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Effect of JTP-2942, a novel thyrotropin-releasing hormone analogue, on pentobarbital-induced anesthesia in rats

Mutsuyoshi Matsushita *, Fumihiko Yonemori, Atsushi Hamada, Katsuo Toide, Kunio Iwata

Central Pharmaceutical Research Institute, Japan Tobacco Inc., 1-1, Murasaki-cho, Takatsuki, Osaka 569, Japan

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Abstract

The effects of a novel thyrotropin-releasing hormone (TRH) analogue, *Nα*-((1*S*,2*R*)-2-methyl-4-oxocyclopentylcarbonyl)-L-histidyl-L-prolinamide monohydrate (JTP-2942), on pentobarbital-induced anesthesia in rats were investigated and compared with those of TRH. Intravenous administration of both JTP-2942 and TRH caused a dose-dependent decrease in the recovery time from pentobarbital-induced anesthesia. The minimum effective doses of JTP-2942 and TRH were respectively 0.03 and 1 mg/kg. The effect of JTP-2942 was antagonized by intraperitoneal scopolamine (0.5 mg/kg). Intraperitoneal JTP-2942 (1 mg/kg) caused an increase of acetylcholine release and a decrease of choline release in the frontal cortex and hippocampus of pentobarbital-treated rats. In addition, JTP-2942 ameliorated the decrease of hemicholinium-3-sensitive high-affinity choline uptake and the increase of acetylcholine in these brain regions. However, JTP-2942 had no effect on choline acetyltransferase activity or the choline content, which were also not changed by pentobarbital. Our results indicate that the effect of JTP-2942 on pentobarbital-induced anesthesia was about 30 times more potent than that of TRH, and suggest that JTP-2942 may act by accelerating acetylcholine turnover.

Keywords: JTP-2942 (*Nα*-((1*S*,2*R*)-2-methyl-4-oxocyclopentylcarbonyl)-L-histidyl-L-prolinamide monohydrate); TRH (thyrotropin-releasing hormone) analog; Acetylcholine turnover

1. Introduction

It is well known that exogenous thyrotropin-releasing hormone (TRH) is an analeptic agent which can reverse the central nervous system (CNS) depressant effect of barbiturates (Breese et al., 1975) and that this action of TRH is independent of its influence on the pituitary gland. Some reports have suggested that the CNS effect of TRH involves cholinergic mechanisms, at least with respect to the TRH-induced reduction of sleeping time and restoration of the righting reflex (Horita and Carino, 1990; Zucker et al., 1985).

We have previously reported that a novel TRH analogue, *Nα*-((1*S*,2*R*)-2-methyl-4-oxocyclopentylcarbonyl)-L-histidyl-L-prolinamide monohydrate (JTP-2942), has a stronger and more persistent CNS effect than TRH. In particular, JTP-2942 has a stronger effect than TRH on the disturbance of consciousness induced by concussive head trauma (Matsushita et al., 1992).

In the present study, we investigated the effect of JTP-2942 on recovery from pentobarbital-induced anesthesia and compared it with that of TRH. Also, to study the mechanism by which JTP-2942 promotes recovery from pentobarbital anesthesia, we investigated its effect on the extracellular concentrations of acetylcholine and choline, the acetylcholine and choline contents, the choline acetyltransferase activity, and the hemicholinium-3-sensitive high-affinity choline uptake in the rat brain.

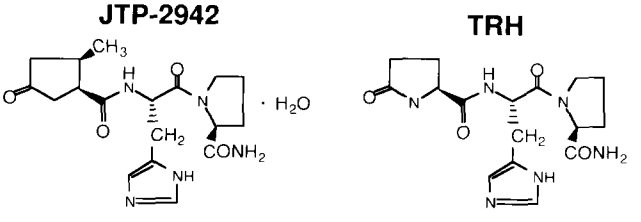

Fig. 1. Chemical structures of JTP-2942 and TRH.

* Corresponding author.

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2. Materials and methods

2.1. Animals and compounds

Male Wistar rats were used. All animals were kept in a room illuminated for 12 h (7:00 a.m.–7:00 p.m.) with the temperature regulated at 23 ± 1°C. They were allowed free access to a pellet diet and water. JTP-2942 (Fig. 1) was synthesized at the Central Pharmaceutical Research Institute of Japan Tobacco (Japan). Other compounds used for this study were as follows: sodium pentobarbital (Tokyo Kasei Co., Japan), scopolamine hydrobromide (Sigma, USA), physostigmine sulfate (Sigma, USA), acetyl-CoA (Sigma, USA), choline chloride (Kanto Chemicals, Japan) and TRH (Peptide Institute, Japan). These compounds were dissolved in saline and the administration volume was 1 ml/kg.

