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Full Paper

Comparison of Short- and Long-Acting Benzodiazepine-Receptor Agonists With Different Receptor Selectivity on Motor Coordination and Muscle Relaxation Following Thiopental-Induced Anesthesia in Mice

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Abstract. In this study, we compared the effects of Type I benzodiazepine receptor–selective agonists (zolpidem, quazepam) and Type I/II non-selective agonists (zopiclone, triazolam, nitrazepam) with either an ultra-short action (zolpidem, zopiclone, triazolam) or long action (quazepam, nitrazepam) on motor coordination (rota-rod test) and muscle relaxation (traction test) following the recovery from thiopental-induced anesthesia (20 mg/kg) in ddY mice. Zolpidem (3 mg/kg), zopiclone (6 mg/kg), and triazolam (0.3 mg/kg) similarly caused an approximately 2-fold prolongation of the thiopental-induced anesthesia. Nitrazepam (1 mg/kg) and quazepam (3 mg/kg) showed a 6- or 10-fold prolongation of the anesthesia, respectively. Zolpidem and zopiclone had no effect on the rota-rod and traction test. Moreover, zolpidem did not affect motor coordination and caused no muscle relaxation following the recovery from the thiopental-induced anesthesia. However, zopiclone significantly impaired the motor coordination at the beginning of the recovery. Triazolam significantly impaired the motor coordination and muscle relaxant activity by itself, and these impairments were markedly exacerbated after the recovery from anesthesia. Nitrazepam and quazepam significantly impaired motor coordination, and the impairments were exacerbated after the recovery. These results suggest that the profile of recovery of motor coordination and muscle flaccidity after co-administration of benzodiazepine-receptor agonists and thiopental is related to the half-life and selectivity for the benzodiazepine-receptor subtypes.

Keywords: hypnotics, thiopental, loss of righting reflex, motor coordination, muscle relaxant

Introduction

A meta-analysis suggested that short-acting hypnotics may be effective for the short-term treatment of situational insomnia (1). However, despite their therapeutic effectiveness, many hypnotics, such as the benzodiazepine derivative triazolam, cyclopyrrolone derivative zopiclone, and imidazopyridine derivative zolpidem, have some side effects, including impaired coordination and balance, cognitive impairment, tolerance, and dependence (2 – 4). In addition, a number of clinical studies have revealed that the impaired coordination and balance induced by hypnotics or benzodiazepines are associated with falls (1, 5). Patient falls are an important issue in risk management for hospitalized patients and elderly people in nursing homes (6). Such accidents may lead to negative outcomes, such as injuries, prolonged hospitalization, reduction in patients' activities of daily living, and increased medical expenses. Previous studies of benzodiazepine regimens, which may be associated with a risk of adverse events, have produced conflicting results. Early investigations of benzodiazepines with different elimination half-lives have suggested that long-acting benzodiazepines are more likely to cause adverse events than short-acting agents (7, 8). However, other studies have found significantly greater risks of adverse

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effects with the use of short-acting agents (1, 9). These results indicate that falls are a complex phenomenon with multifactorial causes such as age, health conditions, and concomitant medications in the clinical settings. Therefore, studies using experimental animals are important to clarify the pharmacological profile of benzodiazepine-receptor agonists.

Several benzodiazepine-receptor agonists with receptor subtypes or different elimination half-lives have been developed. Molecular biological studies have demonstrated that the $GABA_A$ receptor is a pentamer consisting of subunits from at least five different families, of which the α-, β-, and γ-subunits are generally considered necessary for modulation by benzodiazepine-receptor agonists (10, 11). Zolpidem and quazepam bind selectivity to Type-I ($\omega 1$) benzodiazepine receptors ($GABA_A$-receptor subtypes $\alpha 1$ containing subunits) (12 – 14). Type-II (ω_2) benzodiazepine receptors contain α_1-, α_2-, α_3-, and α_5-subunits; and zopiclone, triazolam, and nitrazepam are non-selective for Types I and II benzodiazepine receptors (13, 15). The benzodiazepine-receptor agonists were also classified based on their pharmacokinetic characteristics: ultra-short–acting (half-life <6 h) such as zolpidem, zopiclone, and triazolam; short-acting (half-life 6 – 12 h); intermediate-acting (half-life 12 – 24 h); and long-acting (half-life >24 h) such as nitrazepam and quazepam (16, 17).

