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Comprehensive behavioral analysis of mice repeatedly treated with propofol

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Abstract. Propofol, known as "milk of anesthesia", is used for the induction and maintenance of anesthesia. Recently, propofol has attracted increasing concerns about its safety and abuse potential because of its psychostimulant effects such as euphoria and sexual hallucinations. Previous reports focused on the effects of postoperative and neonatal exposure to propofol. However, the lasting effects of repetitive propofol administration during adulthood have not been well investigated. It is conceivable that prolonged use of propofol affects brain function and the behavioral characteristics of the abused patient. Thus, we performed a comprehensive behavioral analysis of mice exposed to propofol. Adult male C57BL/6J mice were repeatedly administered with propofol (20 or 80 mg/kg/day i.p.), intralipos (vehicle control), or saline only once a day for seven days. We then performed a behavioral test battery to evaluate various behaviors. Afterwards, we resumed the propofol treatment for three days and subsequently conducted contextual and cued fear conditioning tests. In the three-chamber social approach test, propofol treatment attenuated social novelty preference in mice. In the fear conditioning test, high dose-treated mice exhibited impaired long-term cued-dependent memory retention. In the rotarod test, propofol- and intralipos-treated mice tended to have decreased motor coordination than the saline-treated mice. Our results demonstrated that repetitive propofol treatment has the potential to induce some behavioral changes in mice. Additionally, the solvent itself might have effects different from that of propofol. Our findings provide basic data on the safe use and risk of propofol abuse.
Key words: behavioral test battery, propofol, abuse, anemia, social behavior

Highlights

Propofol, known as "milk of anesthesia", has attracted increasing concerns about its safety and abuse potential. The lasting effects of repetitive propofol administration during adulthood have not been well investigated. To clarify the effects of repetitive propofol use on brain function and behavioral characteristics, we performed a comprehensive behavioral analysis of mice exposed to propofol. In this study, propofol treatment attenuated the social novelty preference and the performance of the cued long-term memory task in mice. Additionally, treatment with propofol and intralipos tended to induce decreased motor coordination. Our results demonstrated that repetitive propofol treatment has the potential to induce some behavioral changes in mice. Furthermore, the solvent itself might have effects different from that of propofol. Our findings provide basic data for the safe use and risk of propofol abuse.

Introduction

Propofol is widely used for the induction and maintenance of anesthesia because of its rapid onset of action and short recovery time [1, 2]. Propofol enhances the action of γ-Aminobutyric acid (GABA) through the GABA type A ($GABA_A$) receptor and also blocks the N-methyl-D-aspartate (NMDA) glutamate receptor [3]. Other than anesthesia, propofol has beneficial effects on human health. Previous reports showed the protective effects of propofol against ischemic injury of the adult brain and oxidative stress via $GABA_A$ receptor-mediated signal cascade [4, 5]. Over the past ten years, propofol's risk potential has received increased attention and was highlighted by the death of pop singer, Michael Jackson, in 2009 [6]. The misuse and abuse of propofol among healthcare providers have been reported worldwide, and some misuse has resulted in death [7, 8]. In the United States, 18% of anesthesiology departments reported one or more incidents of propofol abuse or diversion over the past 10 years [7]. These reports suggest that propofol can also have negative effects. The exact mechanisms and effects of propofol on brain function are not well understood. In fact, the memory loss after anesthesia using propofol has been reported as a side effect [9–11]. In some animal models, memory impairment by propofol treatment was demonstrated [12, 13]. These reports revealed the

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single-dose and short-term effects of propofol. However, the effect of repeated doses of propofol over the long-term remains unclear. In addition, because of its poor solubility in water propofol is utilized as an emulsion contains soy oil and egg lecithin. In previous studies in rats, the contents of vehicles such as soy oil and egg lecithin induced anxiolytic behavior [14, 15]. These reagents may affect the results of experiments. Notably, some of the propofol studies have not been standardized by using saline as a control. In this study, we assessed the effects of four different solutions: saline, intralipos (solvent used as a vehicle), and low-dose and high-dose propofol. Therefore, in the present study, C57BL/6J mice treated repeatedly with propofol were evaluated using a comprehensive behavioral test battery to investigate how the repetitive use of propofol affects various behaviors.

Materials and Methods

Animals and experimental design for comprehensive behavioral analysis

Fifty-nine naïve male C57BL/6J mice were transported from Japan SLC, Inc. (Shizuoka, Japan) to the laboratory at University of Toyama when they were seven weeks old. After their arrival, they were group housed (4/cage) in a plastic cage (22.7 × 32.3 × 12.7 cm) in a room maintained at 24 ± 3°C with a 12-hr light/dark cycle (lights on at 7:00 am) and *ad libitum* access to food and water. The mice were randomly assigned to either the saline group (n=13), the intralipos group (n=14), the low-dose (20 mg/kg) propofol group (n=16), or the high-dose (80 mg/kg) propofol group (n=16). Propofol administration begun when the mice were eight weeks old. Their behaviors were assessed with a battery of behavioral tests when the mice were nine weeks old.

