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Effects of *Uncaria tomentosa* Total Alkaloid and its Components on Experimental Amnesia in Mice: Elucidation Using the Passive Avoidance Test

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Abstract

The effects of *Uncaria tomentosa* total alkaloid and its oxindole alkaloid components, uncarine E, uncarine C, mitraphylline, rhynchophylline and isorhynchophylline, on the impairment of retention performance caused by amnesic drugs were investigated using a step-down-type passive avoidance test in mice. In this test, the retention performance of animals treated with the amnesic and test drugs before training was assessed 24 h after training.

Uncaria tomentosa total alkaloid (10–20 mg kg^{-1}, i.p.) and the alkaloid components (10–40 mg kg^{-1}, i.p.), as well as the muscarinic receptor agonist oxotremorine (0·01 mg kg^{-1}, i.p.), significantly attenuated the deficit in retention performance induced by the muscarinic receptor antagonist scopolamine (3 mg kg^{-1}, i.p.). The effective doses of uncarine C and mitraphylline were larger than those of other alkaloid components. Uncarine E (20 mg kg^{-1}, i.p.) also blocked the impairment of passive avoidance performance caused by the nicotinic receptor antagonist mecamylamine (15 mg kg^{-1}, i.p.) and the *N*-methyl-D-aspartate (NMDA) receptor antagonist (±)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP; 7·5 mg kg^{-1}, i.p.), but it failed to affect the deficit caused by the benzodiazepine receptor agonist diazepam (2 mg kg^{-1}, i.p.). Rhynchophylline significantly reduced the mecamylamine-induced deficit in passive avoidance behaviour, but it failed to attenuate the effects of CPP and diazepam.

These results suggest that *Uncaria tomentosa* total alkaloids exert a beneficial effect on memory impairment induced by the dysfunction of cholinergic systems in the brain and that the effect of the total alkaloids is partly attributed to the oxindole alkaloids tested. Moreover, these findings raised the possibility that the glutamatergic systems are implicated in the anti-amnesic effect of uncarine E.

Uncaria tomentosa, a plant known as cat's claw, has been widely used as a folk medicine by native people of the Peruvian rain forest to treat arthritis, asthma, cancer, rheumatism, gastric ulcer and skin diseases (Cabieses 1994; Jones 1995). In-vitro studies have demonstrated that the extract of *Uncaria tomentosa* has potent immunostimulatory (Keplinger et al 1999), anti-inflammatory (Sandoval-Chacon et al 1998) and anti-tumour actions (Sheng et al 1998).

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In terms of chemical constituents, this plant appears to be closely allied to other *Uncaria* species distributed in South-East Asia (Hemingway & Phillipson 1974), since some of the alkaloid components such as rhynchophylline and isorhynchophylline isolated from *Uncaria tomentosa* are also found in *Uncaria rhynchophylla* (MIQ.) Jackson in Japan and *Uncaria sinensis* (Oliv.) Havil in China. However, recent chemical studies revealed the presence of new pentacyclic indole and oxindole alkaloids in *Uncaria tomentosa* but not in other Uncaria species (Wagner & Kreutzkamp 1985; Laus & Keplinger 1994; Keplinger et al 1999).

Uncaria species have been used medicinally and included in a number of traditional Sino-Japanese herbal prescriptions such as Choto-san in Japan or Diao-Teng-San in China. Alkaloids isolated from these plants exert a variety of pharmacological actions such as sedative, antihypertensive and anticonvulsant activity in animals (Du 1987; Tang & Eisenbrand 1992). Moreover, it has been demonstrated that administration of *Uncaria sinensis* (Oliv.) Havil extract improves the disruption of spatial cognition in rats (Egashira et al 1993). However, only a little information is available on the in-vivo neuropharmacological effects of *Uncaria tomentosa* (Reinhard 1999). Thus, in this study to clarify the neuropharmacological profiles of *Uncaria tomentosa*, we examined the effects of total alkaloid and oxindole alkaloid components obtained from this plant on memory disruptions induced by several amnesic drugs using the step-down-type passive avoidance test in mice.

Materials and Methods

Drugs

Powdered Peruvian Uña de Gato (200 g), which had been mixed with $Ca(OH)_2$ and steamed for about 5 h, was extracted with hot toluene. The concentrated toluene extract was dissolved with ethyl acetate and the whole was shaken with diluted sulfuric acid (2%). The acid layer was basified with NH_4OH. The aqueous layer was extracted with $CHCl_3$ to give the crude base (total alkaloid, 698 mg), which was purified by SiO_2 column chromatography to give the alkaloid components (Figure 1), isopteropodine (Uncarine E, 211 mg), pteropodine (Uncarine C, 51 mg), mitraphylline (10 mg), isorhynchophylline (55 mg) and rhynchophylline (6 mg). The purity ($>99\%$) of each component was confirmed by ^{1}H-NMR analysis (500 MHz, $CDCl_3$).

The following drugs were obtained from commercial sources: scopolamine hydrobromide, oxotremorine hydrochloride and mecamylamine hydrochloride (Sigma Chemical Co., St Louis, MO); diazepam (Cercine injection, Takeda Pharmaceutical Co., Osaka, Japan), flumazenil (Anexate, Yamanouchi Pharmaceutical Co., Tokyo, Japan) and pirenzepine dihydrochloride and (±)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP: Research Biochemicals Inc., Natick, MA).