2.2. Pentobarbital-induced anesthesia

Rats aged 7–9 weeks (150–270 g) were used in this experiment. An intraperitoneal injection of pentobarbital was given at a dose of 40 mg/kg. Either TRH or JTP-2942 was administered intravenously at 10 min after pentobarbital and the time when the righting reflex returned was recorded. Integrity of the righting reflex was determined by placing the animal on its back and observing whether it could resume and maintain an upright position. In some animals, a muscarinic acetylcholine receptor antagonist (scopolamine, 0.5 mg/kg) was administered intraperitoneally 10 min before the administration of pentobarbital. All experiments were carried out between 13:00 and 18:00 h in an air-conditioned room. Behavioral observation was performed by an experimenter who did not know to which group the rats had been assigned.

2.3. Determination of the extracellular acetylcholine and choline concentrations by in vivo microdialysis

Brain dialysis was performed according to the method of Toide and Arima (1989). Briefly, rats (190–260 g) were anesthetized with intraperitoneal pentobarbital (40 mg/kg), and mounted in a stereotaxic frame, after which a stainless steel guide cannula was implanted into the left frontal cortex (A +3.2, L −3.0 and V −1.5 mm relative to the bregma) or the left hippocampus (A −6.5, L −4.9 and V −3.5 mm relative to the bregma) according to the coordinates in the atlas of König and Klippel (1963).

An I-shaped dialysis probe (Eicom, Japan) was used, and 2.0 or 3.0 mm of the tip was exposed to the tissue of the frontal cortex and hippocampus, respectively. Perfusion experiments with a unilateral dialysis probe inserted into the brain were carried out between 26 and 48 h after surgery to avoid the effects of anesthesia. Perfusion was performed at a constant rate of 2 μl/min with Ringer's solution (mM: NaCl, 147; $CaCl_2$, 3.4; KCl, 4.0 pH 6.1) containing 10 μM physostigmine. Samples were extracted every 15 min and automatically fed into a high-performance liquid chromatograph with an electrochemical detector (HPLC/ECD apparatus) for determination of the acetylcholine and choline concentrations.

2.4. Acetylcholine and choline contents

The acetylcholine and choline contents were determined on the basis of the method described by Toide and Arima (1989). After microwave irradiation (10 kW, 1.6 s), the brain of each rat was excised and dissected into the frontal cortex and hippocampus by the method of Glowinski and Iversen (1966). Each region was then separately homogenized in a 1 N formic acid-acetone (15:85) solution containing ethylhomocholine as an internal standard. After cooling on ice for 30 min, the samples were centrifuged at 18000 × *g* and 4°C for 15 min, after which acetylcholine and choline were extracted from the supernatant by shaking with an equal quantity of ether followed by centrifugation at 900 × *g* for 5 min. Then the bottom layer of this liquid was mixed with an equal quantity of a heptane-chloroform solution (8:2), and this mixture was shaken and centrifuged under the same conditions as for ether extraction. Next, the bottom layer of this centrifuged sample (100 μl for the hippocampus and 700 μl for the frontal cortex) was subjected to evaporative inspissation under nitrogen gas, following which the residue was dissolved in distilled water (100 μl for the hippocampus and 200 μl for the frontal cortex) and then subjected to centrifugal filtration through a 0.45 μm Millipore filter. Finally, 10 μl of the filtrate was injected into the HPLC/ECD apparatus.

The mobile phase was 0.1 M phosphate buffer (pH 8.2) containing 0.6 mM tetramethylammonium and 1.03 mM decanesulphonic acid. Measurements were performed at an applied potential of 450 mV and a flow rate of 1.0 ml/min, using a platinum electrode 3.0 mm in diameter. The column temperature was 33°C. The mobile phase was degassed by passage through a 0.45 μm Millipore filter before use.

2.5. Hemicholinium-3-sensitive high-affinity choline uptake and choline acetyltransferase activity

Tissue preparation

Crude tissue extract and synaptosomes were prepared by the method of Williams and Pylett (1990). In brief, rats were decapitated at 40 min after the intraperitoneal injection of pentobarbital, after which their brains were quickly removed and rinsed in cold 0.32 M sucrose buffered with 5 mM Tris-HCl (pH 7.4).