Propofol treatment

Mice were treated with propofol (20 or 80 mg/kg, i.p.; Pfizer Inc., Tokyo, Japan) dissolved in intralipos (Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan), intralipos only, or saline only (1% of body weight) once a day. Propofol treatment was initiated when the mice were eight weeks old and continued for seven days. To perform the fear conditioning test under the chronic treatment condition, we resumed treatment three days before the test and interrupted treatment again after the test.

Behavioral tests

The mice were subjected to a battery of behavioral tests in the following sequence: general health and neurologic screening (body weight, body temperature, and grip strength), light/dark transition, open field, elevated plus maze, hot plate, social interaction, rotarod, three-chamber social approach, startle response/prepulse inhibition, Porsolt forced swim test, and contextual/cued fear conditioning and fear extinction tests. The schedule for the comprehensive test battery for this study is shown in Table 1 and Fig. 1A. All the saline-treated, intralipos-treated, and propofol-treated mice underwent the same test battery on the same day and in the same order. The ordinal position in the sequence of removal from the cage and the test chamber were carefully counterbalanced between groups. After each test, the floors and walls of the testing apparatuses were cleaned with 70% ethanol solution or super hypochlorous water to prevent bias caused by olfactory cues. The behavioral tests were performed between 8:30 am and 6:00 pm. Information about each mouse and the behavioral data collected in this study are available in the "Mouse Phenotype Database" (http://www.mouse-phenotype.org/).

General health and neurological screen test (GHNS):

The righting, whiskers twitch, and ear twitch reflexes were evaluated. A number of physical features, including the presence of whiskers or bald hair patches, were also recorded. Body weight and rectal temperature were measured. Neuromuscular strength was assessed using the grip strength and wire hang tests. A grip strength meter (O'Hara & Co., Tokyo Japan) was used to assess forelimb grip strength. In this test, mice were lifted and held by their tail while their forepaws grasped a wire grid. The mice were then gently pulled backward by the tail until they released their grip on the grid. The peak force applied by the forelimbs of the mouse was recorded in Newtons (N). Each mouse was tested three times, and the largest value was used for statistical analysis.

Light/dark transition test (LD):

The light/dark transition test, developed by Crawley and colleagues [16], was performed as previously described [17]. The apparatus comprised a cage (21 × 42 × 25 cm) divided into two sections of equal size by a partition with a door

Table 1. The schedule of the comprehensive behavioral test battery of propofol-treated mice

Order	Test	Age (w)	Table/Figure
1	General health and neurological screen test (GHNS)	9	Table 2
2	Light/dark transition test (LD)	10	Table 2
3	Open-field test (OF)	10	Table 2
4	Elevated plus maze test (EP)	11	Fig. 3C–F
5	Hot plate test (HP)	11	Table 2
6	Social interaction test (SI)	11	Table 2
7	Rotarod test (RR)	12	Fig. 3A and 3B
8	Three-chamber social approach test (CSI)	13	Fig. 1C and 1D
9	Startle response/prepulse inhibition test (PPI)	14	Table 2
10	Porsolt forced swim test (PS)	14	Table 2
11	Contextual and cued fear conditioning and fear extinction test (FZ)	35–43	Fig. 2A–F, Table 2