Previous studies have demonstrated the pharmacological actions of hypnotics at the time of sleep induction and during sleep (18 – 22). For the evaluation of the side effects of benzodiazepine-receptor agonists, rota-rod and traction tests have been used as screening methods for an impairment of motor coordination or muscle relaxant effects in rodents (21). However, little is known about their pharmacological actions just after awakening. On the other hand, thiopental is a rapidly acting barbiturate and a single intravenous injection of thiopental produces only a brief period of hypnosis and anesthesia (22). Therefore, in this study, we applied the thiopental-induced hypnosis to the evaluation of side effects of benzodiazepine receptor agonists after awakening.

In this study, we examined the effects of zolpidem, zopiclone, triazolam, quazepam, and nitrazepam on the impairment of motor coordination using the rota-rod test and muscle relaxant activity using the traction test during the recovery phase from thiopental-induced anesthesia in mice.

Materials and Methods

Animals

Male ddY mice (SLC Co., Ltd., Shizuoka) weighing 20 – 30 g were used in all of the experiments. All of the animals were housed in groups of five per plastic cage (18 × 44 × 27 cm) in a room maintained at 22 ± 2°C under a 12/12-h light/dark cycle with lights on at 7:00 AM. The experimental protocol was conducted according to the Guidelines of the Ethics Review Committee for Animal Experimentation of Ehime University Medical School.

Drugs

The following drugs were used: zolpidem (Astellas Pharmaceutical Co., Ltd., Osaka), zopiclone (Sanofi-Aventis Co., Ltd., Tokyo), nitrazepam and triazolam (Sigma-Aldrich, Inc., St. Louis, MO, USA), quazepam (Mitsubishi Pharma Co., Ltd., Tokyo), and thiopental sodium (Ravonal®; Tanabe Seiyaku Co., Osaka). Zopiclone, triazolam, nitrazepam, and quazepam were dissolved in 0.5% polypropylene glycol (PPG), and zolpidem and thiopental sodium were dissolved in physiological saline (0.9% sodium chloride). The thiopental sodium was injected intravenously, and the hypnotics were injected intraperitoneally at a volume of 0.1 ml per 10 g body weight.

Loss of righting reflex

The mice were injected with thiopental (20 mg/kg, i.v.) in order to induce hypnosis, which was defined as a loss of the righting reflex. In a preliminary study, we investigated which doses of ultra-short acting hypnotics with thiopental were required to induce a similar loss of the righting reflex. Zolpidem (3 and 10 mg/kg, i.p.), zopiclone (3 and 6 mg/kg, i.p.), and triazolam (3 mg/kg, i.p.) were administered 10 min before the injection of thiopental, and the long-acting hypnotics, nitrazepam (0.6 and 1 mg/kg, i.p.) and quazepam (3 mg/kg, i.p.), were administered 20 min before the injection of thiopental. The control mice were administered thiopental and vehicle containing the hypnotic solution (saline or 0.5% PPG). Loss of the righting reflex was induced immediately after the injection of thiopental. Thereafter, the mice were placed in a V-shaped support in the supine position until recovery under a heat lamp to maintain normal temperature, and the duration of the loss of the righting reflex was measured. In the rota-rod and traction tests, the mice were administered zolpidem (3 mg/kg, i.p.), zopiclone (6 mg/kg, i.p.), triazolam (0.3 mg/kg, i.p.) nitrazepam (1 mg/kg, i.p.), or quazepam (3 mg/kg, i.p.); and these tests were performed at 15, 30, and 60 min after recovery from the loss of the righting reflex.

Traction test

The grip strength of each mouse was measured with a

traction apparatus (23). The forepaws of the mouse were placed on the attached bar (2-mm diameter, 30-cm-long, 30-cm above the bench level) and the latency until falling occurred was monitored for 60 s.

Rota-rod test

The rota-rod test (24) has been used to assess motor coordination and balance alterations in rodents. In this study, we used a rotating rod (3-cm diameter) apparatus (UGO BASILE, Comerio, Italy). The rota-rod test was performed under accelerating conditions: each mouse was placed on the rod rotating from 3 to 30 rpm over 3 min. The time the animal was able to maintain its balance walking on top of the rod was measured. The mice were given two trials with a maximum time of 180 s. Prior to the start of all experiments, the riding ability of the animals on the rota-rod was checked.