Crude tissue extracts were prepared by homogenizing the brain tissues in 3 ml of cold sucrose buffer using a glass-Teflon homogenizer. An aliquot of the total homogenate was stored at −80°C for the analysis of choline acetyltransferase activity. The remaining homogenate was centrifuged at 1000 × *g* for 10 min and the supernatant was centrifuged at 12000 × *g* for 20 min to yield the crude synaptosomal pellet. The pellet was resuspended in 3 ml of Hepes-Krebs Ringer (Hepes-KR) buffer (124 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 1.3 mM $MgCl_2$, and 20 mM Hepes-NaOH, pH 7.4) and 10 mM glucose.

Hemicholinium-3-sensitive high-affinity choline uptake

Hemicholinium-3-sensitive high-affinity choline uptake was measured according to the method of Zucker et al. (1985) with some modifications. In brief, 0.1 ml of the synaptosomal suspension was added to 0.9 ml of the incubation medium (Hepes-KR buffer containing 0.5 μM choline Cl and 0.65 μCi of radiolabeled choline Cl (75 Ci/mmol, Amersham)), and then incubated at 37°C for 5 min in the absence or presence of 10 mM hemicholinium-3. The uptake of choline in the presence of hemicholinium-3 was subtracted from the total uptake in its absence to obtain the hemicholinium-3-sensitive high-affinity choline uptake. The reaction was terminated by transferring the samples rapidly to an ice bath, after which synaptosomes were collected by centrifugation at 1000 × *g* for 5 min and were washed twice with cold Hepes-KR buffer. The pellet was solubilized in 0.1 ml of 0.1% Triton X-100, 1 ml of scintillator was added, and the radioactivity was counted with a liquid scintillation counter. All uptake experiments were performed in duplicate and the protein concentration was determined by the method of Lowry et al. (1951).

Choline acetyltransferase activity

Choline acetyltransferase activity was measured by a modification of the method of Fonnum (1969), as described by Rylett (1989). In brief, 0.1 ml of the crude tissue extract was diluted with 1 ml of 25 mM sodium phosphate buffer (pH 7.4) containing 0.1% Triton X-100. Then 20 μl of the diluted extract was used for the determination of choline acetyltransferase activity. The choline acetyltransferase assay mixture (50 mM sodium phosphate buffer (pH 7.4) containing 300 mM NaCl, 10 mM choline Cl, 0.2 μM eserine sulfate, 0.2 μM acetyl-CoA, and 8 nCi of [^{14}C]acetyl-CoA (5 mCi/mmol, Amersham)) was mixed with the diluted extract, and then incubation was done at 37°C for 15 min. The reaction was terminated by adding 0.5 ml of a stop solution, which consisted of 0.1 mM *p*-chloromercurybenzoate and 5 mg/ml sodium tetraphenylborate in a 5:2 mixture of 2 mM sodium phosphate (pH 7.4) and acetonitrile. Then 1 ml of toluene scintillator was added, the tube was mixed vigorously, and was centrifuged at 1000 × *g* for 5 min. Finally, the radioactivity was counted with a liquid scintillation counter.

2.6. Statistics

Results are expressed as the mean ± S.E. Differences between the groups treated with vehicle or the test compounds were evaluated using Duncan's multiple comparison following analysis of variance (ANOVA).

3. Results

3.1. Effect on pentobarbital-induced anesthesia

Intravenous administration of JTP-2942 and TRH caused a significant dose-dependent decrease of the sleeping time induced by pentobarbital (Fig. 2). The minimum effective doses of JTP-2942 and TRH were 0.03 mg/kg and 1 mg/kg, respectively. Thus, JTP-2942 was about 30 times more potent than TRH with regard to its effects on pentobarbital anesthesia. This effect of JTP-2942 was antagonized by the intraperitoneal administration of scopolamine (0.5 mg/kg) at 20 min before JTP-2942. The single administration of scopolamine alone did not have any effect on pentobarbital-induced anesthesia (Fig. 3).