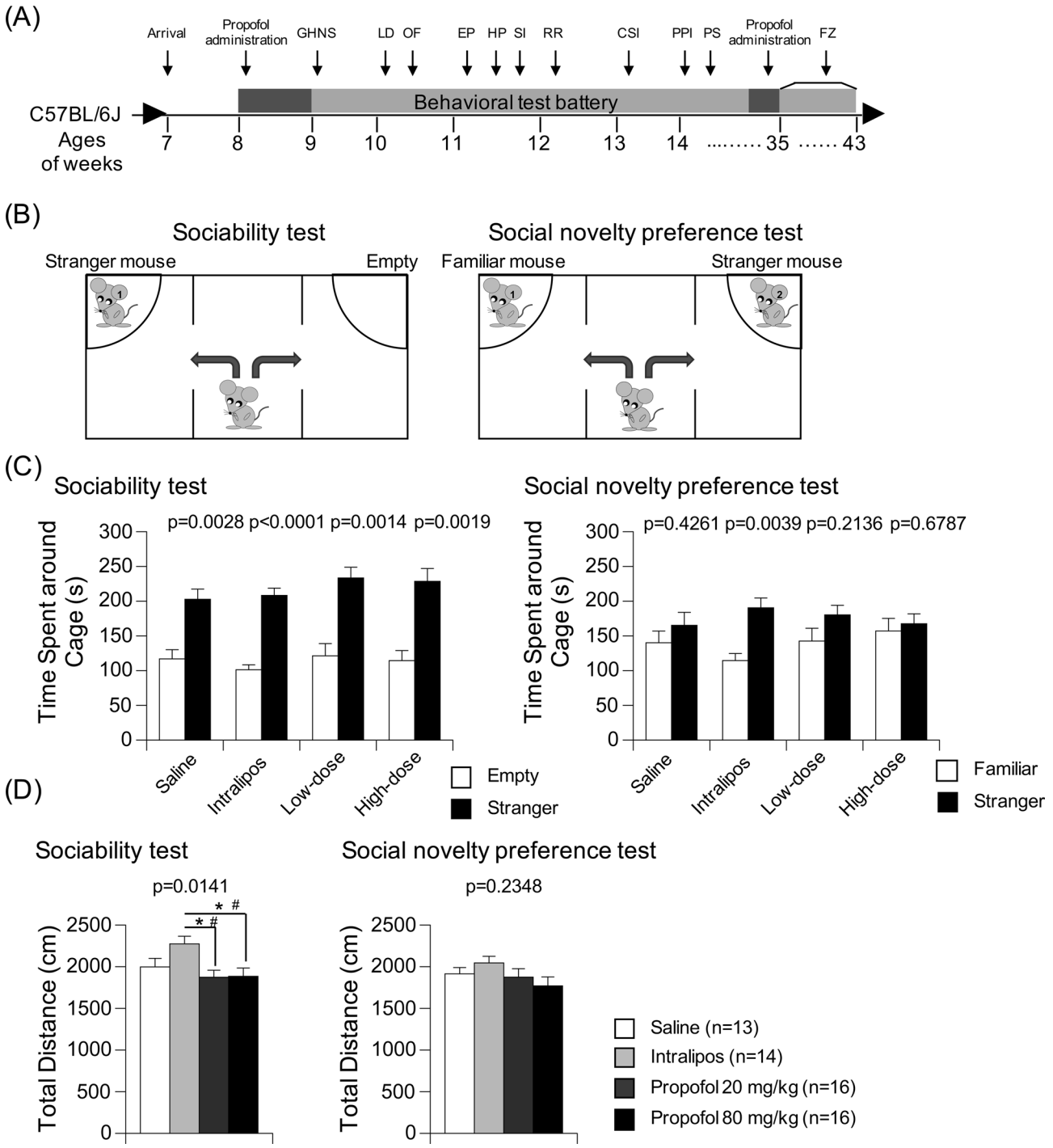

Fig. 1. Propofol-treated mice exhibited abnormal social novelty in the three-chamber social approach test. The propofol treatment and the behavioral test battery were performed in the schedule as described above (A). Schematic diagram of the three-chamber social approach test (B). In the first session, one of the cages contains a stranger mouse (stranger 1), and the other cage is empty. In the second session, the test mouse becomes familiar with the stranger mouse (stranger 1), and the other stranger mouse (stranger 2) is put in the other cage. The time spent around the cage with stranger 1 or around the empty cage, time spent around the cages with stranger 1 and stranger 2 (C), and total distance traveled (D) are shown. In the social novelty test, intralipos-treated mice showed more preference for stranger 2. On the other hand, there are no significant differences between preference for stranger 1 and stranger 2 in propofol-treated mice. Data are presented as means ± SEM for the indicated numbers of animals. The paired *t*-test (C) and one-way analysis of variance (D) were used to test for statistical significance. The *P* values indicate the treatment effect in each statistical analysis. The asterisk indicates a nominally significant difference for comparisons between treatment groups ($P<0.05$) and the number sign indicates significance after Bonferroni correction ($P<0.05/6$).

(O'Hara & Co.). One chamber was brightly illuminated (390 lux), whereas the other was dark (2 lux). Mice were placed in the dark chamber and were allowed to move freely between the two chambers for 10 min with the door open. The distance traveled (cm), total number of transitions between compartments, latency to the first entry into the light chamber (sec), and time spent in the light chamber (sec) were recorded automatically using the ImageLD program.

Open-field test (OF):

Locomotor activity was measured using an open-field test. Each mouse was placed in the corner of the open-field apparatus (40 × 40 × 30 cm: AccuScan Instruments, Columbus, OH, USA). The center of the floor was illuminated at 100 lux. Total distance traveled (cm), vertical activity (rearing measured by counting the number of photobeam interruptions), time spent in the center area (20 × 20 cm), and beam-break counts for stereotypic behaviors were recorded. Data were collected for a period of 120 min.

Elevated plus maze test (EP):

The elevated plus maze test, which is widely used to assess anxiety-like behavior, was performed as previously described [18]. The apparatus comprised two arms without walls (open arms, 25 × 5 cm), two arms of the same size with 15-cm-high transparent walls (closed arms), and a central square (5 × 5 cm) connecting the arms, which were at 90° to each other (O'Hara & Co.). The arms and central square were made of white plastic plates and were elevated to a height of 55 cm above the floor. The open arms were surrounded by a raised ledge (3 mm thick and 3 mm high) to prevent mice from falling off the open arms. Arms of the same type were located opposite one another. Each mouse was placed in the central square of the maze facing one of the closed arms. The number of arm entries, distance traveled (cm), percentage of entries into the open arms, and percentage of time spent in the open arms were measured during a 10-min test period. Data acquisition and analysis were performed automatically using the ImageEP program.

