector (CMA/140, Carnegie Medicine) at 10-min intervals over a 120-min period. To achieve stable baseline readings, microdialysis was allowed to proceed for 30 min before the collection of fractions. The sampled ACh concentration was then measured by the HPLC-ECD system as described above.

Histology

After completion of the experiment, the test animals were injected with 2 μ l cresyl violet dye to confirm the cannula placement. Ten minutes after this microinjection, the test animals were anesthetized with ether and decapitated. After the brain was removed, it was frozen and cut into 40- μ m-thick slices. The diffusion of dye to the lateral ventricle

was confirmed by macroscopic examination. Only data from animals in which the injections were made into the desired site were analyzed.

Drugs

Scopolamine-HBr and pirenzepine-dihydrochloride (Research Biochemicals, Inc., Natick, MA, USA) were dissolved in saline. AF-DX116 (a generous gift from Boehringer-Ingelheim, Inc., Ingelheim, Germany) was initially dissolved in a minimum volume of 0.1 N HCl solution and its pH was adjusted to about 7.0 by adding NaHCO_3 .

Statistical analyses

Significant differences for the data analysis of the 8-arm radial maze task were evaluated by the Wilcoxon's rank sum test in two groups and by the Kruskal-Wallis test followed by the Bonferroni's (Dunn's) test for non-parametric multiple comparisons. Student's *t*-test was used to determine significance for any changes in the ACh contents. The regional ACh release was analyzed by two-way (with repeated measures) analysis of variance (ANOVA) followed by the Contrast test for differences among the groups when the overall significance was observed in repeated measures of two-way ANOVA. The Spearman correlation coefficient by rank between the behavioral score of an 8-arm radial maze task and the ACh releases of the 6th fraction (this fraction obtained at 30 min after scopolamine treatment corresponds to the i.p. peak injection time in the 8-arm radial maze) was determined in the scopol-

amine-treated rats. The criterion for statistical significance was considered to be $P < 0.05$ in all statistical evaluations.

RESULTS

Effects of muscarinic receptor antagonists on spatial cognition in the 8-arm radial maze task

All test animals demonstrated a successful performance of the maze task within a maximum of 20 days. When the rats were subjected to cannula implantation and drug injection, they were again re-trained and confirmed to still maintain their previous performance level.

As shown in Fig. 1, the i.p. (0.5 mg/kg) and i.c.v. (20 $\mu\text{g}/\text{brain}$) injections of scopolamine significantly decreased the correct choices and increased errors 30 and 10 min after the injection, respectively. The i.p. injection of pirenzepine and AF-DX116 did not affect spatial cognition (data not shown). For this reason, the i.c.v. injection of both drugs was carried out 10, 30 and 60 min after the treatment. The i.c.v. injection of pirenzepine (80 $\mu\text{g}/\text{brain}$) most severely disrupted the spatial cognition 30 min after the injection. On the other hand, AF-DX116 (40 and 80 $\mu\text{g}/\text{brain}$) did not disrupt the spatial cognition 10, 30 and 60 min after the injection. At 10 min after the higher dose of AF-DX116 (80 $\mu\text{g}/\text{brain}$), 4 of 9 rats could not even move when attempting to perform the 8-arm radial maze task. These rats were excluded from the experimental data because we can not determine whether the spatial cognition was selectively impaired or motor function decreased due to sedative ac-

Fig. 1. Effects of i.c.v. injection with muscarinic receptor antagonists on spatial cognition in the 8-arm radial maze task. Scopolamine and AF-DX116 were injected i.c.v. 10 min prior to the test, and pirenzepine was given i.c.v. 30 min prior to the test. Values are the mean $\pm$ S.E.M. Numbers in parentheses represent number of rats tested. * $P < 0.05$ vs control (Bonferroni's test).

Fig. 2. Effects of scopolamine, pirenzepine and AF-DX116 on the ACh release from the ventral hippocampus into Ringer's solution containing 0.1 mM eserine sulfate in intact rats. Scopolamine and pirenzepine or AF-DX116 was injected i.p. or i.c.v., respectively, into the highly trained rats immediately after sampling the 3rd fraction (F3). The ACh concentrations are expressed as a % of the F3 sample taken immediately before the administration. Values are the mean $\pm$ S.E.M. ○, scopolamine, 0.5 mg/kg ($n=12$); ●, pirenzepine, 80 μ g/10 μ l ($n=5$); ▲, AF-DX116, 80 μ g/10 μ l ($n=5$).

tion by AF-DX116.