3.2. Effect of JTP-2942 on the extracellular acetylcholine and choline concentrations

Intraperitoneal pentobarbital (40 mg/kg) decreased the extracellular acetylcholine concentrations in the frontal cortex and hippocampus (Fig. 4). In contrast, the choline concentration was not affected by pentobarbital in either brain region. In the frontal cortex

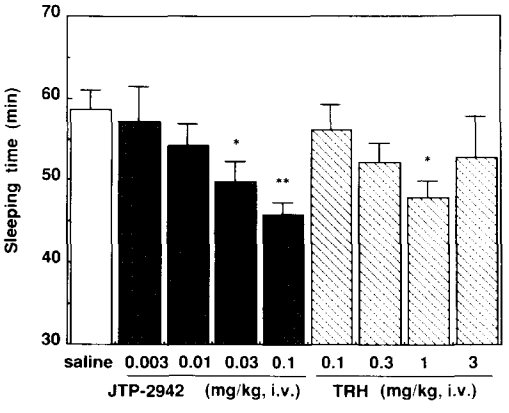

Fig. 2. Effect of JTP-2942 and TRH on pentobarbital-induced anesthesia in rats. * $P < 0.05$, ** $P < 0.01$ vs. the control group (Duncan's multiple comparison following ANOVA).

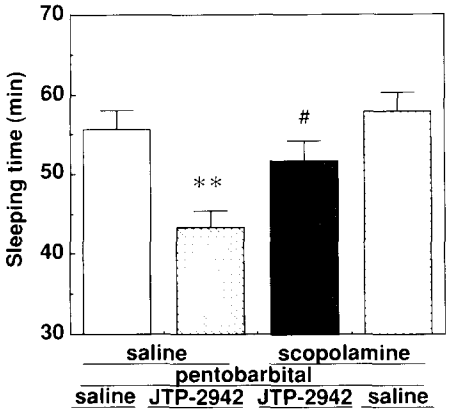

Fig. 3. Antagonistic effect of scopolamine (0.5 mg/kg i.p.) on the action of JTP-2942 (0.1 mg/kg i.v.). $^{**}P < 0.01$ vs. the saline-saline group (Duncan's multiple comparison following ANOVA); $^{\#}P < 0.05$ vs. the saline-JTP-2942 group (Duncan's multiple comparison following ANOVA).

and hippocampus, intraperitoneal JTP-2942 (0.03 and 0.3 mg/kg) significantly reversed the effect of pentobarbital on the extracellular acetylcholine concentration in a dose-dependent manner. JTP-2942 (0.03 and 0.3 mg/kg) also significantly decreased the extracellular choline concentration in the hippocampus after

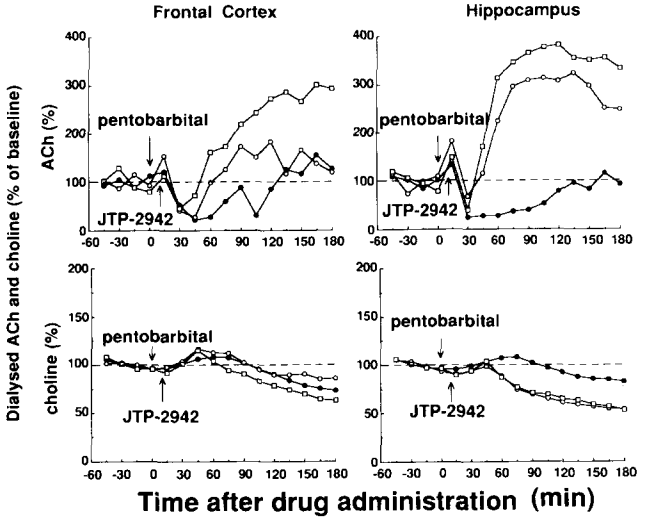

Fig. 4. Effect of JTP-2942 on the pentobarbital-induced decrease in extracellular acetylcholine and choline concentrations in the frontal cortex (left) and hippocampus (right). JTP-2942 at a dose of 0.03 (○) or 0.3 (□) mg/kg and saline (●) was injected at 10 min after the administration of pentobarbital. Data ($n = 3$–5) represent the acetylcholine and choline contents of each 15-min fraction, expressed as a percentage of the average baseline levels. Differences were evaluated by Duncan's multiple comparison test following ANOVA. Administration of pentobarbital plus JTP-2942 (0.03 and 0.3 mg/kg) had a significantly different effect from administration of pentobarbital alone on the acetylcholine and choline concentration levels in the hippocampus and on the acetylcholine content of the frontal cortex.