Social interaction test (SI):

The social interaction test was conducted to measure social behavior in a novel environment, as previously described [19]. Weight-matched (within 2 g) mice of the same treatment group that had been housed in different cages were placed together into an acrylic box (40 × 40 × 30 cm) and allowed to explore freely for 10 min. The total number of contacts, total duration of contacts (sec), total duration of active contacts (sec), mean duration per contact (sec), and total distance traveled (cm) were recorded and analyzed automatically using the ImageSI program. Active contact was defined as contact between two mice followed by one or both mice moving with a velocity of at least 10 cm/sec.

Three-chamber social approach test (CSI):

The three-chamber social approach test is a well-designed test to investigate sociability and preference for social novelty in mice [20]. The apparatus comprised a rectangular, three-chambered box and a lid with a video camera (O'Hara & Co.). Each chamber was 20 × 40 × 47 cm, and the dividing walls were made from clear Plexiglas with a small square opening (5 × 3 cm) allowing access to each chamber. The tests were performed as previously described [21], with slight modification as follows: subject mice were placed in the three-chambered box and allowed to explore for 10 min before the sociability test was conducted (habituation session), and during the session, empty wire cages (9 cm in diameter, 11 cm in height, with vertical bars 0.5 cm apart) were located in the corner outside each compartment. During the following session, an unfamiliar C57BL/6J male mouse (stranger 1) that had had no prior contact with the subject mouse was put into a wire cage located in one of the side chambers. The location of the stranger mouse in the left vs right chamber was systematically alternated between trials. The subject mouse was placed in the central compartment and allowed to explore the entire box for 10 min to assess sociability (sociability test). Next, a second stranger male mouse was placed into the wire cage in the other compartment that had been empty during the first 10-min session to evaluate social preference for a new stranger (social novelty preference test). Thus, the subject mouse had a choice between the first, already investigated and now familiar mouse (stranger 1) and the novel unfamiliar mouse (stranger 2). The amount of time spent in each chamber and time spent around each cage were automatically calculated from video images using the ImageCSI program. The male C57BL/6J mice used as stranger 1 and 2 were transported from Japan SLC, Inc. (Shizuoka, Japan) to the laboratory in University of Toyama when they were four weeks old. After their arrival, the mice were group housed (4/cage) and used for the three-chamber social approach test when they were 10–11 weeks old.

Rotarod test (RR):

Motor coordination and balance were tested with the rotarod test. The rotarod test was performed using an accelerating rotarod (UGO Basile Accelerating Rotarod, Varese, Italy). It was performed by placing mice on a rotating drum (3 cm diameter) and measuring how long each animal was able to maintain its balance on the drum. The speed of the rotarod increased from 4 to 40 rpm over a 5-min period.

Hot plate test (HP):

The hot plate test was used to evaluate sensitivity to a painful stimulus. The mice were placed on a 55.0 (± 0.3)°C hot plate (Columbus Instruments, Columbus, OH, USA), and the latency to the first hind-paw response was recorded. The hind-paw response was defined as either a foot shake or a paw lick.

Startle response/prepulse inhibition test (PPI):

The startle response and prepulse inhibition test were performed as previously described [22]. A startle reflex measurement system (O'Hara & Co.) was used. The mice were placed in a Plexiglas cylinder and left undisturbed for 10 min. The test comprised two test trials involving only the startle stimulus, followed by four test trials for prepulse inhibition. White noise (40 msec) was used as the startle stimulus for all trials. The startle response was recorded for 140 msec (while measuring the response every 1 msec) starting with the onset of the prepulse stimulus. The background noise level in each chamber was 70 dB. The peak startle amplitude recorded during the 140-msec sampling window was used as the dependent variable. The intensity of the startle stimulus was 110 or 120 dB. The prepulse sound was presented 100 msec before the startle stimulus, and its intensity was 74 or 78 dB. Four combinations of prepulse

and startle stimuli were employed (74–110, 78–110, 74–120, and 78–120 dB). The mean inter-trial interval was 15 sec (range 10–20 sec).

Porsolt forced swim test (PS):

The Porsolt forced swim test [23] was performed to assess depression-related behavior. Mice were placed into a Plexiglas cylinder (20 cm height × 10 cm diameter, O'Hara & Co.) filled with water (approximately 23°C) up to a height of 7.5 cm for 10 min per day for two consecutive days. The percentage of time spent immobile was recorded automatically using the ImagePS program.