Effects of muscarinic receptor antagonists on ACh release in the VH

Ringer's solution containing 0.1 mM eserine sulfate was used in the present study. The baseline of ACh release was not changed in vehicle-treated groups (data not shown). As shown in Fig. 2, scopolamine (0.5 mg/kg, i.p.) and AF-DX116 (80 μ g/brain) caused marked increases in the ACh release of the VH to $488.8 \pm 83.1\%$ and $408.0 \pm 218.4\%$ within 30 min (6th fraction) and 10 min (4th fraction) after injection, respectively, with the peak fraction of ACh release corresponding to the peak injection time in 8-arm radial maze task. However, pirenzepine did not change the ACh release in the VH at 30 min (6th fraction) after injection. Repeated measurements of ANOVA revealed a group difference [$F(2,20)=3.938$, $P<0.05$], effect of fraction [$F(9,180)=2.624$, $P<0.01$] as well as a group $\times$ fraction interaction [$F(18,180)=3.554$, $P<0.001$]. The contrast test revealed the ACh release in the scopolamine-treated group was significantly increased compared to that in pirenzepine-treated group ($P<0.05$), but no significant difference was observed between the another groups.

Effects of the microinjection of scopolamine in various brain regions of rats on the spatial cognition in the 8-arm radial maze task

As shown in Table 2, the bilateral injection of 2 μ g/site of scopolamine into the VH and dorsomedial thalamus nucleus (DMT), significantly decreased the number of correct choices and increased the number of errors in comparison with each vehicle-treated group. When the 8-arm radial maze task was again examined at 30 min after treatment,

the spatial cognition was impaired by the injection of scopolamine into the VH, DH, basal forebrain (BF) and medial raphe nucleus (MR).

Changes in the brain ACh contents in the scopolamine-treated rats

As shown in Table 3, scopolamine (0.5 mg/kg, i.p.) significantly decreased the ACh contents in all brain regions measured in the scopolamine-treated rats compared with the vehicle-treated rats. The most pronounced decrease of the ACh contents was observed in the VH (42.6%, $P<0.001$, Student's t -test).

The correlation between the in vivo changes in brain ACh release and the 8-arm radial maze task

To determine the relationship between the behavioral score in the 8-arm radial maze and ACh release from each brain region in the scopolamine-treated rats, a new experimental schedule was planned as shown in Fig. 3A. In order to test the behavioral and biochemical changes using the same animal, each rat was trained for 2–3 weeks and then a microdialysis probe was implanted into the target brain region. As shown in Fig. 3B, scopolamine (0.5 mg/kg, i.p.) caused a marked increase in the ACh release, like it did in the data shown in Fig. 2. The peak ACh releases of all brain regions were observed at 30 to 40 min after the i.p. injection of scopolamine (6th fraction, FC: $181.5 \pm 42.3\%$, DH: $446.1 \pm 101.1\%$, VH: $617.2 \pm 131.4\%$ and ABL: $269.9 \pm 47.0\%$ of baseline), and this period was the same time when spatial cognition was impaired in the 8-arm radial maze task. Repeated measurements of ANOVA revealed a group difference [$F(3,44)=4.140$, $P<0.05$], effect of fraction [$F(9,396)=20.675$, $P<0.001$]

Table 2. The microinjection of scopolamine in various brain regions of rats undergoing the 8-arm radial maze task

