Protective Effect of GTS-21, a Novel Nicotinic Receptor Agonist, on Delayed Neuronal Death Induced by Ischemia in Gerbils

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Received September 4, 1997 Accepted October 7, 1997

ABSTRACT—The neuroprotective effects of GTS-21 [3-(2,4-dimethoxybenzylidene)-anabaseine dihydrochloride] were studied and compared with those of nicotine, 9-amino-1,2,3,4-tetrahydroacridine hydrochloride hydrate (THA) and pentobarbital-Na (PB) using a cerebral ischemia model in Mongolian gerbils. The learning performance and memory retention were elucidated by a step-through passive avoidance task at 2 and 3 days after ischemia-reperfusion. In this task, the ischemia-operated gerbils showed impairment of learning performance and memory retention. Neuronal cell death in the hippocampal CA1 area was observed at 7 days after ischemia. When administered i.p. 30 min before ischemia, GTS-21 (5 mg/kg), (−)-nicotine (1.5 mg/kg), THA (5 mg/kg) and PB (50 mg/kg) significantly attenuated the impairment of passive avoidance performance and the neuronal cell death induced by the ischemia. When administered orally twice daily for 2 weeks prior to the ischemia, GTS-21 (10 mg/kg) significantly suppressed both amnesia and neuronal cell death, while (−)-nicotine (10 mg/kg) and THA (10 mg/kg) suppressed only the amnesia. These results suggest that GTS-21 exerts a protective activity on not only impairment of learning and memory but also delayed neuronal death and that the underlying mechanism of GTS-21 differs from that of nicotine or THA.

Keywords: GTS-21, THA, Ischemia, Mongolian gerbil, Nicotinic agonist

Cerebral ischemia produces severe neuronal damage of the central nervous system in humans (1, 2). A hemiplegia or a speech impediment has been induced in patients suffering from cerebrovascular diseases as a sequel of ischemia. Hachinski et al. (3) reported that vascular disease is responsible for dementia, as it gives rise to multiple small and large infarcts. Neurons in the central nervous system have different vulnerability to ischemic injury, and the hippocampus is susceptible to ischemic damage (4). Wallin et al. (2) reported that disturbances in the cholinergic systems were found in subcortical and cortical gray matter in a vascular dementia. Disturbances in the cholinergic systems in brain tissue from patients with Alzheimer's disease were found to be similar to those of vascular dementia (2, 5).

We have previously reported that subchronically administered GTS-21, a selective nicotinic agonist, attenuates the neuron loss in the layers II-III of the parietal cortex in the nucleus basalis magnocellularis (nBM)-lesioned rats (6). In addition, GTS-21 has been found to enhance eyeblink classical conditioning in rabbits, to improve the learning performance in aged or nBM-lesioned rats, and to facilitate the induction of long-term potentiation (LTP) in rat hippocampal slices (7-11). A recent study indicates that nicotine and nicotinic agonists exert a protective activity against glutamate neurotoxicity in vitro (12, 13). However, pharmacological effects of nicotinic agonists on brain function, such as neuroprotective effects, are less investigated in models of cerebral ischemia. To clarify if GTS-21 has a protective effect on neuronal degeneration induced by ischemia, we planned to use gerbils subjected to cerebral ischemia. The gerbil bilateral carotid occlusion model has been widely used to induce global ischemia of the brain (14), since this animal lacks an interconnection between the carotid and vertebro-basilar circulation. The gerbils that received transient occlusion of bilateral carotid arteries exhibit neuronal cell death in the hippocampal CA1 area and passive

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avoidance impairment (15).

In this study, we investigated the effects of GTS-21 on neuropathological and behavioral changes caused by transient occlusion of bilateral carotid arteries in the gerbil and compared them with the effects of nicotine and 9-amino-1,2,3,4-tetrahydroacridine hydrochloride hydrate (THA).

MATERIALS AND METHODS

Animals

Adult male Mongolian gerbils (11- to 13-week-old; Seiwa Experimental Animals Co., Ltd., Fukuoka) were housed 4–5 per cage at least for 1 week before the start of the experiments. The housing was thermostatically maintained at 23±3℃ with a constant humidity (30–70%) and a 12 hr light-dark cycle (lights on 06:00–18:00). The animals were given free access to food and water.