Contextual and cued fear conditioning test and extinction test (FZ):

The fear conditioning test was conducted using an automated video-analysis system as previously described [24]. Mice were placed in a conditioning chamber (26 × 34 × 29 cm) in a sound-attenuated room and allowed to explore freely for 2 min. The animals were presented with an auditory cue (55 dB white noise) that served as a conditioned stimulus (CS) for 30 sec. During the last 2 sec of the CS, mice were given a foot shock (0.3 mA, 2 sec) as an unconditioned stimulus (US). Two more CS-US pairings were presented at 120-sec intervals. One day and 30 days after the conditioning session, a context test was performed in the conditioning chamber. A cued test in an altered context was performed after the context test using a triangular box (35 × 35 × 40 cm) made of white opaque plastic, which was located in a different sound-attenuated room. In the cued test, after the initial 3-min period of no CS presentation, the CS was presented during the last 3-min period of the test. The cued tests were performed more than 30 min later after the contexts test were completed.

The fear extinction test was also conducted using an automated video-analysis system 55 days after the conditioning session (Table 1). The animals were placed inside the conditioning chamber. After the initial 4-min period of no cue presentation, the cue was presented during the last 14-min period of the test. Next, mice were exposed to the altered context after the initial 3-min period of no CS presentation, and the CS was presented during the last 3-min period of the test. Freezing during each minute of the test was measured automatically using the ImageFZ program in the same manner as previously described [24] [23]. In the fear conditioning test, the data of one mouse from the saline group and two mice from the high-dose group were excluded from statistical analysis because the mice died in the home cage before the test. In the fear extinction test, the data of another mouse were excluded from statistical analysis because it died in the home cage before the test. In addition, due to technical problems with the video analysis system, we failed to obtain the data for one mouse from the intralipos group, two mice from the low-dose group, and one mouse from the high-dose group, and therefore excluded the data for these mice from the statistical analysis.

Data analysis in behavioral tests

Behavioral data were obtained automatically using applications (ImageLD [17], ImageEP [18], ImageSI [19], ImageCSI [21], ImagePS [25], and ImageFZ [24]) based on the public domain NIH Image program and ImageJ program. These applications were modified for each test. The plugins are freely available on the "Mouse Phenotype Database" website (http://www.mouse-phenotype.org/software.html) [26].

Statistical analysis

Statistical analyses were performed using StatView (SAS Institute, Cary, NC, USA). A priori planned comparisons were performed to examine the effect of drug treatment (vehicle vs. propofol). In addition, we examined the effect of nutritive materials in the vehicle as a priori planned comparisons (saline vs. vehicle). A priori planned multiple comparisons were performed using the *t*-test with Bonferroni correction. After a priori comparisons, data were analyzed using either the one-way or two-way analysis of variance (ANOVA) followed by Fisher's Protected Least Significant Difference (PLSD) test, two-way repeated measures ANOVA, or paired *t*-test, where appropriate. Values in graphs are presented as mean ± S.E.M. For multiple comparisons in the behavioral test battery, we defined study-wide significance as statistical significance after controlling for the false discovery rate (FDR) [27, 28]. Nominal significance was defined as a statistically significant difference in an index ($P<0.05$) that did not survive FDR and Bonferroni corrections. For a priori multiple comparisons, $P<0.05/3$ was defined as statistically significant (2-sided). For the post-hoc multiple comparisons, $P<0.05/6$ was defined as statistically significant (2-sided). The results of the statistical analyses are described in Table 2.

Results

Abnormal social behavior in propofol-treated mice

We examined the social behavior of propofol-treated mice using the social interaction test in a novel environment and the three-chamber social approach test. In the social interaction test conducted in a novel environment, there were no significant differences among groups in all parameters (Table 2). The three-chamber social approach test was used to assess sociability and social novelty preference. In the sociability test, the preference for a novel mouse is quantified based on the time spent around a wire cage containing a stranger mouse versus an empty cage. In the social novelty preference test, the preference for a stranger mouse versus a familiar mouse is tested (Fig. 1B). In all four groups, the time spent around the cage with stranger 1 was significantly longer than the time spent around the empty cage (Fig. 1C). The total distance traveled by propofol-treated mice significantly decreased compared with intralipos-treated mice (treatment effect, $P=0.0141$; saline group vs intralipos group, $P=0.0528$; intralipos group vs low-dose group, $P=0.0038$; intralipos group vs high-dose group, $P=0.0048$; Fig. 1D). In the social novelty preference test, the intralipos-treated mice spent a significantly longer time around the cage of the stranger mouse compared to that of the familiar mouse (Fig. 1C). In contrast, the other three groups did not show a significant preference for the stranger mouse (Fig. 1C). These results suggest that propofol treatment attenuated social novelty preference in mice.