Brain region	Scopolamine ($\mu\text{g}/\text{brain}$)	No. of rats	10 min		30 min	
			Correct	Error	Correct	Error
Ventral hippocampus	vehicle	7	7.4 $\pm$ 0.3	0.9 $\pm$ 0.5	7.6 $\pm$ 0.3	0.7 $\pm$ 0.5
	2 $\mu\text{g} \times 2$	8	6.5 $\pm$ 0.2*	3.3 $\pm$ 0.6*	6.5 $\pm$ 0.4*	5.3 $\pm$ 1.8*
Dorsal hippocampus	vehicle	6–9	7.2 $\pm$ 0.1 (9)	1.1 $\pm$ 0.3 (9)	7.3 $\pm$ 0.2 (6)	0.9 $\pm$ 0.3 (6)
	2 $\mu\text{g} \times 2$	9	7.0	2.0 $\pm$ 0.5	6.2 $\pm$ 0.4*	5.3 $\pm$ 1.9*
Frontal cortex	vehicle	9	7.2 $\pm$ 0.1	1.1 $\pm$ 0.3	7.3 $\pm$ 0.2	0.9 $\pm$ 0.3
	2 $\mu\text{g} \times 2$	4	7.8 $\pm$ 0.3	0.8 $\pm$ 0.8	7.3 $\pm$ 0.3	1.3 $\pm$ 0.6
Parietal cortex	vehicle	10	7.2 $\pm$ 0.1	1.1 $\pm$ 0.2	7.4 $\pm$ 0.2	0.8 $\pm$ 0.3
	2 $\mu\text{g} \times 2$	3	7.0 $\pm$ 0.6	1.0 $\pm$ 0.6	7.0 $\pm$ 0.6	1.7 $\pm$ 0.9
Mammillary body	vehicle	12	7.4 $\pm$ 0.1	0.8 $\pm$ 0.2	7.3 $\pm$ 0.2	0.9 $\pm$ 0.3
	2 $\mu\text{g} \times 2$	10	6.9 $\pm$ 0.2	3.1 $\pm$ 1.1	6.8 $\pm$ 0.3	3.1 $\pm$ 0.9
Dorsomedial thalamus nucleus	vehicle	9	7.2 $\pm$ 0.1	1.1 $\pm$ 0.3	7.3 $\pm$ 0.2	0.9 $\pm$ 0.3
	2 $\mu\text{g} \times 2$	6	6.2 $\pm$ 0.4*	6.0 $\pm$ 2.1*	6.7 $\pm$ 0.3	4.8 $\pm$ 2.4
Central amygdaloid nucleus	vehicle	10	7.3 $\pm$ 0.2	1.0 $\pm$ 0.3	7.3 $\pm$ 0.2	0.9 $\pm$ 0.3
	2 $\mu\text{g} \times 2$	7	7.1 $\pm$ 0.1	2.7 $\pm$ 1.1	6.7 $\pm$ 0.3	2.6 $\pm$ 0.7
Basolateral amygdaloid nucleus	vehicle	11	7.3 $\pm$ 0.1	1.2 $\pm$ 0.3	7.4 $\pm$ 0.2	0.8 $\pm$ 0.3
	2 $\mu\text{g} \times 2$	9	7.7 $\pm$ 0.2	0.9 $\pm$ 0.5	7.3 $\pm$ 0.2	1.9 $\pm$ 0.9
Medial caudate putamen	vehicle	11	7.4 $\pm$ 0.2	0.9 $\pm$ 0.3	7.4 $\pm$ 0.2	0.8 $\pm$ 0.3
	2 $\mu\text{g} \times 2$	5	7.6 $\pm$ 0.2	0.8 $\pm$ 0.6	7.0 $\pm$ 0.3	3.4 $\pm$ 1.4
Lateral hypothalamus	vehicle	6	7.5 $\pm$ 0.2	0.8 $\pm$ 0.5	7.2 $\pm$ 0.2	2.7 $\pm$ 1.0
	2 $\mu\text{g} \times 2$	3	8.0	0	7.3 $\pm$ 0.3	1.7 $\pm$ 0.9
Basal forebrain	vehicle	10	7.3 $\pm$ 0.2	1.0 $\pm$ 0.3	7.4 $\pm$ 0.2	0.8 $\pm$ 0.3
	2 $\mu\text{g} \times 2$	8	7.4 $\pm$ 0.3	3.9 $\pm$ 1.7	6.1 $\pm$ 0.4*	7.1 $\pm$ 2.3*
Medial septal nucleus	vehicle	12	7.4 $\pm$ 0.1	0.8 $\pm$ 0.2	7.3 $\pm$ 0.2	0.9 $\pm$ 0.3
	2 μg	10	7.6 $\pm$ 0.2	0.9 $\pm$ 0.4	6.8 $\pm$ 0.4	3.5 $\pm$ 1.6
Dorsal raphe nucleus	vehicle	7	7.0 $\pm$ 0.2	1.3 $\pm$ 0.5	7.3 $\pm$ 0.2	1.1 $\pm$ 0.4
	2 μg	11	7.2 $\pm$ 0.2	3.3 $\pm$ 1.1	6.9 $\pm$ 0.2	2.8 $\pm$ 1.0
Medial raphe nucleus	vehicle	6	7.3 $\pm$ 0.3	1.0 $\pm$ 0.6	7.5 $\pm$ 0.2	0.8 $\pm$ 0.5
	2 μg	11	7.4 $\pm$ 0.2	1.5 $\pm$ 0.5	6.7 $\pm$ 0.2*	2.8 $\pm$ 0.5*