Uncaria tomentosa total alkaloid and alkaloid components were suspended in saline containing 2% dimethylsulphoxide. Diazepam was dissolved

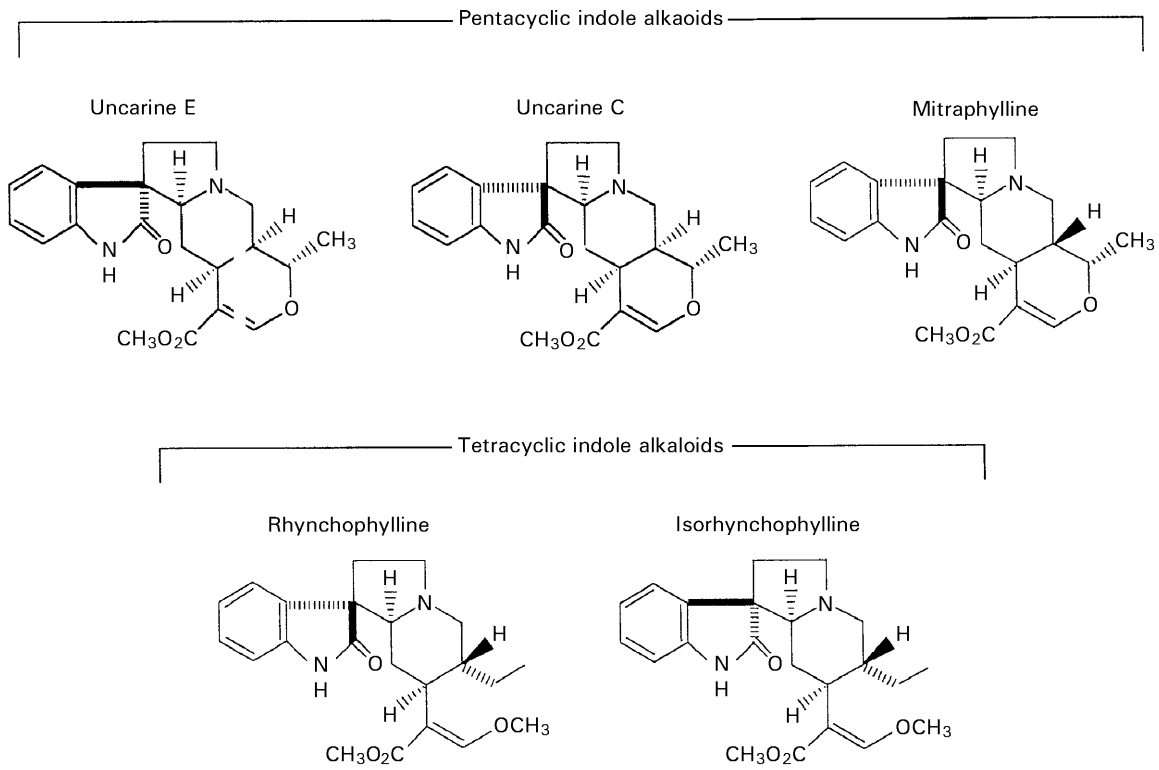

Figure 1. Chemical structure of *Uncaria tomentosa* alkaloids, uncarine E, uncarine C, mitraphylline, rhynchophylline and isorhynchophylline.

in saline containing 40% propylene glycol and flumazenil was dissolved in saline containing 0·1% Tween 80. The other test drugs were dissolved in saline. All drug solutions were prepared just before the start of the experiments. Intraperitoneal injections were performed in a volume of 1·0 mL/100 g of animal weight, and intracerebroventricular injections were made in a volume of 5 μL according to the methods of Haley & McCormick (1957).

Animals

Male ddY mice, 8–9 weeks old (Japan SLC Inc., Hamamatsu, Japan), were used. Mice were housed in groups of 20 per cage (35 × 30 × 16 cm) for at least one week before the experiments. Housing conditions were thermostatically maintained at 24 ± 2°C with constant humidity (55 ± 5%) and a 12-h light–dark cycle. Animals were given free access to food and water. These studies were conducted in accordance with the standards established by the Guide for the Care and Use of Laboratory Animals of Toyama Medical and Pharmaceutical University.

Step-down passive avoidance test

The apparatus used for the step-down-type passive avoidance test consisted of a chamber equipped with a grid floor (25 × 25 × 30 cm), through which scrambled electric shocks were delivered by a shock generator (SGS-002, Muromachi Kikai Co. Ltd, Tokyo, Japan), and an escape rubber platform (4·5 × 4·5 cm) located in the corner of the chamber. The experiments were performed by slightly modifying the method described in a previous report (Nomura et al 1994). In the training trial of the passive avoidance test, a mouse was placed on the platform and allowed to explore for 8 min. When the mouse stepped down from the platform and all paws touched the grid floor, electric foot-shocks (0·4 mA) were delivered. During the last 3 min of the training trial period, the time that elapsed before the mouse step-down, was recorded as the step-down latency in the training trial. Twenty-four hours after training, mice were tested for their retention performance. In the retention trial, latency to step-down from the platform was measured up to a maximum of 180 s. Amnesic drugs were given intraperitoneally or intracerebroventricularly 30 min or 15 min, respectively, before the training trial. *Uncaria tomentosa* total alkaloid, the alkaloid components, oxotremorine, and flumazenil were given intraperitoneally 30 min before the injection of the amnesic drugs.