Table 2. Statistical analyses of behavioral data

Test	Measure	Figure	Means ± SEM				*P*-value	
			Saline	Intralipos	Low-dose treatment	High-dose treatment	Treatment	Treatment × trial/ time
General health and neurological screen test	Body weight (g)	-	25.022 ± 0.29	24.872 ± 0.33	24.72 ± 0.406	24.683 ± 0.377	0.914	-
	Body temperature (°C)	-	36.031 ± 0.128	35.814 ± 0.229	35.831 ± 0.198	35.862 ± 0.174	0.8454	-
	Grip strength (N)	-	0.623 ± 0.043	0.721 ± 0.047	0.68 ± 0.049	0.664 ± 0.041	0.5372	-
	Wire hang (sec)	-	34.377 ± 6.477	33.108 ± 5.927	39.045 ± 5.869	34.233 ± 5.788	0.8951	-
Light/dark transition test	Distance traveled (cm) in light chamber	-	559.877 ± 61.503	582.614 ± 59.466	530.894 ± 47.316	582.65 ± 44.757	0.8851	-
	Distance traveled (cm) in dark chamber	-	1410.208 ± 73.882	1441.65 ± 55.02	1397.706 ± 41.236	1436.794 ± 52.794		-
	Transitions	-	28.154 ± 3.23	27.643 ± 3.203	26.125 ± 2.439	28.563 ± 2.448	0.9263	-
	Latency to light chamber (sec)	-	105.538 ± 35.213	95.929 ± 25.65	72.688 ± 18.084	59.375 ± 10.528	0.4702	-
	Time spent in light chamber (sec)	-	139.038 ± 14.425	147.464 ± 13.612	139.688 ± 11.356	153.344 ± 10.673	0.8173	-
Open-field test	Distance traveled (cm)	-	1263.308 ± 112.058	1251.643 ± 144.29	922.938 ± 127.814	968.125 ± 128.787	0.1343	0.9695
	Vertical activity	-	1325.923 ± 119.609	1297.786 ± 149.744	962.938 ± 133.449	1006.063 ± 134.151	0.1347	0.3603
	Center time (sec)	-	1244.315 ± 133.007	1185.293 ± 106.407	1275.738 ± 194.204	1044.031 ± 138.501	0.6896	0.7016
	Stereotypic counts	-	12554.462 ± 771.543	11351.786 ± 846.737	10281.563 ± 725.498	10941.875 ± 832.878	0.2525	0.6812
Elevated plus maze test	Distance traveled (cm)	Fig. 3C	2149.231 ± 108.58	2025.536 ± 124.893	1873.647 ± 117.705	1865.488 ± 127.512	0.3136	-
	Number of entries	Fig. 3D	54.846 ± 3.728	48.857 ± 3.38	44.333 ± 3.814	44.813 ± 4.4	0.2299	-
	Entries into open arms (%)	Fig. 3E	41.823 ± 2.037	36.686 ± 1.683	33.94 ± 2.572	36.513 ± 3.523	0.2366	-
	Time on open arms (%)	Fig. 3F	16.308 ± 2.432	10.007 ± 1.201	10.6 ± 2.264	12.1 ± 2.182	0.174	-
Hot plate test	Hot plate latency (sec)	-	10.532 ± 0.7	11.499 ± 0.606	10.578 ± 0.648	12.016 ± 0.632	0.2882	-
Social interaction test	Distance traveled (cm)	-	2977.95 ± 182.021	3313.643 ± 278.336	3027.9 ± 212.557	2990.713 ± 254.708	0.7375	-
	Number of contacts	-	47.833 ± 4.269	49.714 ± 4.75	48.75 ± 3.783	49.375 ± 3.932	0.991	-
	Total duration of contacts (sec)	-	67.667 ± 8.694	67.671 ± 8.346	79.3 ± 9.516	82.663 ± 10.31	0.5747	-
	Total duration of active contacts (sec)	-	13.1 ± 1.464	14.457 ± 1.471	13.713 ± 0.884	13.75 ± 1.314	0.9131	-
	Mean duration of contact (sec)	-	1.4 ± 0.082	1.429 ± 0.234	1.688 ± 0.284	1.813 ± 0.374	0.6943	-
Rotarod test	Latency to fall (sec) in 1st trial*	Fig. 3A and 3B	142.923 ± 23.977	95.929 ± 16.89	93.688 ± 20.203	63.25 ± 13.47	0.4156	0.2707
	Latency to fall (sec) in 2nd trial		153.923 ± 26.038	162.071 ± 23.171	198.875 ± 16.906	143.438 ± 19.69		
	Latency to fall (sec) in 3rd trial		188.615 ± 26.925	197.5 ± 25.401	187.563 ± 22.687	158.188 ± 22.889		
	Latency to fall (sec) in 4th trial		200.846 ± 20.075	191.5 ± 21.91	219.688 ± 19.412	193.625 ± 21.1		
	Latency to fall (sec) in 5th trial		238.615 ± 16.728	211.429 ± 19.503	246.813 ± 16.049	212.563 ± 20.524		
	Latency to fall (sec) in 6th trial		255.385 ± 20.037	223.571 ± 13.826	236.188 ± 16.551	242.25 ± 15.091		