Drugs

GTS-21 [3-(2,4-dimethoxybenzylidene)-anabaseine dihydrochloride] was synthesized at Taiho Pharmaceutical Co., Ltd. as described previously (16). GTS-21, (−)-nicotine ditartrate salt (Wako Pure Chemical Industries, Ltd., Osaka) and THA (Sigma Chem., St. Louis, MO, USA) were dissolved in saline or distilled water. Pentobarbital-Na (PB) was obtained from Abbot Laboratories (North Chicago, IL, USA). For examination of the acute effects of test drugs, GTS-21 (1 and 5 mg/kg), nicotine (0.3 and 1.5 mg/kg), THA (1 and 5 mg/kg) or PB (50 mg/kg) were injected i.p. 30 min before forebrain ischemia. For examination of the effects of long-term treatment with test drugs, GTS-21 (0.1, 1 and 10 mg/kg), nicotine (10 mg/kg) or THA (10 mg/kg) was orally administered twice daily for a 2-week period before the ischemia. Animals received the surgery for carotid ligation 24 hr after the last administration of test drugs. The doses are expressed in terms of the salts.

Surgery

Gerbils were anesthetized with 2% halothane mixed with 70% nitrous oxide (the balance being oxygen). The bilateral common carotid arteries were exposed and occluded with Sugita aneurysm clips. Following 3-min ischemia, the clips were removed to restore blood flow through the arteries, and then the skin incision was closed with wound clips. During the occlusion period, the animals were kept on a thermostat-equipped warming plate to maintain body temperature at 37℃ until the animal's righting reflex was restored, in order to prevent hypothermia during anesthesia and surgery. The animals that received the same surgical operation without carotid ligation served as the sham-operated control.

Passive avoidance task

A step-through type passive avoidance apparatus was used for the evaluation of memory. This apparatus consists of two compartments, the light and dark compartment (each same size: 16.5×16.0×14.7 cm), and each compartment was separated by a guillotine door. The dark compartment had a stainless grid floor. A scrambled electric shock is delivered through the floor grids by a shock generator (Daiichi Kikai, Inc., Tokushima). The learning trial of passive avoidance was carried out 2 days after the surgery. Each animal was placed initially in the light compartment. When the animals stepped into the dark compartment, they received a electric footshock through the grid floor (0.6 mA, 3 sec). Immediately after receiving the footshock, the gerbils went back to the light compartment. Mongolian gerbils, unlike rats or mice, could not learn the one-trial passive avoidance task, so the multi-trial passive avoidance task was adopted. If the animals re-entered the dark compartment, they received a foot-shock again. The number of shocks that the gerbil was subjected to for 8-min training period was recorded as a parameter for learning ability.

The retention test was performed 24 hr after the training sessions. In the retention test, the gerbil was placed in the light compartment, and the latency to enter the dark compartment was measured. If the gerbil did not enter the dark compartment within 180 sec, a ceiling score of 180 sec was assigned.

Histology

The gerbils were anesthetized with 50 mg/kg of PB at 7 days after surgery, and they were perfused intracardially with heparinized saline and then with 10% formalin solution. The whole brain was removed and a tissue block containing the hippocampus area was dissected out and embedded in paraffin. Coronal sections (5-μm-thick) were taken at the level of the hippocampus (approximately 1.5-mm caudal to the bregma) using a frozen-stage microtome and stained with 1% Cresyl violet for microscopic observation. The degree of neuronal cell damage at the hippocampal CA1 area was expressed as the density of surviving CA1 pyramidal cells according to the following equation: Density = the number of surviving CA1 pyramidal cells / the length of CA1 region (mm).

Statistics

Values are expressed as the mean±S.E.M. Data obtained by behavioral and histological studies were analyzed by the Dunnett's test. A difference with $P<0.05$ was considered statistically significant.