Statistical analyses

The results obtained were expressed as the mean ± S.E.M. A repeated measures analysis of variance (two-way ANOVA) with drug factor and time factor was used. Whenever the drug factor or the interaction of drug factor × time factor was significant, *post hoc* comparisons were performed with the Dunnett test. The data regarding the duration of the loss of the righting reflex were analyzed using one-way ANOVA followed by the Dunnett test. The significance level was set at $P<0.05$. We used the JMP 5J (SAS Institute, Cary, NC, USA) statistical analysis software package.

Results

Table 1 shows the duration of the loss of the righting reflex induced by the combination with thiopental (20 mg/kg, i.v.) and hypnotics in mice. The ultra-short–acting hypnotics, zolpidem (3 mg/kg, i.p.), zopiclone (6 mg/kg, i.p.), and triazolam (0.3 mg/kg, i.p.), produced a slight but not significant prolongation of the duration of the loss of the righting reflex induced by thiopental; and the three hypnotics approximately 2-fold prolonged the duration of the loss of the righting reflex in a similar degree. However, zolpidem (10 mg/kg, i.p.) significantly ($P<0.01$) prolonged the duration of the loss of the righting reflex. On the other hand, the long acting hypnotics, nitrazepam (1 mg/kg, i.p.) and quazepam (3 mg/kg, i.p.), approximately 6- or 10-fold significantly prolonged the duration of the loss of the righting reflex, respectively. However, there was no significant difference in the duration of the loss of the righting reflex between the two groups.

Figure 1 illustrates the effects of the ultra-short–acting hypnotics on the rota-rod and traction test. Two-way ANOVA revealed that triazolam (0.3 mg/kg, i.p.) significantly impaired motor coordination in the rota-rod test [drug: $F(1,30) = 18.481$, $P<0.001$; time: $F(2,30) = 0.212$, $P>0.05$; interaction: $F(2,30) = 0.201$, $P>0.05$] and produced muscle relaxant activity in the traction test [drug: $F(1,30) = 3.225$, $P>0.05$; time: $F(2,30) = 3.225$, $P<0.05$; interaction: $F(2,30) = 3.786$, $P<0.05$]. However, zolpidem (3 mg/kg, i.p.) and zopiclone (6 mg/kg, i.p.) had no effect on the rota-rod and traction test by itself.

Table 1. The duration of the loss of the righting reflex induced by the combination of thiopental and hypnotics

Treatment	Dose	n	Duration of the loss of righting reflex (s)
Saline	1 ml/kg	7	113.1 ± 24.5
Zolpidem	3 mg/kg	7	205.3 ± 62.6
	10 mg/kg	7	1109.1 ± 122.1[a]
0.5% PPG	1 ml/kg	7	117.3 ± 15.0
Zopiclone	3 mg/kg	7	146.3 ± 41.6
	6 mg/kg	9	227.2 ± 67.4
Triazolam	0.3 mg/kg	7	234.8 ± 39.5
Nitrazepam	0.6 mg/kg	6	225.5 ± 35.5
	1 mg/kg	7	655.6 ± 172.4[b]
Quazepam	3 mg/kg	7	1080.4 ± 524.3[b]

Hypnotics or vehicle (saline or 0.5% PPG, 1 ml/kg) were administered intraperitoneally 10 or 20 min before the injection of thiopental (20 mg/kg, i.v.). Data represents the mean time with S.E.M. Data were analyzed using one way-ANOVA followed by the Dunnett test. [a]$P<0.05$ *vs* saline and [b]$P<0.05$ *vs* 0.5% PPG.

Figure 2 illustrates the effects of the long-acting hypnotics, nitrazepam and quazepam. Nitrazepam (1 mg/kg, i.p.) significantly impaired motor coordination in the rota-rod test [drug: $F(1,30) = 10.837$, $P<0.01$; time: $F(2,30) = 3.569$, $P<0.05$; interaction: $F(2,30) = 4.307$, $P<0.05$] and muscle relaxant activity in the traction test [drug: $F(1,30) = 5.753$, $P<0.05$; time: $F(2,30) = 0.425$, $P>0.05$; interaction: $F(2,30) = 0.410$, $P>0.05$]. Quazepam (3 mg/kg, i.p.) significantly impaired motor coordination in the rota-rod test [drug: $F(1,30) = 9.365$, $P<0.01$; time: $F(2,30) = 3.178$, $P>0.05$; interaction: $F(2,30) = 3.718$, $P<0.05$]. However, quazepam showed no muscle relaxant activity.