Table 2. Continued

Test	Measure	Figure	Means ± SEM				*P*-value	
			Saline	Intralipos	Low-dose treatment	High-dose treatment	Treatment	Treatment × trial/ time
Three-chamber social approach test (Sociability test)	Distance traveled (cm)*	Fig. 1D	1997.538 ± 102.413	2273.786 ± 91.004	1873.469 ± 84.252	1884.375 ± 99.415	0.0141	-
	The ratio of time spent around cage with stranger 1	-	0.634 ± 0.035	0.67 ± 0.026	0.664 ± 0.038	0.662 ± 0.04	0.8039	-
Three-chamber social approach test (Social novelty preference test)	Distance traveled (cm)	Fig. 1D	1914 ± 75.207	2045.25 ± 78.877	1874.813 ± 101.048	1769.838 ± 107.613	0.2348	-
	The ratio of time spent around cage with stranger 2	-	0.544 ± 0.048	0.62 ± 0.034	0.568 ± 0.038	0.529 ± 0.034	0.3091	-
Startle response test	Startle response (110 dB)	-	1.817 ± 0.232	2.152 ± 0.243	2.382 ± 0.328	1.922 ± 0.362	0.4637	-
	Startle response (120 dB)	-	2.615 ± 0.302	3.228 ± 0.393	3.078 ± 0.329	2.46 ± 0.364		-
Prepulse inhibition test	PPI (74–110 dB)	-	27.234 ± 6.085	30.4 ± 5.661	28.918 ± 5.708	18.29 ± 7.986	0.9052	-
	PPI (78–110 dB)	-	37.404 ± 5.786	42.155 ± 5.558	37.261 ± 4.825	43 ± 4.71		-
	PPI (74–120 dB)	-	30.434 ± 4.337	20.888 ± 4.91	20.358 ± 6.066	16.244 ± 5.748	0.377	-
	PPI (78–120 dB)		34.27 ± 5.421	36.81 ± 4.112	29.868 ± 4.641	25.469 ± 5.169		-
Porsolt forced swim test	Immobility (%) on Day 1	-	26.464 ± 3.772	32.678 ± 2.43	25.198 ± 2.741	27.751 ± 1.933	0.2474	0.3115
	Immobility (%) on Day 2	-	33.811 ± 3.288	33.476 ± 2.213	34.258 ± 2.65	33.051 ± 2.517	0.9895	0.6385
Fear conditioning test (Conditioning)	Freezing (%)	Fig. 2A	-	-	-	-	0.1946	0.7706
Fear conditioning test (Context test 1 day after conditioning)	Freezing (%)	Fig. 2B	-	-	-	-	0.4042	0.2574
Fear conditioning test (Cued test 1 day after conditioning (pre-CS))	Freezing (%)	Fig. 2C	-	-	-	-	0.4673	0.0944
Fear conditioning test (Cued test 1 day after conditioning (CS))	Freezing (%)	Fig. 2C	-	-	-	-	0.9602	0.2351
Fear conditioning test (Context test 30 days after conditioning)	Freezing (%)	Fig. 2D	-	-	-	-	0.8299	0.8971
Fear conditioning test (Cued test 30 days after conditioning (pre-CS))	Freezing (%)	Fig. 2E	-	-	-	-	0.6951	0.1099
Fear conditioning test (Cued test 30 days after conditioning (CS))	Freezing (%)*	Fig. 2E	-	-	-	-	0.0256	0.0393
Fear conditioning test (foot shock 1)	Distance traveled (cm)	Fig. 2F	-	-	-	-	0.6096	0.9862
Fear conditioning test (foot shock 2)	Distance traveled (cm)	Fig. 2F	-	-	-	-	0.5785	0.7142
Fear conditioning test (foot shock 3)	Distance traveled (cm)	Fig. 2F	-	-	-	-	0.8955	0.9988
Fear training (pre-CS)	Freezing (%)	-	33.63 ± 7.241	35.593 ± 6.543	40.523 ± 5.316	37.936 ± 6.206	0.8792	0.2078
Fear training (CS)	Freezing (%)	-	33.527 ± 6.161	33.135 ± 3.65	39.159 ± 3.296	37.492 ± 5.076	0.7142	0.2219
Fear extinction test (pre-CS)	Freezing (%)	-	27.021 ± 5.306	40.636 ± 4.98	42.1 ± 5.587	43.813 ± 5.586	0.1542	0.3107
Fear extinction test (CS)	Freezing (%)	-	43.491 ± 5.654	54.149 ± 6.187	49.276 ± 5.207	52.474 ± 7.207	0.6573	0.2952

Nominal significance: *$P<0.05$.