The measurement was done 10 and 30 min after the microinjection of scopolamine into each brain region. Scopolamine was injected bilaterally ($\times 2$). Values are the mean $\pm$ S.E.M. * $P < 0.05$ vs the vehicle (Wilcoxon's rank sum test).

Table 3. Changes in the brain ACh contents of the scopolamine-treated rats showing an impairment of spatial cognition

Brain region	ACh content (pmol/mg tissue)	
	vehicle	scopolamine 0.5 mg/kg, i.p.
Ventral hippocampus	59.70 $\pm$ 4.95	25.43 $\pm$ 3.54 (42.6%)*
Dorsal hippocampus	55.71 $\pm$ 8.39	46.00 $\pm$ 4.44 (82.6%)*
Frontal cortex	31.96 $\pm$ 3.64	19.86 $\pm$ 1.25 (62.1%)*
Temporal cortex	21.22 $\pm$ 0.84	10.44 $\pm$ 1.58 (49.2%)*
Parietal cortex	25.48 $\pm$ 2.10	16.50 $\pm$ 2.39 (64.8%)*
Caudate putamen	120.03 $\pm$ 6.49	66.64 $\pm$ 3.43 (55.5%)*
Lateral hypothalamus	48.57 $\pm$ 1.16	35.22 $\pm$ 1.34 (72.5%)*

The data and values in parentheses represent the mean of the ACh contents and % change of vehicle-treated groups in each region, respectively. Values are the mean $\pm$ S.E.M. of 6–8 animals. * $P < 0.05$, *** $P < 0.001$ vs the vehicle (Student's *t*-test).

as well as a group $\times$ fraction interaction [$F(27,396) = 3.601$, $P < 0.001$]. The contrast test revealed the ACh release in the VH was significantly increased compared to that in the FC ($P < 0.01$) and the ABL ($P < 0.05$), but no significant differ-

ence was observed between the other regions. We further investigated the relationship between the behavioral score of the 8-arm radial maze and ACh release. As shown in Fig. 4, the number of correct choices and errors observed at 30 min after scopolamine treatment significantly correlated with the ACh release in the VH during the 6th fraction ($r = 0.867$ for correct choices, $P < 0.01$ and $r = -0.777$ for errors, $P < 0.01$). In all other regions, no significant correlation was observed. Thus spatial cognition (number of correct choices decreased and errors increased) was severely impaired in the 8-arm radial maze task if ACh release decreased in the VH.

DISCUSSION

The present results showed that the i.p. and i.c.v. injections of scopolamine produced a severe impairment of spatial cognition in the 8-arm radial maze, and the degree of the impairment by the i.c.v. injection was similar to that of the i.p. injection previously reported (12). We found that scopolamine acted in the central nervous system. Musca-

Fig. 3. Effects of scopolamine on the ACh release from the ventral and dorsal hippocampus, frontal cortex and basolateral amygdala nucleus into Ringer's solution containing 0.1 mM eserine sulfate in highly trained rats. Scopolamine was injected i.p. into the highly trained rats immediately after sampling the 3rd fraction (F3). The ACh concentrations are expressed as a % of the F3 sample taken immediately before scopolamine administration. Values are the mean $\pm$ S.E.M. ○, VH (n = 12); ●, DH (n = 12); ▲, FC (n = 12); ◆, ABL (n = 13).