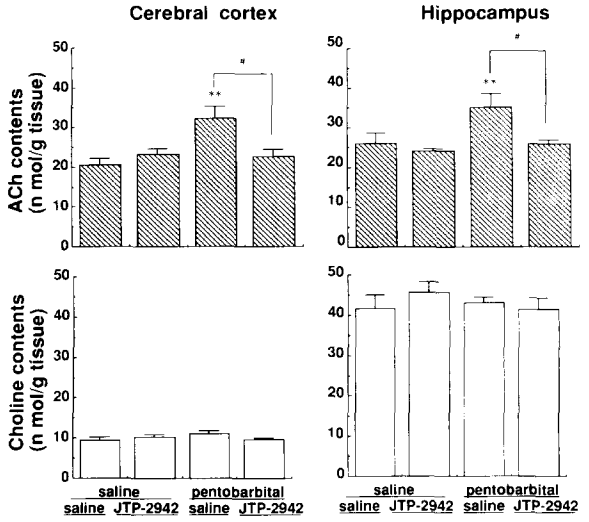

Fig. 5. Effect of JTP-2942 on the acetylcholine and choline contents of two brain regions in rats after pretreatment with pentobarbital. JTP-2942 (1 mg/kg i.p.) was administered at 10 min after pentobarbital (40 mg/kg i.p.) injection. Rats were killed 30 min after administration of JTP-2942. Data represent the mean ± S.E. for groups of 6 rats. $^{**}P < 0.01$ vs. the control group (Duncan's multiple comparison following ANOVA); $^{\#}P < 0.05$ vs. pentobarbital-treated rats (Duncan's multiple comparison following ANOVA).

pretreatment with pentobarbital, but had no effect on the frontal cortex (Fig. 4).

3.3. Effect of JTP-2942 on brain acetylcholine and choline contents

Intraperitoneal pentobarbital (40 mg/kg) significantly increased the acetylcholine content without any change of the choline level in both the frontal cortex and the hippocampus (Fig. 5). Intraperitoneal JTP-2942 (1 mg/kg) antagonized the increase of acetylcholine in the frontal cortex and hippocampus following pentobarbital administration, although it did not change the acetylcholine and choline levels in both brain regions when administered alone.

3.4. Effect of JTP-2942 on hemicholinium-3-sensitive high-affinity choline uptake and choline acetyltransferase activity

Intraperitoneal pentobarbital (40 mg/kg) significantly decreased hemicholinium-3-sensitive high-affinity choline uptake by 80% when administered at 40 min before decapitation. Intraperitoneal administration of JTP-2942 (1 mg/kg) at 10 min after pentobarbital caused the recovery of hemicholinium-3-sensitive high-affinity choline uptake, while administration of JTP-2942 alone had no effect on hemicholinium-3-sensitive high-affinity choline uptake (Fig. 6). Both JTP-2942 and pentobarbital had no effect on choline

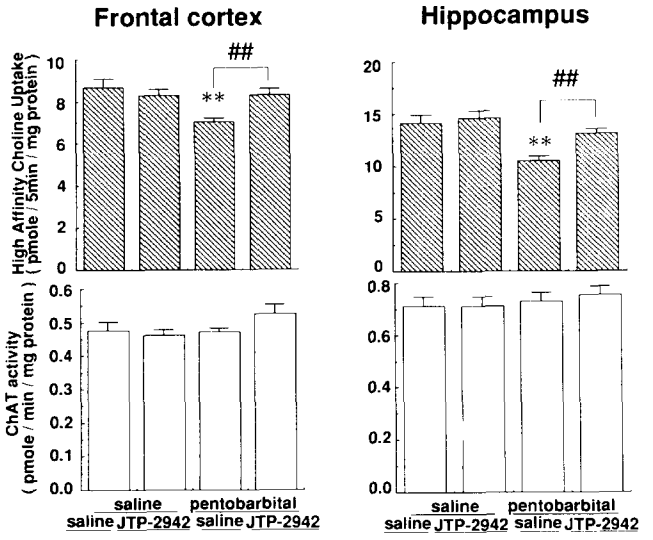

Fig. 6. Hemicholinium-3-sensitive high-affinity choline uptake and choline acetyltransferase activity into the cortical or hippocampal synaptosomes in conscious rats, JTP-2942 (1 mg/kg i.p.) treated rats, pentobarbital (40 mg/kg i.p.) treated rats and pentobarbital (40 mg/kg i.p.) plus JTP-2942 (1 mg/kg i.p.) treated rats. Data represent mean ± S.E. for groups of 9–10 rats. ** $P < 0.01$: significantly different from the control group (Duncan's multiple comparison following ANOVA); ## $P < 0.01$: significantly different from values for pentobarbital-treated rats (Duncan's multiple comparison following ANOVA).

acetyltransferase activity in either brain region (Fig. 6).