Measurement of spontaneous motor activity in mice

An Animex activity meter (MK-110, Muromachi Kikai Co., Tokyo) was used to measure spontaneous motor activity in the mice. The administration schedule of drugs was the same as that used in the passive avoidance test. Briefly on day 1, mice received either an injection of vehicle or total alkaloid (20 mg kg^{-1}, i.p.). Thirty minutes later, the vehicle-treated mice received saline or scopolamine (3 mg kg^{-1}, i.p.), and the total-alkaloid-treated mice received scopolamine (3 mg kg^{-1}, i.p.). Thirty minutes after the last injection, three mice from each group were placed in a cage (35 × 40 × 35 cm) and their motor activity was measured at 10-min intervals over a 30-min period. On day 2 (24 h after the last injection), the motor activity of these mice was measured again for a 30-min period. Each group consisted of 18 mice.

Statistical analysis

Data were analysed by Kruskal-Wallis analysis of variance followed by Dunn's test for multiple comparisons among different groups. Differences with $P < 0.05$ were considered significant.

Results

Effect of Uncaria tomentosa *total alkaloid on scopolamine-induced deficit in passive avoidance response*

As shown in Figure 2A, pre-training treatment with scopolamine (0·5–3 mg kg^{-1}, i.p.) had no effect on the step-down latency in the training trial, but at a dose of 3 mg kg^{-1} (i.p.), it significantly reduced the step-down latency in the retention trial. When administered 30 min before scopolamine (3 mg kg^{-1}, i.p.), *Uncaria tomentosa* total alkaloid (10–20 mg kg^{-1}, i.p.), as well as the cholinergic muscarinic receptor agonist oxotremorine (0·01 mg kg^{-1}, i.p.), dose dependently attenuated the deficit in passive avoidance response induced by scopolamine (Figure 2B).

To test if the effect of *Uncaria tomentosa* total alkaloid on scopolamine-induced deficit in the passive avoidance response is apparently due to motor dysfunction caused by scopolamine and *Uncaria tomentosa* total alkaloid, the spontaneous motor activity of mice was measured 30 min (day 1) and 24 h (day 2) after administration of scopolamine (3 mg kg^{-1}, i.p.) alone or with *Uncaria tomentosa* total alkaloid (20 mg kg^{-1}, i.p.). As summarized in Table 1, neither scopolamine alone nor scopolamine plus *Uncaria tomentosa* total