rinic receptors have been divided pharmacologically into five subtypes (M_1 to M_5) based on their affinity to such antagonists as pirenzepine, AF-DX116 and hexahydro-sila-difenidol (16). M_1 receptors predominate in the hippocampus and cortex, in which cholinergic transmission is thought to play an important role in the learning and memory processes. Pirenzepine, a selective M_1 receptor antagonist, has been reported to disrupt the cognitive function in rats (17, 18). On the other hand, presynaptic autoreceptors in the hippocampus and cortex are thought to predominantly appear in the M_2 subtype (19). Therefore, we examined the effects of pirenzepine, an M_1 antagonist, and AF-DX116, an M_2 antagonist, on the spatial cognition in the 8-arm radial maze task. The present study showed that the i.c.v. injection of pirenzepine, but not AF-DX116, disrupted the spatial cognition of this task in a dose-dependent manner. These facts suggested the involvement of M_1 receptors in the spatial cognition of the 8-arm radial maze task in rats.

It is known that an increase in ACh release from the ter-

minal causes a positive feedback mechanism by prolonged blockade of the postsynaptic M_1 receptor and disinhibition by presynaptic M_2 blockade (20–22). However, the positive feedback mechanism by prolonged blockade of the postsynaptic M_1 receptor can be neglected in the present study because of an increase in ACh concentration by cholinesterase inhibition of eserine in Ringer's solution. We examined the effects of scopolamine, pirenzepine and AF-DX116 on ACh release in the VH using brain microdialysis with Ringer's solution containing 0.1 mM eserine sulfate because the VH is the most important region of scopolamine-induced impairment of spatial cognition as shown in Table 2. The present biochemical study shows that scopolamine and AF-DX116 significantly increased extracellular ACh release in all regions tested. In contrast, pirenzepine did not affect the ACh release. Our data revealed that scopolamine and AF-DX116 increased the ACh release by disinhibition of the M_2 receptor blocking, although eserine, cholinesterase inhibitor, was contained in Ringer's solution. In addition, the increase in ACh release from

Fig. 4. Correlation between the degree of spatial cognition impairment and regional ACh concentrations in scopolamine-treated rats. The Spearman's correlation coefficients by rank are shown as R-values.

the presynaptic terminal also resulted in a decrease in the ACh content in the terminal (23, 24).

We next focused on which brain region was most related to the observed behavioral changes. We examined the changes in ACh contents of various brain regions in scopolamine-treated rats and the effect of microinjection of scopolamine into various brain regions on the spatial cognition. At 10 min after the microinjection of scopolamine into the VH and DMT, the spatial cognition was markedly impaired in the 8-arm radial maze task. The numbers of errors increased in the DMT, rather than the VH, because perseverative behavior, in which the DMT-treated group produced a repetitive response pattern choosing two arms away (90°) from the one currently visited, was observed in the DMT. The abnormal behavior continued 24 and 48 h after the treatment. On the other hand, such perseverative behavior was not observed in the VH. Therefore the spatial cognition impairment in the DMT was interpreted as abnormal behavior. Furthermore, at 30 min after treatment, the spatial cognition was also impaired by the injection of scopolamine into the VH, DH, BF and MR. It is difficult to explain spatial cognition impairment at 30 min after the injection of scopolamine into the DH, BF and MR, but the impairment may not be a direct action of scopolamine in these regions. The biochemical findings also showed marked and prolonged changes in the ACh content and release, especially in the VH, although ACh release from the DMT was not measured because of the induction of perseverative behavior after scopolamine in the 8-arm radial maze. These results suggest that the VH may thus play an important role behaviorally and biochemically as an action site of scopolamine-induced impairment of spatial cognition in the 8-arm radial maze task. Several reports have provided evidence for higher levels in such cholinergic markers such as ChAT (25), ACh contents and release (26), the QNB binding site (27), and the co-existence of the neuropeptide galanin (28) in the VH. On the other hand, other reports have also demonstrated the importance of the DH, not the VH, in the hippocampal area. For example, such studies were compared with several brain regions regarding the learning behavior impairment by brain lesions (29) and by the microinjection of anticholinergic drugs (17), hippocampal place unit (30) and long-term potentiation (31). It was reported that there are two pathways of the ACh system in the hippocampus: the DH (septal end) receives fibers from both the septum-diagonal band area through the fimbria-fornix/supracallosal and the lateral entorhinal area, while the VH (temporal end) receives fibers from both diagonal band areas through the pyriform lobe and the amygdala and the hypothalamus (32, 33). However, much evidence supports the importance of the VH by showing that the propagation of the neural activity increased along the septotemporal (VH connecting area) axis