Figure 3 shows the effects of the co-administration of the ultra-short–acting hypnotics and thiopental (20 mg/kg, i.v.). The co-administration of triazolam and thiopental markedly impaired motor coordination [drug: $F(1,36) = 207.364$, $P<0.001$; time: $F(2,36) = 7.571$, $P<0.01$; interaction: $F(2,36) = 7.518$, $P<0.01$] and muscle relaxant activity [drug: $F(1,36) = 57.438$, $P<0.001$; time: $F(2,36) = 8.429$, $P<0.001$; interaction:

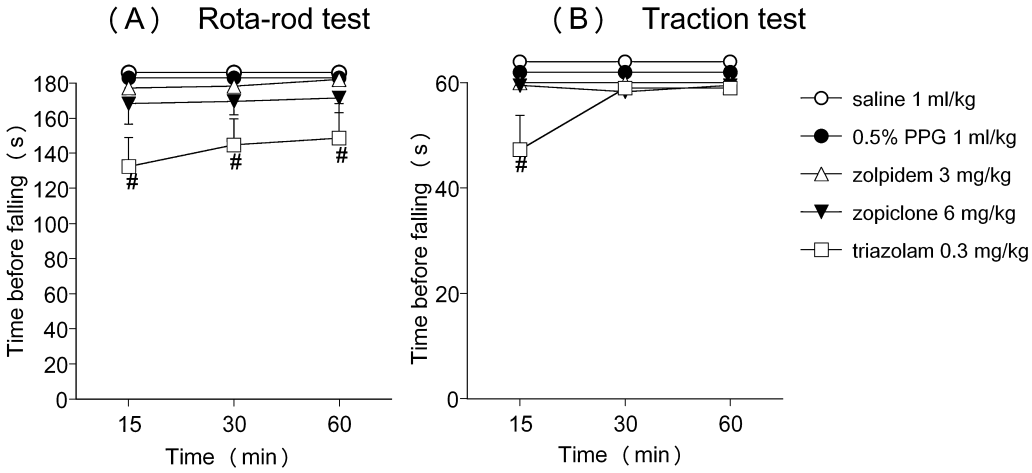


Fig. 1. Effects of the ultra-short–acting hypnotics, zolpidem, zopiclone, and triazolam, on the rota-rod and traction tests. The mice were intraperitoneally administered these hypnotics 15 min before the start of the tests. Each point represents the mean time of 6 mice with S.E.M. before falling in the rota-rod test (A) and the traction test (B). Data were analyzed using a repeated measures two way-ANOVA followed by the Dunnett test. $^{\#}P<0.05$: in comparison to vehicle (0.5% PPG).

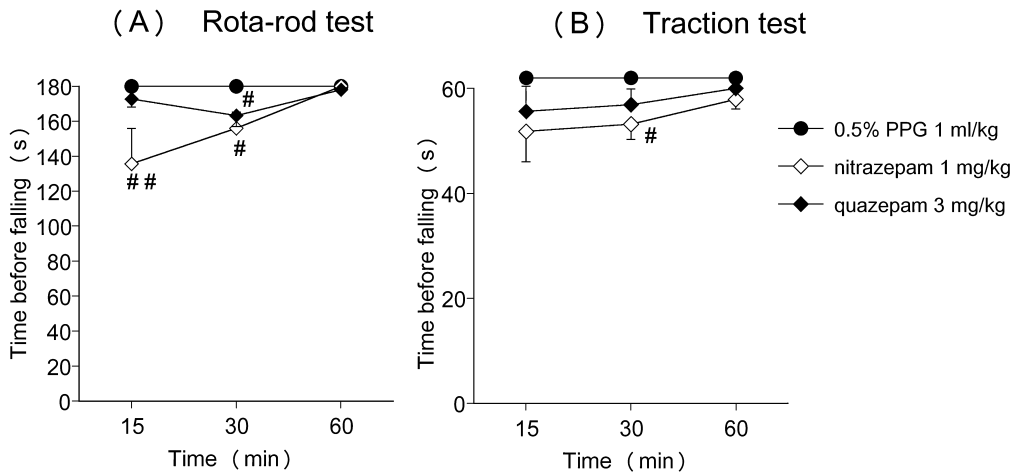


Fig. 2. Effects of the long-acting hypnotics, nitrazepam and quazepam, on the rota-rod and traction test in mice. The mice were intraperitoneally administered these hypnotics 15 min before the start of the tests. Each point represents the mean time of 6 mice with S.E.M. before falling in the rota-rod test (A) and the traction test (B). Data were analyzed using a repeated measures two way-ANOVA followed by the Dunnett test. $^{\#}P<0.05$, $^{\#\#}P<0.01$: in comparison to vehicle (0.5% PPG).