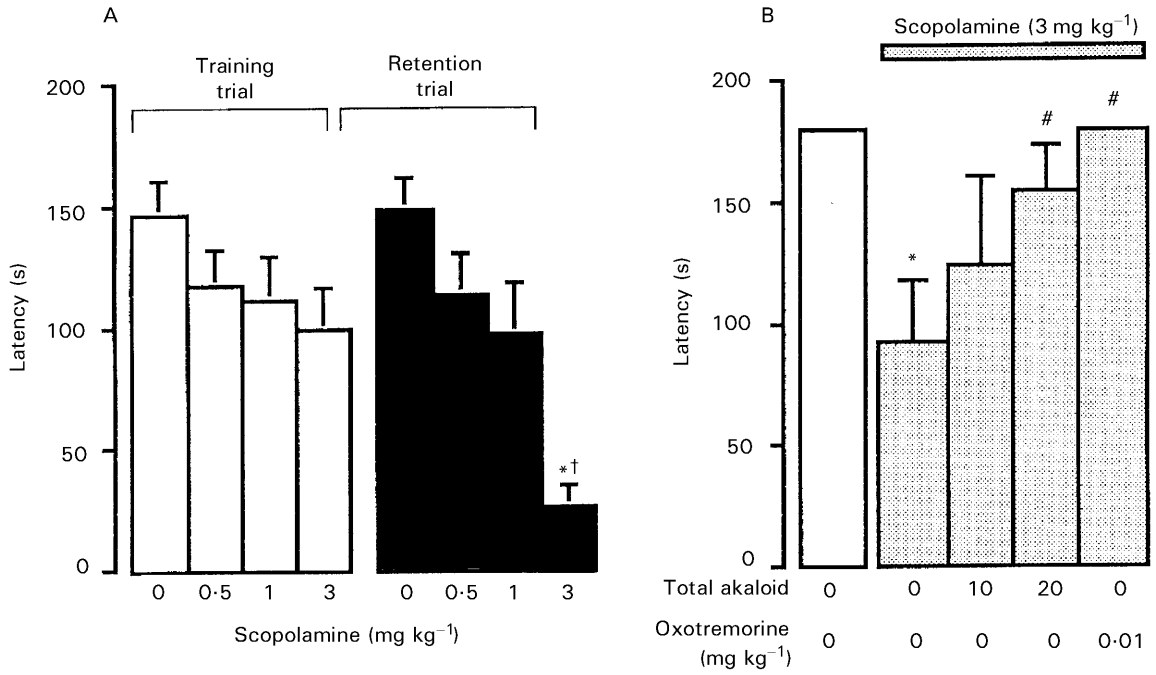

Figure 2. Effect of *Uncaria tomentosa* total alkaloid and oxotremorine on scopolamine-induced impairment of retention performance in the passive avoidance test in mice. A. Scopolamine (0·5–3 mg kg^{-1}, i.p.) was injected 30 min before the training trials. Twenty-four hours after the training trials, the retention trials were performed. *$P < 0·05$ compared with vehicle control and †$P < 0·05$ compared with the performance of 3 mg kg^{-1} scopolamine-pretreated mice in the training trials. B. Total alkaloid (10–20 mg kg^{-1}, i.p.) or oxotremorine (0·01 mg kg^{-1}, i.p.) was administered 30 min before scopolamine injection in the training trials, and the memory acquisition was tested 24 h after the training trial. *$P < 0·05$ compared with vehicle alone. #$P < 0·05$ compared with scopolamine alone. Each column represents the mean ± s.e.m., n = 13–19.

Table 1. Effect of scopolamine on the locomotor activity of *Uncaria tomentosa* total alkaloid-treated mice.

Drug treatment	No. of movements (counts)	
	Day 1	Day 2
Vehicle + saline	952·50 ± 182·21	1012·00 ± 220·48
Vehicle + scopolamine (3 mg kg^{-1})	1571 ± 368	1052·00 ± 138·36
Uncaria tomentosa total alkaloid (20 mg kg^{-1}) + scopolamine (3 mg kg^{-1})	1350·5 ± 398·0	999·00 ± 98·49

Data represent the mean counts/30 min/3 mice ± s.e.m., of 6 independent experiments.

alkaloid significantly affected the motor activity of the mice measured on days 1–2.

Effect of Uncaria tomentosa *alkaloid components on muscarinic receptor antagonist-induced deficit in passive avoidance response*

Pre-treatment with uncarine E, rhynchophylline and isorhynchophylline (20 mg kg^{-1}, i.p.) significantly reversed scopolamine-induced behavioural deficit in the retention trial (Figure 3). Uncarine C and mitraphylline also attenuated the effect of scopolamine (only significant at a dose of 40 mg kg^{-1}, i.p.).

Pretraining administration of pirenzepine (3 μg/mouse, i.c.v.), a selective muscarinic M_1 receptor antagonist, had no effect on step-down latency in the training trial but significantly reduced the latency assessed in the retention trial (Figure 4A). Pre-treatment of the mice with uncarine E (20 mg kg^{-1}, i.p.) before intracerebroventricular pirenzepine also significantly reduced the memory impairment induced by pirenzepine (Figure 4B).

Effect of uncarine E and rhynchophylline on mecamylamine-induced deficit in passive avoidance response

When administered before training, the nicotinic receptor antagonist mecamylamine (15 mg kg^{-1}, i.p.) significantly reduced step-down latency in the retention trial but not in the training trial (Figure 5A). Pre-treatment with uncarine E and rhynchophylline (20 mg kg^{-1}, i.p.) significantly attenuated the deficit in passive avoidance response induced by mecamylamine (Figure 5B).