Table 1. Effects of single administration of GTS-21, nicotine, THA and pentobarbital (PB) on the learning performance of transient ischemic gerbils in the step-through passive avoidance task

Drugs	Dose (mg/kg, i.p.)		No. of footshocks
Ischemic vehicle	—	(9)	28.0±1.7
GTS-21	1	(10)	31.0±3.9
	5	(9)	25.2±2.7
Nicotine	0.3	(9)	42.2±3.8**
	1.5	(8)	36.8±3.7
THA	1	(10)	35.0±3.0
	5	(10)	20.0±2.6
PB	50	(9)	16.8±2.5

In the sham-operated gerbils, the number of footshocks was 13.5±1.1 (10). Test drugs were injected i.p. 30 min before cerebral ischemia. **P<0.01, significantly different from ischemic vehicle by Dunnett's *t*-test. Each value is the mean±S.E.M. The numbers of animals used are shown in parentheses.

Table 2. Effects of subchronic treatment with GTS-21, nicotine or THA on the learning performance of transient ischemic gerbils in the step-through passive avoidance task

Drugs	Dose (mg/kg, p.o.)		No. of footshocks
Ischemic vehicle	—	(10)	29.2±5.4
GTS-21	0.1	(14)	23.9±3.1
	1	(13)	25.1±2.8
	10	(14)	23.9±2.7
Nicotine	10	(12)	27.2±2.7
THA	10	(13)	33.8±3.8

In the sham-operated gerbils, the number of footshocks was 10.9±0.9 (14). Test drugs were orally administered (twice/daily) for 2 weeks before the ischemia. Each value is the mean±S.E.M. The numbers of animals used are shown in parentheses.

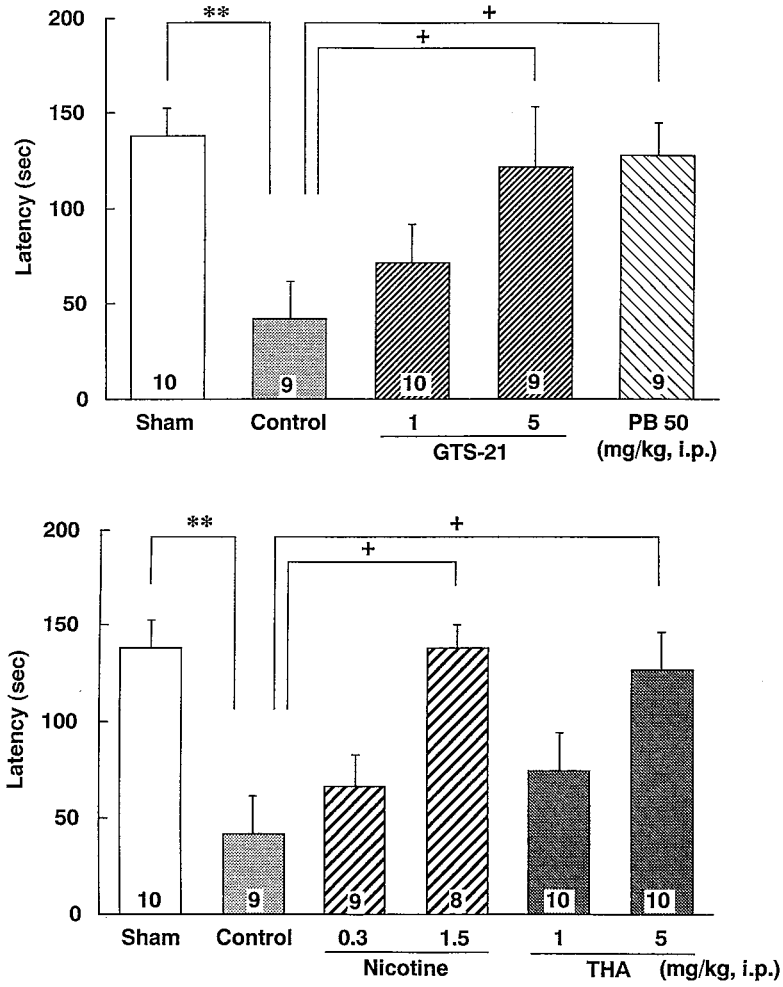

Fig. 1. Effects of GTS-21, nicotine, THA and pentobarbital (PB) on the latency of the transient ischemic gerbils in the step-through passive avoidance task. Drugs were injected i.p. 30 min before ischemia. Each column with a vertical bar represents the mean±S.E.M. +P<0.05, **P<0.01, significantly different from ischemic vehicle with Dunnett's *t*-test. The number of animals used is shown in each column.