$F(2,36) = 4.353$, $P<0.001$] in comparison to the co-administration of vehicle (0.5% PPG) and thiopental. The impairment of motor coordination continued until 60 min after the recovery from the loss of the righting reflex. The co-administration of zopiclone (6 mg/kg i.p.) and thiopental significantly impaired motor coordination [drug: $F(1,42) = 18.464$, $P<0.001$; time: $F(2,42) = 6.728$, $P<0.01$; interaction: $F(2,42) = 4.282$, $P<0.05$] in the beginning of the recovery until 30 min from the loss of the righting reflex. However, there was no effect on the muscle relaxant activity. On the other hand, the co-administration of zolpidem (3 mg/kg, i.p.) and thiopental did not have any effect on either the rota-rod or traction tests.

Figure 4 shows the combined effects of the long-acting hypnotics and thiopental. Co-administration of nitrazepam (1 mg/kg, i.p.) impaired motor coordination [drug: $F(1,36) = 31.037$, $P<0.001$; time: $F(2,36) = 14.791$, $P<0.001$; interaction: $F(2,36) = 31.157$, $P<0.001$] and muscle relaxant activity [drug: $F(1,36) = 4.803$, $P<0.05$; time: $F(2,36) = 0.255$, $P>0.05$; interaction: $F(2,36) = 0.245$, $P>0.05$] in comparison to the co-administration of the vehicle (0.5% PPG). However, a *post hoc* comparison using the Dunnett test revealed no significant difference in muscle relaxant activity at 15, 30, and 60 min. The co-administration of quazepam

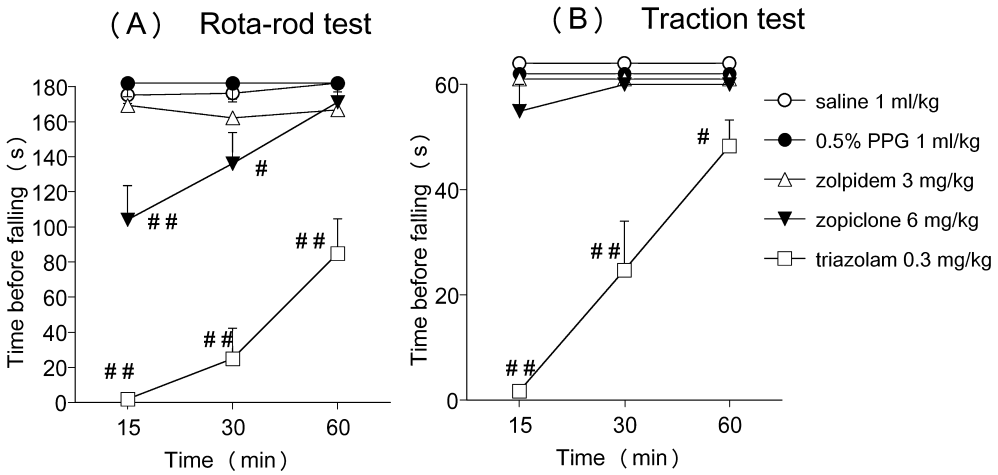


Fig. 3. The time course of changes in motor incoordination and muscle relaxant activity after recovery from the loss of the righting reflex by thiopental (20 mg, i.v.) and ultra-short-acting hypnotics. Zolpidem (3 mg/kg, n = 7), zopiclone (6 mg/kg, n = 7), and triazolam (0.3 mg/kg, n = 7) were administered intraperitoneally 10 min before the injection of thiopental (20 mg/kg, i.v.). These ultra-short–acting hypnotics similarly caused an approximately 2-fold prolongation of the thiopental-induced anesthesia (Table 1). Each point represents the mean time with S.E.M. before falling in the rota-rod test (A) and the traction test (B). Data were analyzed using a repeated measures two way-ANOVA followed by the Dunnett test. $^{\#}P<0.05$, $^{\#\#}P<0.01$: in comparison to vehicle (0.5% PPG).