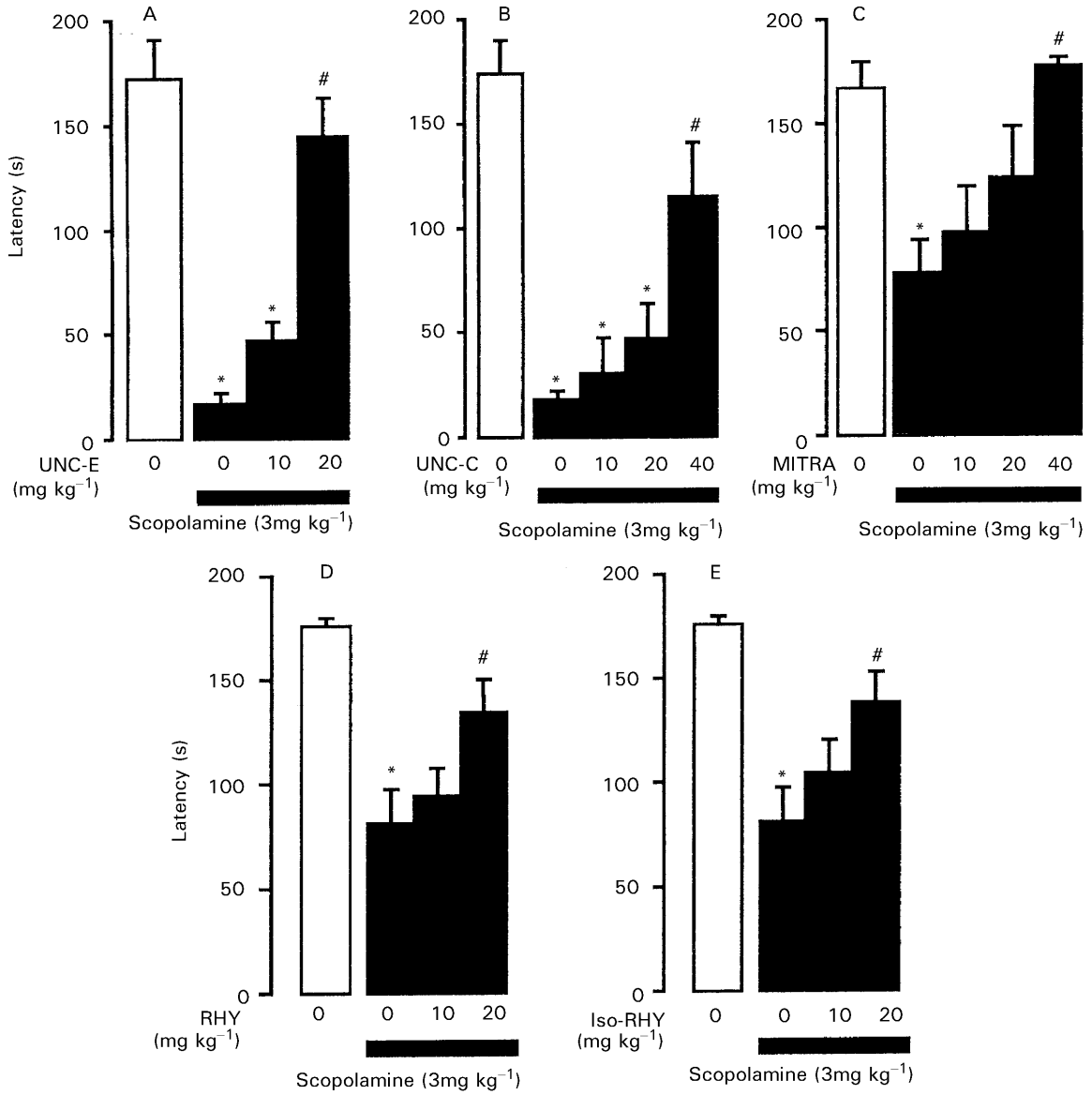

Figure 3. Effects of *Uncaria tomentosa* alkaloid components, uncarine E (A: UNC-E), uncarine C (B: UNC-C), mitraphylline (C: MITRA), rhynchophylline (D: RHY) and isorhynchophylline (E: Iso-RHY) on scopolamine-induced impairment of retention performance in mice. Each alkaloid component was administered intraperitoneally 30 min before scopolamine (3 mg kg^{-1}, i.p.) injection in the training trials. In the retention trial, performed 24 h after the training trials, the passive avoidance performance was tested. $*P < 0{\cdot}05$ compared with vehicle alone. $\#P < 0{\cdot}05$ compared with scopolamine alone. Each data column represents the mean ± s.e.m., n = 10–12.

Effect of uncarine E and rhynchophylline on deficit in the passive avoidance response caused by CPP and diazepam

Pre-training administrations of the selective NMDA receptor antagonist CPP (Figure 6A) and the benzodiazepine receptor agonist diazepam (Figure 6C) significantly impaired the passive avoidance performance in the retention trial in a dose-dependent manner. Uncarine E (20 mg kg^{-1}, i.p.) but not rhynchophylline (20 mg kg^{-1}, i.p.) significantly reversed the deficit caused by CPP (7·5 mg kg^{-1}, i.p.) (Figure 6B). The selective benzodiazepine receptor antagonist flumazenil (10 mg kg^{-1}, i.p.), however, significantly reversed the diazepam (2 mg kg^{-1}, i.p.)-induced impairment of retention performance, while uncarine E or rhynchophylline failed to attenuate the effect of diazepam (Figure 6D).

Discussion

This study demonstrates that *Uncaria tomentosa* total alkaloid produces an ameliorative effect on