RESULTS

Effects of GTS-21, nicotine and THA on the passive avoidance task

The sham-operated group received 11−14 footshocks in the learning trial, and the ischemic group received footshocks 2−3 times more than the sham-operated group (Tables 1 and 2). The latency of the ischemic animals to enter the dark compartment (41.4±19.7 sec) was significantly shorter than that of sham-operated animals (138.2±14.9 sec) in the retention test (Fig. 1). The administration of GTS-21 (1 and 5 mg/kg), (−)-nicotine (1.5 mg/kg) and THA (1 and 5 mg/kg) 30 min before ischemic operation had no effect on learning behavior, whereas nicotine (0.3 mg/kg, i.p.) increased the number of shocks given to the ischemic animals in the learning trial (Table 1). GTS-21 (5 mg/kg), (−)-nicotine (1.5 mg/kg) and THA (5 mg/kg) significantly increased ($P<0.05$) the latency of the ischemic animals in the retention test (Fig. 1). Treatment with 50 mg/kg (i.p.) PB decreased the number of shocks given to the ischemic animals in the learning trial and significantly prolonged step-through latency to the level of the sham-operated animals (Fig. 1, Table 1). When test drugs were administered orally twice daily for 2 weeks, GTS-21, nicotine and THA had no effect on learning behavior (Table 2). However, GTS-21 (0.1 and 10 mg/kg), (−)-nicotine (10 mg/kg) and THA (10 mg/kg) significantly increased the step-through latency of the ischemic animals in the retention test (Fig. 2).

Effect on neuronal cell death

Degeneration of the CA1 neurons in the hippocampal area was detected in the vehicle-treated animals at 7 days