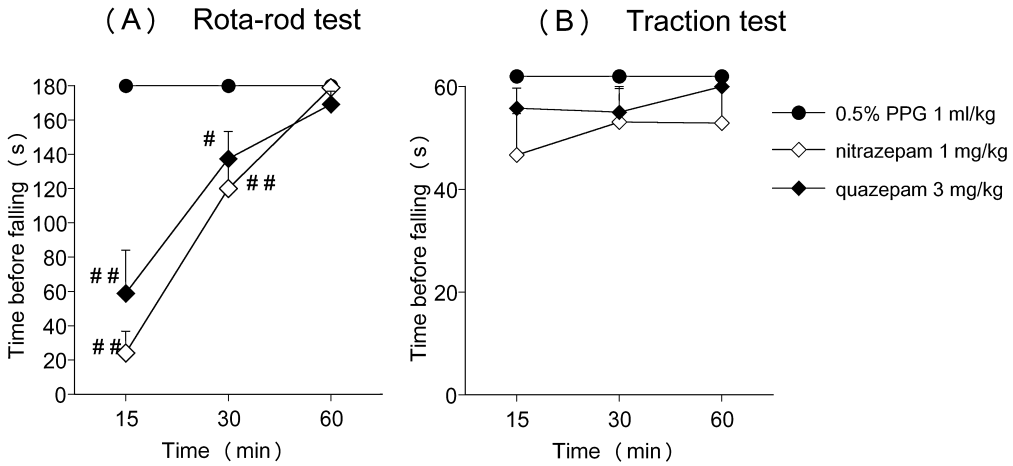


Fig. 4. The time course of changes in motor incoordination and muscle relaxant activity after recovery from the loss of the righting reflex by thiopental (20 mg, i.v.) and long-acting hypnotics. Nitrazepam (1 mg/kg, n = 9) and quazepam (3 mg/kg, n = 7) were administered intraperitoneally 20 min before the injection of thiopental (20 mg/kg, i.v.). The doses of nitrazepam and quazepam caused a 6- or 10-fold prolongation of the thiopental-induced anesthesia, respectively (Table 1). Each point represents the mean time with S.E.M. before falling in the rota-rod test (A) and the traction test (B). Data were analyzed using a repeated measures two way-ANOVA followed by the Dunnett test. $^{\#}P<0.05$, $^{\#\#}P<0.01$: in comparison to vehicle (0.5% PPG).

(3 mg/kg, i.p.) impaired motor coordination [drug: $F(1,36) = 21.232$, $P<0.001$; time: $F(2,36) = 8.479$, $P<0.001$; interaction: $F(2,36) = 10.323$, $P<0.001$]. However, co-administration of quazepam had no significant effect on the muscle relaxant activity in the traction test.

Discussion

Thiopental is an ultra-short–acting depressant of the central nervous system, which induces hypnosis and anesthesia. Following the intravenous injection of thiopental, the drug rapidly reaches the brain and causes unconsciousness. At 1 min, the drug attains a peak concentration of about 60% of the total dose in the brain. Thereafter, the drug is distributed to the rest of the

body and the concentration is low enough in the brain such that consciousness returns (22). However, both drowsiness and staggering tend to still remain for as long as 15 min after an individual regains consciousness (25). Therefore, in this study, we applied the thiopental-induced sleep model to evaluate the side effects of benzodiazepine-receptor agonists after awaking from sleep. In the present study, the intravenous injection of thiopental (20 mg/kg) produced a brief period of loss of the righting reflex for about 2 min, and marked impairments of motor coordination and muscle relaxant activity were observed just after recovery from the loss of the righting reflex (data not shown). However, these impairments completely disappeared 15 min after the recovery of the righting reflex.