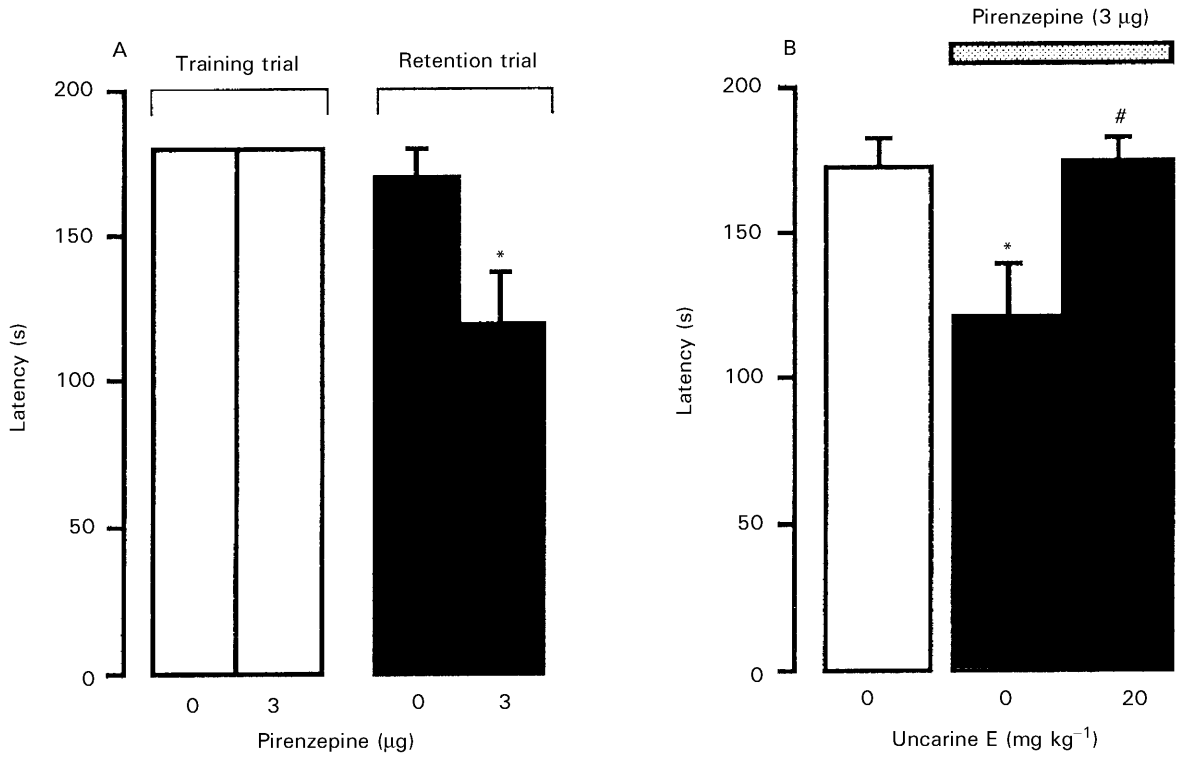

Figure 4. Effect of uncarine E on impairment of retention performance caused by the selective muscarinic M_1 receptor antagonist pirenzepine. A. Pirenzepine (3 μg/mouse) was injected intracerebroventricularly 15 min before the training trials. Twenty-four hours after the training trials, the retention trials were performed. $^*P < 0{\cdot}05$ compared with the vehicle control. B. Uncarine E (20 mg kg^{-1}, i.p.) was administered 30 min before pirenzepine injection in the training trial, and the memory acquisition was tested 24 h after the training trials. $^*P < 0{\cdot}05$ compared with the vehicle alone. $\#P < 0{\cdot}05$ compared with pirenzepine alone. Each data column represents the mean $\pm$ s.e.m., n = 8–10.

scopolamine-induced impairment of memory acquisition in mice and that its oxindole alkaloid components, uncarine E, uncarine C, mitraphylline, rhynchophylline and isorhynchophylline, at least partly, contribute to the effect of the total alkaloid.

The systemic administration of scopolamine before the training trial significantly impaired the passive avoidance responses in the retention trial, and this amnesic effect of scopolamine was abolished by oxotremorine, a muscarinic receptor agonist. These results agree with previous findings (Rush 1988; Verloes et al 1988; Nomura et al 1994) and indicate that the dysfunction of central cholinergic systems is attributed to the scopolamine-induced amnesia assessed by the passive avoidance test. Moreover, we found that the systemic administration of *Uncaria tomentosa* total alkaloid significantly antagonized the amnesic effect of scopolamine. It is possible that *Uncaria tomentosa* total alkaloid ameliorated the scopolamine-induced amnesia by producing a sedative effect on scopolamine-treated mice since drugs capable of causing motor dysfunction can prolong the step-down latency. This possibility, however, seems slight because no significant difference in spontaneous motor activity was found between groups of mice treated with scopolamine alone and scopolamine plus *Uncaria tomentosa* total alkaloid. Thus, the results suggest that the total alkaloid of this plant is able to exert an activity in the CNS, and that the reversal of scopolamine-induced amnesia by the total alkaloid is due to modulation of the central cholinergic systems.

It is interesting that the oxindole alkaloid components also exerted an ameliorative effect on scopolamine-induced impairment of retention performance in the passive avoidance test and that the effective dose range of the components, except uncarine C and mitraphylline, was similar to that of the total alkaloid. From these findings, it is conceivable that the anti-amnesic action of the total alkaloid is at least partly due to the effects of the alkaloid components tested and that the central muscarinic cholinergic systems are implicated in the effects of *Uncaria tomentosa* alkaloids. This idea seems further supported by the antagonism between uncarine E and the selective muscarinic M_1 receptor antagonist pirenzepine in the passive avoidance test. In this study, when administered before training, pirenzepine as well as scopolamine impaired only the retention performance, and this deficit was significantly reversed by uncarine E.