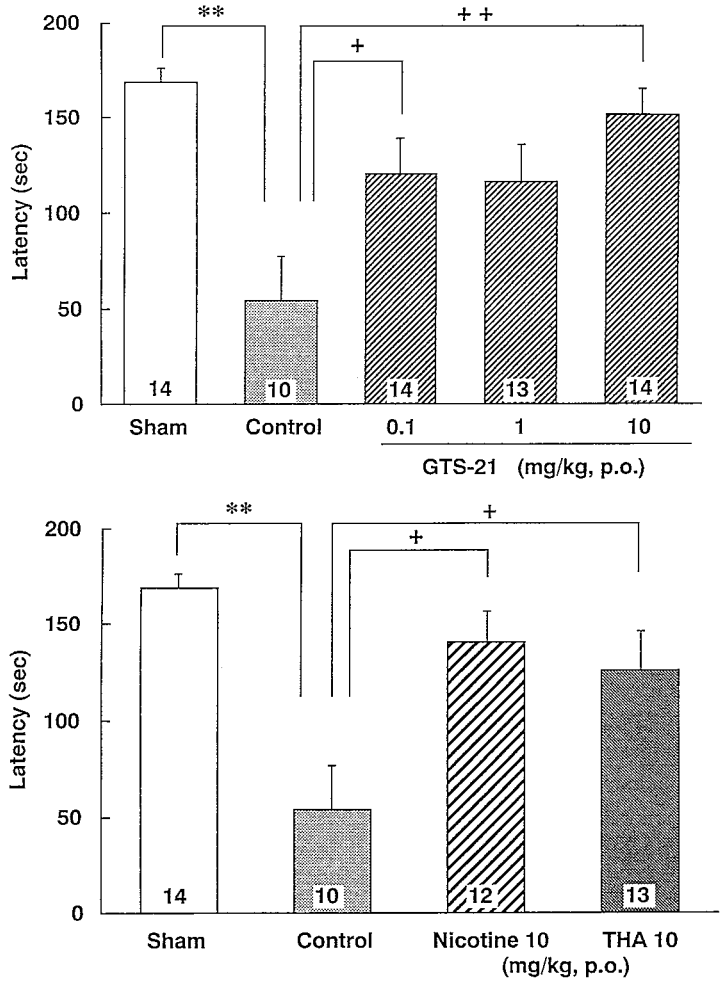

Fig. 2. Effects of subchronic treatment with GTS-21, nicotine and THA on the latency of the transient ischemic gerbils in the step-through passive avoidance task. Drugs were orally administered (twice/day for 2 weeks) before ischemia. Each column with a vertical bar represents the mean±S.E.M. $^{+}P<0.05$, $^{**,++}P<0.01$, significantly different from ischemic vehicle with Dunnett's *t*-test. The number of animals used is shown in each column.