Thiopental is a rapidly acting barbiturate (22). Barbiturates and benzodiazepines are agonists for the $GABA_A$-receptor complex, and the combinations of benzodiazepines with barbiturates exhibit synergistic interactions (22, 26). On the other hand, the pharmacological relevance of the $GABA_A$-receptor subtypes was identified by using gene–knock-out mice, and α_1-$GABA_A$ receptors were found to mediate sedation, anterograde amnesia, and seizure protection (27). α_2-$GABA_A$ receptors are highly specifically expressed in the spinal cord, predominately in the superficial layer of the dorsal horn and in motor neurons (28), with the latter being most clearly implicated in muscle relaxation. The in vivo [^{3}H]flumazenil binding assays showed that zopiclone, triazolam, and nitrazepam interact with high affinity at both the Type I (containing α_1-subunit) and Type II (containing α_1-, α_2-, α_3-, and α_5-subunits) benzodiazepine receptors, while the selectivity ratio of zopiclone for the Type I receptors was 2- to 6-fold more potent than that of triazolam and nitrazepam, respectively (29, 30). In contrast, zolpidem and quazepam have been shown to have a high selectivity for Type I benzodiazepine receptors (13, 14). In the present study, triazolam (0.3 mg/kg, i.p.) and nitrazepam (1 mg/kg, i.p.) produced a slight but significant impairment of the muscle relaxant action in the traction test. However, the single administration of zolpidem (3 mg/kg i.p.), quazepam (3 mg/kg i.p.), and zopiclone (6 mg/kg i.p.) did not produce any muscle relaxant action.

The potentiation of thiopental-induced anesthesia has been used as a screening method for hypnotic action in mice (31 – 33). In this study, ultra-short–acting hypnotics, zolpidem (3 mg/kg), zopiclone (6 mg/kg), and triazolam (0.3 mg/kg), approximately 2-fold prolonged the duration of the loss of the righting reflex induced by thiopental (20 mg/kg, i.v.) in a similar degree. On the other hand, the long-acting agents, nitrazepam (1 mg/kg) and quazepam (3 mg/kg), approximately 6- or 10-fold prolonged the duration of the loss of the righting reflex, respectively.

The co-administration of triazolam and thiopental markedly exacerbated the impairments of both motor coordination and muscle relaxant activity in comparison to triazolam alone. The co-administration of zopiclone significantly impaired motor coordination in the beginning of the recovery from the loss of the righting reflex. However, there was no significant effect on the muscle relaxant action, suggesting a faster improvement of the muscle relaxant action than the impairment of motor coordination during the recovery from the loss of the righting reflex. On the other hand, the co-administration with zolpidem, which has high selectivity for Type I benzodiazepine receptors, did not have any effects on either the rota-rod or the traction tests. These findings indicated that zolpidem is short-acting and highly selective for Type I benzodiazepine receptors and shows a faster recovery from impairments of motor coordination and muscle flaccidity induced by the co-administration with thiopental.

Regarding long-acting hypnotics, the co-administration of thiopental and quazepam (Type I benzodiazepine receptor–selective agonist) or nitrazepam (Type I/II non-selective agonist) produced marked impairments of the motor coordination without muscle relaxant action, and there is no significant difference in impairments of motor coordination between nitrazepam and quazepam. In addition, the impairments of motor coordination by co-administration of long-acting hypnotics were markedly more than those by the short-acting hypnotics, except for triazolam. These results indicated that the impairment of motor coordination during the recovery phase from anesthesia might be related to the half-life of hypnotics. However, further studies will be necessary, since quazepam caused a more prolonged duration of the loss of the righting reflex induced by thiopental in comparison to nitrazepam, but no significant difference was observed between the two groups. Taken together, the results of the current study suggest that zolpidem that is short-acting and highly selective for Type I benzodiazepine receptors shows faster recovery from impairment of motor coordination induced by the co-administration with thiopental.

In the present study, benzodiazepine-receptor agonists more markedly impaired motor coordination in the rota-rod test than muscle relaxant activity in the traction test during the recovery from the loss of the righting reflex induced by thiopental. In this study, we examined the muscle relaxant effects using the traction test, which measures the grip strength of the fore paws. However, there is a need to investigate the muscle relaxant activities of all four limbs using another method such as the

climbing test or inclined screen test (34). On the other hand, one of the side effects of benzodiazepine-receptor agonists is delirium (35) and this is thought to be associated with falls (36). Therefore, it is necessary to examine the effect of benzodiazepine-receptor agonists on the level of consciousness in order to achieve a more detailed understanding of the factors that contribute to prevent hypnotics-related falls in patients treated with these drugs.

In summary, the major findings of the present study were that the impairment of the movement disorder was markedly exacerbated after the recovery from the loss of the righting reflex induced by thiopental and that the profile of recovery from the impairments in both motor coordination and muscle flaccidity may be related to the half-life of the hypnotics and their selectivity for Type I benzodiazepine receptors. Further studies will be necessary to clarify the pharmacological profile of hypnotics after awakening using another hypnotic model.

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