4. Discussion

We demonstrated that JTP-2942, a novel TRH analogue, significantly inhibited pentobarbital anesthesia at a minimum effective intravenous dose of 0.03 mg/kg in rats. JTP-2942 was about 30 times more potent in this action than TRH. TRH is mainly inactivated by hydrolysis at the pGlu-His bond, but JTP-2942 has a cyclopentanone structure at the pyroglutamyl residue, which would be expected to increase its resistance to hydrolysis. It therefore appears that JTP-2942 may have a stronger action than TRH on pentobarbital anesthesia in rats due to greater resistance to breakdown, though JTP-2942 has a 10-fold lower affinity on TRH receptors in brain than TRH. In fact, JTP-2942 shows a higher stability than TRH in both brain synaptic membrane fractions and partially purified pyroglutamyl aminopeptidase II fractions (unpublished data).

Since TRH produces an antagonistic effect on pentobarbital anesthesia in hypophysectomized animals and since thyroid hormones such as triiodothyronine and thyroxine have no anti-anesthetic effect, the anti-anesthetic action of TRH is not considered to be related to the hypothalamic-pituitary-thyroid axis (Breese et al., 1975; Cott et al., 1976). In addition, we have previously reported that JTP-2942 has a more persistent action in CNS models such as concussive head trauma than TRH itself, and a 3-fold lower potency in potentiating TSH release (Matsushita et al., 1993). These findings suggest that JTP-2942, like TRH, produces its anti-anesthetic effect via the CNS.

To clarify the mechanism of action of JTP-2942, we studied its effect on the cholinergic system in pentobarbital-treated rats. Scopolamine antagonized the promoting effect of JTP-2942 on the recovery from pentobarbital-induced anesthesia, suggesting that this action was regulated by the cholinergic system.

It is well known that pentobarbital inhibits the CNS cholinergic system (Schmidt, 1977). To clarify the acute effects of pentobarbital on the extracellular acetylcholine content in the CNS, we monitored extracellular acetylcholine concentrations in the frontal cortex and hippocampus using microdialysis. As shown in Fig. 4, pentobarbital decreased acetylcholine release in both the frontal cortex and hippocampus of rats. JTP-2942 antagonized this effect of pentobarbital on acetylcholine in both brain regions, and its action was stronger than that of TRH. In normal rats, JTP-2942 strongly promoted acetylcholine release in the frontal cortex and hippocampus, with its effect being more prolonged and greater than that of TRH. The increase of acetylcholine release by JTP-2942 is reported to be completely antagonized by perfusion with tetrodotoxin (1 μM), suggesting that the effect of JTP-2942 on cholinergic neurons is mediated via neuronal activity (Toide et al., 1993).

Since JTP-2942 strongly promotes acetylcholine release in the frontal cortex and hippocampus, we studied its effect on acetylcholine turnover as an index of the brain acetylcholine and choline contents, and also determined the choline acetyltransferase activity and hemicholinium-3-sensitive high-affinity choline uptake. Pentobarbital significantly decreased hemicholinium-3-sensitive high-affinity choline uptake and increased

Table 1
CNS effects of JTP-2942 in rats with pentobarbital-induced anesthesia

	ChAT	ACh content	Ch content	ACh release	Ch release	HACU
Pentobarbital	±	↑	±	↓	±	↓
Pentobarbital + JTP-2942	±	±	±	↑↑	↓	±

ACh: acetylcholine, Ch: choline, ChAT: choline acetyltransferase, HACU: hemicolinium-3-sensitive high-affinity choline uptake.

the acetylcholine content in both brain regions. These observations agree well with those of Horita and Carino (1990), who found that TRH ameliorated the changes in acetylcholine content and hemicholinium-3-sensitive high-affinity choline uptake induced by pentobarbital. Yamamura et al. (1991) reported that the antagonistic effect of TRH on pentobarbital anesthesia is due to the involvement of not only acetylcholine but also other neurotransmitters, such as dopamine, serotonin, and noradrenaline. However, since JTP-2942 has a particularly strong effect on the central cholinergic system and since scopolamine almost completely antagonizes this effect, it appears that the activity of JTP-2942 is chiefly due to acceleration of the cholinergic system, at least with respect to pentobarbital-induced anesthesia.

In conclusion, the present results indicate that the antagonistic effect of JTP-2942 on pentobarbital-induced anesthesia is stronger than that of TRH. This action of JTP-2942 might be due to acceleration of acetylcholine turnover in the cerebral cortex and hippocampus, which is depressed by pentobarbital (Table 1). Therefore, JTP-2942 may perhaps be more useful than TRH for clinically treating disturbance of consciousness.

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