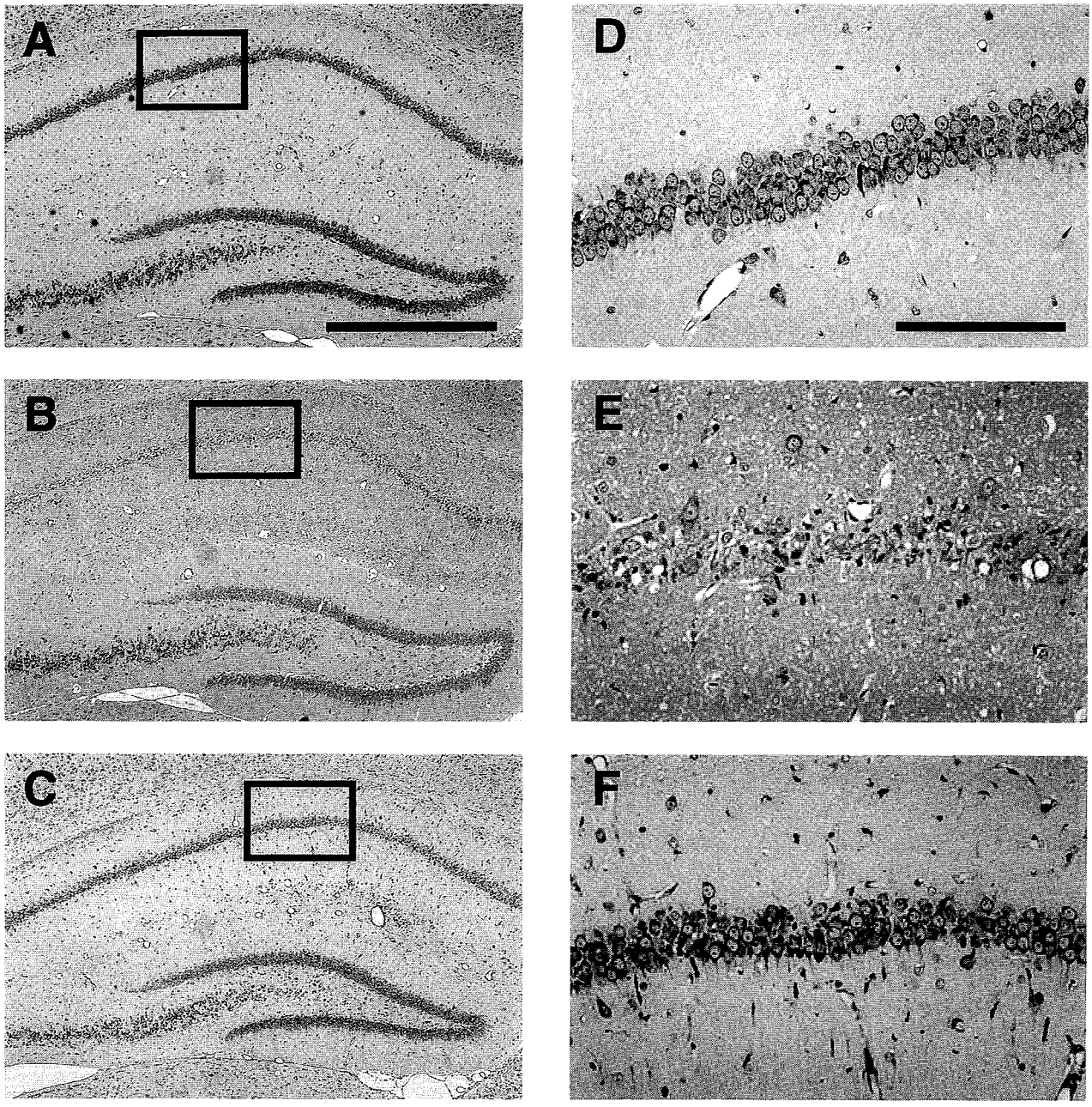

Fig. 3. Histopathological changes of dorsal hippocampus 7 days after 3-min common carotid artery occlusion in gerbils. A and D: sham-operation; B and E: control; C and F: GTS-21, 10 mg/kg-treated animal. Scale bars represent 500 μm in A, B and C. Scale bars represent 100 μm in D, E and F.

after the carotid artery occlusion. Most of the pyramidal cells in CA1 were destroyed and many glial cells appeared (Fig. 3). This finding agrees with those previously reported (14). The number of surviving neurons in the CA1 area was significantly decreased.

As shown in Table 3, neuronal cell death in the hippocampus CA1 region of the vehicle-treated animals was approximately 90%; GTS-21 prevented the destruction or disappearance of CA1 neurons induced by the ischemia, at doses of 1 and 5 mg/kg. Nicotine (0.3 mg/kg) and THA (5 mg/kg) also attenuated ischemia induced neuronal degeneration. In the PB-treated ischemia group, there were no alteration in CA1 neurons. When administered orally twice daily for 2 weeks, GTS-21 (1 and 10 mg/kg) significantly decreased the neurodegeneration (Table 4). The injection of GTS-21 at a dose of 0.1

Table 3. Effects of single administration of GTS-21, nicotine, THA and pentobarbital (PB) on the hippocampal CA1 pyramidal cell death following transient ischemic gerbils

Drugs	Dose (mg/kg, i.p.)		Neuronal density (No./mm)	(% of sham)
Ischemic vehicle	—	(9)	13.7±0.6	9.7
GTS-21	1	(10)	31.2±6.6*	22.1
	5	(9)	45.3±5.8**	32.1
Nicotine	0.3	(9)	32.9±5.3*	23.3
	1.5	(8)	32.7±3.6	23.2
THA	1	(10)	28.2±1.6	20.0
	5	(10)	68.0±13.0**	48.2
PB	50	(9)	129.6±9.4**	91.9

In the sham-operated gerbils, the neuronal density was 141.0±2.5 (10). Test drugs were injected i.p. 30 min before cerebral ischemia. *$P<0.05$, **$P<0.01$, significantly different from ischemic vehicle by Dunnett's *t*-test. Each value is the mean±S.E.M. The numbers of animals used are shown in parentheses.

Table 4. Effects of subchronic treatment with GTS-21, nicotine or THA on the hippocampal CA1 pyramidal cell death following transient ischemic gerbils

Drugs	Dose (mg/kg, p.o.)		Neuronal density (No./mm)	(% of sham)
Ischemic vehicle	—	(10)	11.8±1.9	10.0
GTS-21	0.1	(14)	34.9±8.6	29.8
	1	(13)	46.1±11.3**	39.6
	10	(14)	43.3±8.9*	36.9
Nicotine	10	(12)	24.3±2.5	20.7
THA	10	(13)	21.8±2.4	18.6

In the sham-operated gerbils, the neuronal density was 117.3±3.4 (14). Test drugs were orally administered (twice/daily) for 2 weeks before the ischemia. *$P<0.05$, **$P<0.01$, significant different from ischemic vehicle by Dunnett's *t*-test. Each value is the mean±S.E.M. The numbers of animals used are shown in parentheses.

mg/kg, nicotine (10 mg/kg) or THA (10 mg/kg) had no effect on the neuronal degeneration of CA1 neurons.