of the hippocampus by optical monitoring (34). Furthermore, as described above, higher neural activities of the VH have also been reported, especially using cholinergic makers (25–28). These results all closely agree with our present findings regarding the importance of the VH rather than the DH in the scopolamine-induced impairment of spatial cognition.

It is possible that scopolamine-induced disruption of spatial cognition may thus be influenced by the balance between the postsynaptic M_1 receptor blocking action and presynaptic M_2 receptor blocking action, especially in the VH. To overcome this problem, we designed the new experimental schedule for measurements in the 8-arm radial maze task and ACh release by microdialysis using the same animal as shown in Fig. 3A. We performed a statistical analysis to determine if there are any correlations between the behavioral and biochemical changes. A significant correlation between scopolamine-induced impairment of spatial cognition and regional ACh release was only found in the VH. This finding indicates that the rats with a slightly impaired spatial cognition induced by scopolamine demonstrated a marked increase in ACh release in the VH, whereas markedly impaired rats exhibited a lower ACh release in the VH. The above findings suggest that the degree of the ACh release in the VH may thus be influenced by the induction of spatial cognitive impairment by scopolamine. Although few previous reports about the relationship between behavioral and biochemical changes have been described, ChAT (3) and HACU (4) have been reported to increase after daily training, while ACh release increased during the performance of learning and memory tasks (6, 7). The findings of these reports also closely agree with our present results. Moreover, it is reported that AF-DX384, an M_2/M_4 receptor antagonist, improved scopolamine-induced impairment of passive avoidance and object recognition (35), and low doses of scopolamine alone facilitated consolidation and retrieval of passive avoidance in mice (36). These reports suggest that the facilitatory effect is attributed to an increase in ACh release by the blockade of presynaptic M_2/M_4 receptors and also support our results that the scopolamine-induced disruption of spatial cognition may be influenced by the balance between the postsynaptic M_1 receptor blocking action and presynaptic M_2 receptor blocking action.

It is known that cognitive enhancers show “bell-shaped” dose-dependencies in learning and memory tasks, especially when cognitive enhancers are administered to animals with scopolamine-induced disruption of learning and memory (12). The biochemical change responsible for the “bell-shaped” dose-dependency still remains undetermined, although it has been psychologically interpreted as changes in arousal and attentional processes influenced by cognitive enhancers. In the present study, scopolamine im-

paired spatial cognition in the 8-arm radial maze task by blocking not only the postsynaptic M_1 receptor but also by blocking the presynaptic M_2 receptor, thus resulting in the increase in ACh release by inhibiting its feedback. These results suggest that the scopolamine-induced disruption of spatial cognition may thus be influenced by the balance between the postsynaptic M_1 receptor blocking action and presynaptic M_2 receptor blocking action. This may be related to the generally observed "bell-shaped" dose-dependency when cognitive enhancers improved scopolamine-induced impairment of learning and memory.

In the present study, a close relationship between the induction of the impairment of spatial cognition and the degree of ACh release in the VH was found in scopolamine-treated rats. These results suggest that the blocking balance between M_1 and M_2 muscarinic receptor in the VH therefore plays a major role in the spatial cognition impairment induced by scopolamine in the 8-arm radial maze task.

Acknowledgments

A part of this study was supported by a Grant-in-Aid for Scientific Research from Japanese Ministry of Education, Science, Sports and Culture. We are grateful to Dr. Y. Ohgami for skillful and thorough technical assistance and Dr. B. Quinn for proofreading the manuscript.

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