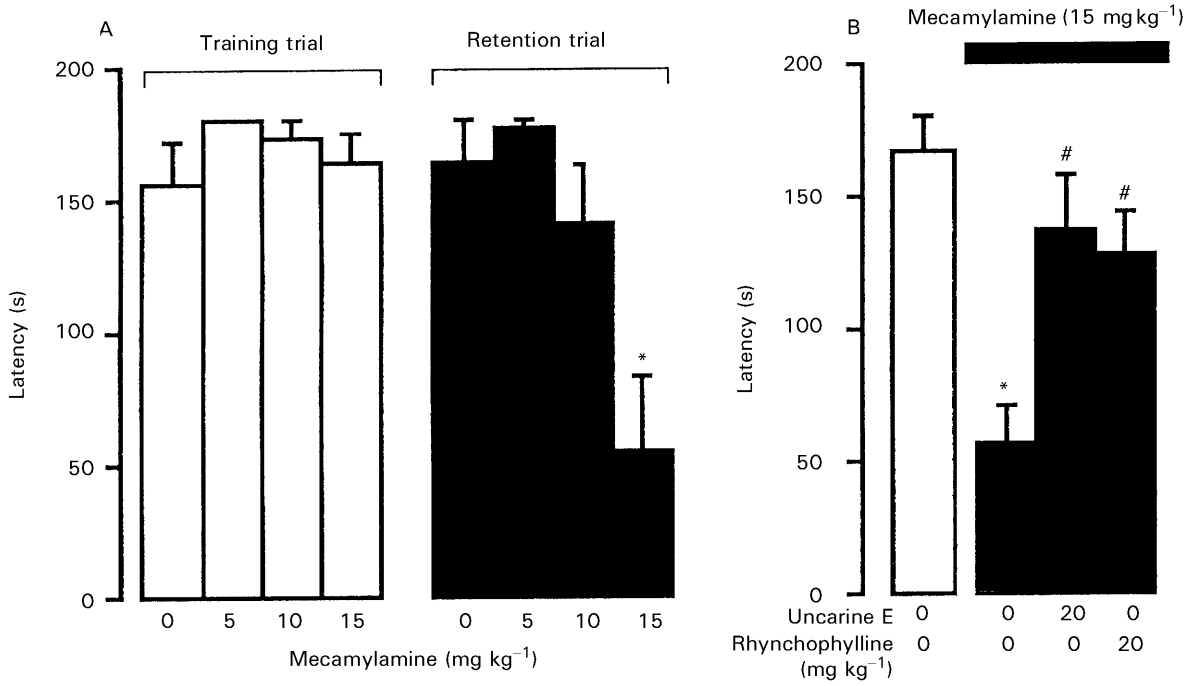

Figure 5. Effect of uncarine E and rhynchophylline on the impairment of memory acquisition caused by the nicotinic receptor antagonist mecamylamine. A. Mecamylamine (5–15 mg kg^{-1}) was injected intraperitoneally 30 min before the training trials. Twenty-four hours after the training trials, the retention trials were performed. *$P < 0.05$ compared with the vehicle control. B. Uncarine E (20 mg kg^{-1}, i.p.) or rhynchophylline (20 mg kg^{-1}, i.p.) was administered 30 min before mecamylamine injection in the training trial, and the memory acquisition was tested 24 h after the training trials. *$P < 0.05$ compared with the vehicle alone. #$P < 0.05$ compared with pirenzepine alone. Each data column represents the mean ± s.e.m., n = 10–12.

Considering the yield of each alkaloid component from the total alkaloid fraction, we can not exclude the involvement of other components, or synergistic interactions among the alkaloid components, in the effect of the total alkaloid fraction on scopolamine-induced deficit in retention performance. Further experiments are needed to clarify these possibilities.

Moreover, our study has revealed that mecamylamine, a nicotinic receptor antagonist, given before training produces a deficit in the retention performance but not the training performance of mice and that uncarine E and rhynchophylline significantly antagonize the effect of mecamylamine. It has been demonstrated that stimulation of central nicotinic receptors reverses the experimental amnesia caused by the blockade of central muscarinic receptors via activation of the nicotinic cholinergic and dopaminergic systems (Nitta et al 1994). Recently Hiramatsu et al (1998) showed that the blockade of nicotinic receptors by mecamylamine reduces acetylcholine release in the brain and causes impairment of passive avoidance performance in rats. Taken together, a speculative explanation for the mechanisms of the action of uncarine E and rhynchophylline is that these compounds may enhance the central cholinergic transmission by increasing the acetylcholine level in the cholinergic synapses or by modulating the activity of other neuronal systems such as dopaminergic systems capable of enhancing the central cholinergic function. Further experiments are needed to test our hypothesis.

In this study, the competitive NMDA receptor antagonist CPP and the benzodiazepine receptor agonist diazepam given before training significantly reduced the step-down latency in the retention trial but not in the training trial. These findings are consistent with previous reports (Patel et al 1979; Venault et al 1986; Izquierdo et al 1990; Parada-Turska & Turski 1990; Venable & Kelly 1990; Reddy & Kulkarni 1998). We found that uncarine E also significantly attenuated the CPP-induced deficit in retention performance at a dose of 20 mg kg^{-1} (i.p.), while at the same dose, failed to reverse the deficit caused by diazepam. Since it has been reported that the benzodiazepine-induced impairment of passive avoidance is mainly mediated by GABAergic systems in mice (Tohyama et al 1991), it is likely that this alkaloid is also capable of interacting with glutamatergic systems but not with GABAergic systems in the brain. Moreover, this study revealed that unlike uncarine E, rhynchophylline had no effect on the retention performance of CPP- or diazepam-treated animals at a dose that significantly ameliorated the scopolamine- and mecamylamine-induced impairment of passive avoidance behaviour.