DISCUSSION

In this study, administration of GTS-21, nicotine and THA improved the passive avoidance impairments induced by cerebral ischemia. Many studies have demonstrated that nicotine and nicotinic agonists cause improvements in memory function in Alzheimer's patients, adult rats and experimental or aging animals (7, 8, 10, 17, 18). The cholinergic hypothesis of aging and dementia attributes impairment of memory and cognition to reduced central cholinergic function (1). Recently, there is increasing evidence that cerebral ischemia results in disruption of central cholinergic systems (2, 19) It is therefore likely that GTS-21, nicotine and THA improved the impaired learning behavior by activating functional cholinergic receptor in the gerbil brain after ischemia. When these drugs were administered subchronically 24 hr before ischemia, nicotine and THA showed a tendency to suppress the neuronal cell damages but the effect was not significant. The exact reason for the difference in the neuroprotective activity between GTS-21 and another drugs remains unclear, but several factors seem to explain this difference. First, nicotine, THA and PB have a decreasing effect on rectal temperature, and these hypothermia are associated with the neuroprotective action against cerebral ischemia and postischemia hyperthermia (15, 20–22). Based on these data, except for the suppression of postischemic hyperthemia, we studied the cerebral protective effects at 24 hr after the final injection of these drugs. Subchronic oral administration of GTS-21 could block the neuronal damage, but other drugs had no effect on the neurodegeneration. The present results suggest that the protective effect of GTS-21 was not associated with hypothermia and that its pharmacological mechanism was different from those of nicotine and THA. Secondly, the difference in the selectivity for the nicotinic receptor subtypes between GTS-21 and nicotine may be implicated in the lack of cytoprotective activity of nicotine in the present study. GTS-21 has been reported to have 4 times higher affinity than nicotine for the nicotinic receptor subtype which consists of α7-subunits (23). GTS-21 or nicotine have a protective effect against glutamate-induced neurotoxicity in cultured cortical neurons (12, 13), which was antagonized by α-bungarotoxin, an α7-selective antagonist. Therefore, the protective action of nicotine and GTS-21 were associated with α7-nicotine receptor. It could be speculated that the α7-subunit-composed nicotinic receptor subtype may play a dominant role in the cytoprotection caused by GTS-21.

The previous finding demonstrated that GTS-21 facilitated induction of LTP in the rat hippocampus via stimulation of nicotinic acetylcholine receptors (nAChRs) (9). LTP is an intensively studied physiological model of learning and memory formation. Taken together, it is possible that stimulation of nAChRs is also involved in the neuroprotective activity of GTS-21 against the cerebral ischemia and that both the protective effect on neuronal cell loss and the facilitatory effect on LTP induction may contribute to the ameliorative effect of GTS-21 on the impairment of learning and memory caused by cerebral ischemia. However, further investigation will be required to test this possibility.

We recently reported that GTS-21 significantly attenuated the neuronal cell loss in layers II–III of the parietal cortex caused by nucleus basalis nBM lesion (6). After 2

weeks of bilateral nBM excitotoxic lesion, GTS-21 was orally administered once daily for 20 weeks. In this model, the degenerative change of neurons in the parietal cortex was very slow, becoming apparently by light microscopy 3 months after the nBM lesion. In the CA1 subfield of the gerbil hippocampus, clefts in the cytoplasm were noticed at two days after ischemia, and four days later, almost all of the pyramidal cells have degenerated (14). From these data, GTS-21 attenuated the neuronal cell loss caused by bilateral nBM lesion in rats and the transient ischemia in gerbils.

In conclusion, chronic oral or acute i.p. administration of GTS-21 actually attenuated the destruction of CA1 neurons and improved the passive avoidance deficits, suggesting that GTS-21 may be useful for treatment of neurodegenerative diseases.

Acknowledgments

The authors gratefully acknowledge Dr. Kinzo Matsumoto and Mr. Hong-Bin Lee (Toyama Medical and Pharmaceutical University) for comment on the manuscript.

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