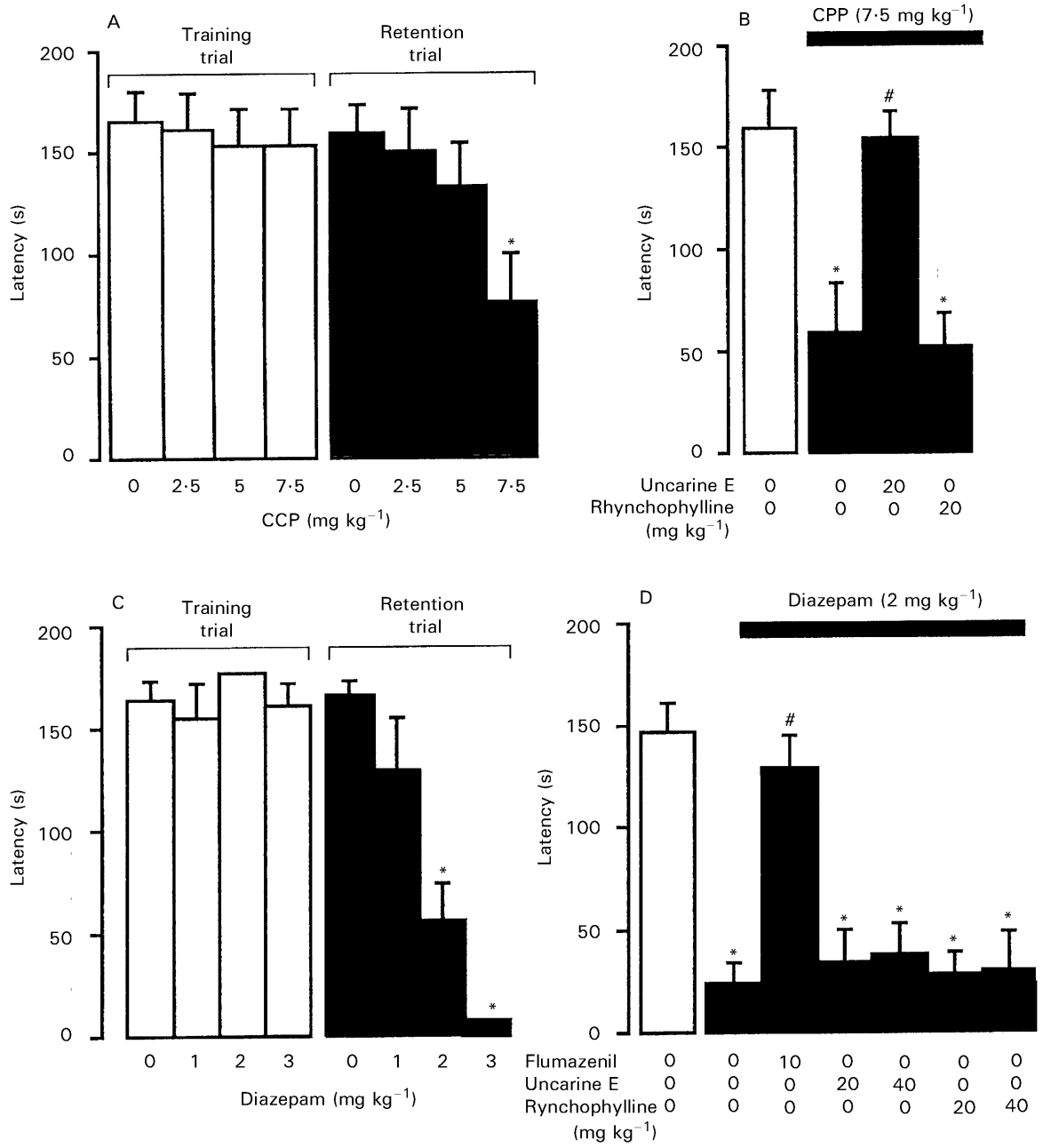

Figure 6. Effects of uncarine E (UNC-E) and rhynchophylline (RHY) on the impairment of memory acquisition caused by the NMDA receptor antagonist CPP and the benzodiazepine receptor agonist diazepam. A and C. CPP (2·5–7·5 mg kg^{-1}, i.p.; A) or diazepam (1–3 mg kg^{-1}, i.p.; C) was injected 30 min before the training trials. The retention trials were performed 24 h after the training trial. *$P < 0{\cdot}05$ compared with the vehicle control. B and D. Uncarine E (20 mg kg^{-1}, i.p.; B) or rhynchophylline (20 mg kg^{-1}, i.p.; D) was administered 30 min before pretraining administration of CPP (B) or diazepam (D). The benzodiazepine receptor antagonist flumazenil (10 mg kg^{-1}) was administered 30 min before diazepam in the training trials. The retention performance was elucidated 24 h after the training trial. *$P < 0{\cdot}05$ compared with the vehicle alone. #$P < 0{\cdot}05$ compared with CPP or diazepam alone. Each column represents the mean ± s.e.m., n = 8–12.

Thus, it might be anticipated that rhynchophylline would be able to more selectively modify the activity of central cholinergic systems than uncarine E. Further investigations are needed to assess this possibility.

Pharmacological evidence has demonstrated functional co-operation between nicotinic cholinergic and glutamatergic systems in the brain (i.e. stimulation of nicotinic receptors facilitates the release of glutamate, while NMDA receptor stimulation

enhances acetylcholine release in the brain (Snell & Johnson 1986; Nishimura & Boegman 1990; Vidal 1994)). Thus, from the finding that uncarine E is capable of attenuating both mecamylamine- and CPP-induced amnesia in the passive avoidance test, one might infer that uncarine E selectively interacts with one of these neuronal systems in the brain and apparently reverses the effect of the selective antagonist for the other neuronal system. This possibility remains to be elucidated by further experiments.

In conclusion, *Uncaria tomentosa* total alkaloid may have a beneficial effect on amnesia caused by cholinergic dysfunction. Moreover, it is likely that not only cholinergic systems but also glutamatergic systems are implicated in the anti-amnesic effect of uncarine E, one of the alkaloid components that is present in *Uncaria tomentosa* but not in other *Uncaria* species.

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