Impaired performance of cued long-term memory task in propofol-treated mice

The cognitive functions of propofol-treated mice were examined in a cued and contextual fear conditioning test. During conditioning, there was no significant difference in the levels of freezing among all groups (Fig. 2A). With the exception of the cued test performed 30 days after conditioning where differences in the levels of freezing were observed, there were no significant differences in the levels of freezing among all groups (Fig. 2B–D). In the cued test performed 30 days after conditioning, mice in the propofol group showed decreased levels of freezing than those in the other groups (pre-CS; treatment effect, P=0.6951; treatment ×time, P=0.1099; CS; treatment effect, P=0.0256; treatment ×time, P=0.0393; intralipos group vs low-dose group, P=0.0447; intralipos group vs high-dose group, P=0.0044; Fig. 2E). The distance traveled by mice in all groups after the foot shocks were not significantly different among the treatment groups (Fig. 2F). Our findings suggest that repeated high-dose propofol treatment impaired long-term memory.

Effects of propofol treatment on other behaviors

There were no significant differences among the four groups of mice in terms of their physical characteristics (body weight and temperature) and muscle strength (grip strength and wire hang) (Table 2). In the first trial of the rotarod test, there was a significant difference among the groups (Treatment effect, P=0.0397; Fig. 3A and 3B). The high-dose propofol-treated mice showed decreased motor coordination compared to the saline group (saline group vs high-dose group, P=0.0044; Fig. 3B). In addition, the trend in motor coordination was not different in the low-dose propofol- and intralipos-treated mice (saline group vs low-dose group, P=0.0715; saline group vs intralipos group, P=0.0947). In the elevated plus maze test, the treatment effect did not reach statistical significance. However, the saline group tended to spend a greater percentage of the time in the open arms compared to the other groups (saline group vs low-dose group, P=0.0632; saline group vs high-dose group, P=0.1616; saline group vs intralipos group, P=0.0442; Fig. 3F). The results obtained using the rotarod and elevated plus maze tests suggest that the solvent, intralipos, potentially causes deficits in motor coordination and increases anxiety-like behavior in mice. There were no significant differences in behavior among groups in the other behavioral tests (Table 2).

Discussion and Conclusion

In the present study, mice that received repetitive propofol treatment underwent a comprehensive behavioral test battery to assess the effect of repeated use of the anesthetic agent on brain functions. Our findings revealed that the repeated administration of propofol reduced the preference for social novelty and impaired long-term memory in the cued condition. There were no significant differences among the four groups in the other tests that were performed in this study (Table 2).

Propofol administration is suggested to affect social behavior. However, the effects appear to vary with the timing and duration of administration. In a previous report, repeated exposure of neonatal mice to propofol reduced sociability and social novelty preference in the three-chamber social approach test [29]. In our study, both low- and high-dose propofol-treated mice, and saline-treated mice showed no significant preference for the stranger mouse (Fig. 1C). Our results suggested that the repetitive administration of propofol in adult mice may also induce a deficit in the preference for social novelty. In contrast, repeated propofol treatment did not affect sociability in our study. Another study showed that the single injection of adult mice with propofol did not induce any significant difference in social behaviors compared to control mice. Furthermore, in the same report, the impaired social preference in a mouse model of autism was improved by propofol treatment [30]. This report suggests that propofol treatment does not reduce, but rather improves sociability in adult mice. It is reported that when animals have an olfactory impairment, abnormal social behaviors can be observed in the three-chamber social approach test since the mouse undergoing the test will need to identify the mouse in the wire cage based on olfactory cues [31]. In fact, some studies showed that propofol induces olfactory deficits [29, 32]. Furthermore, ventral hippocampus neurons are important for social memory [33]. It was reported that propofol affects synaptic transmission, neuron maturation, and neuron survival in the hippocampus [34, 35]. Considering the reports of previous studies, the lack of preference for social novelty may be associated with the altered olfactory or hippocampus function.

Propofol administration induced impairments in the performance of a memory task. In studies involving animal models and human patients, the amnestic effects of propofol were described [2, 10, 11, 13]. These reports focused on the acute effect of propofol. Repeated propofol treatment during the neonatal and early postnatal periods induced neurotoxicity in the hippocampus and behavioral deficits during adulthood [12, 29, 36–38]. In our study, propofol-treated mice showed decreased levels of freezing in the cued fear conditioning test (Fig. 2E). During the first 4 min in all the cued tests, all groups of mice increased their freezing time even when no cue was presented. This phenomenon may be induced by fear generalization. In our study, the cued test was performed after the contextual test. Since mice were exposed to the fear-conditioned context before the cued test, mice might have exhibited increased fear response even without the cue.-However, there was no significant difference among the groups before the cue was presented. We, therefore, considered that the differences among the groups were based on the response to the CS. Our results suggested that propofol not only has acute effects but also long-lasting effects on memory function, and the effects are not restricted to the period of development. In the present study, propofol treatment affected cued, but not contextual, fear memory. Contextual associative learning is mediated by the hippocampus and amygdala [39]. On the other hand, cued associative learning is mediated mainly by the amygdala [40]. Previous studies showed propofol treatment effects on the hippocampus [12, 34, 35, 41]. However, in this study, propofol treatment affected the performance

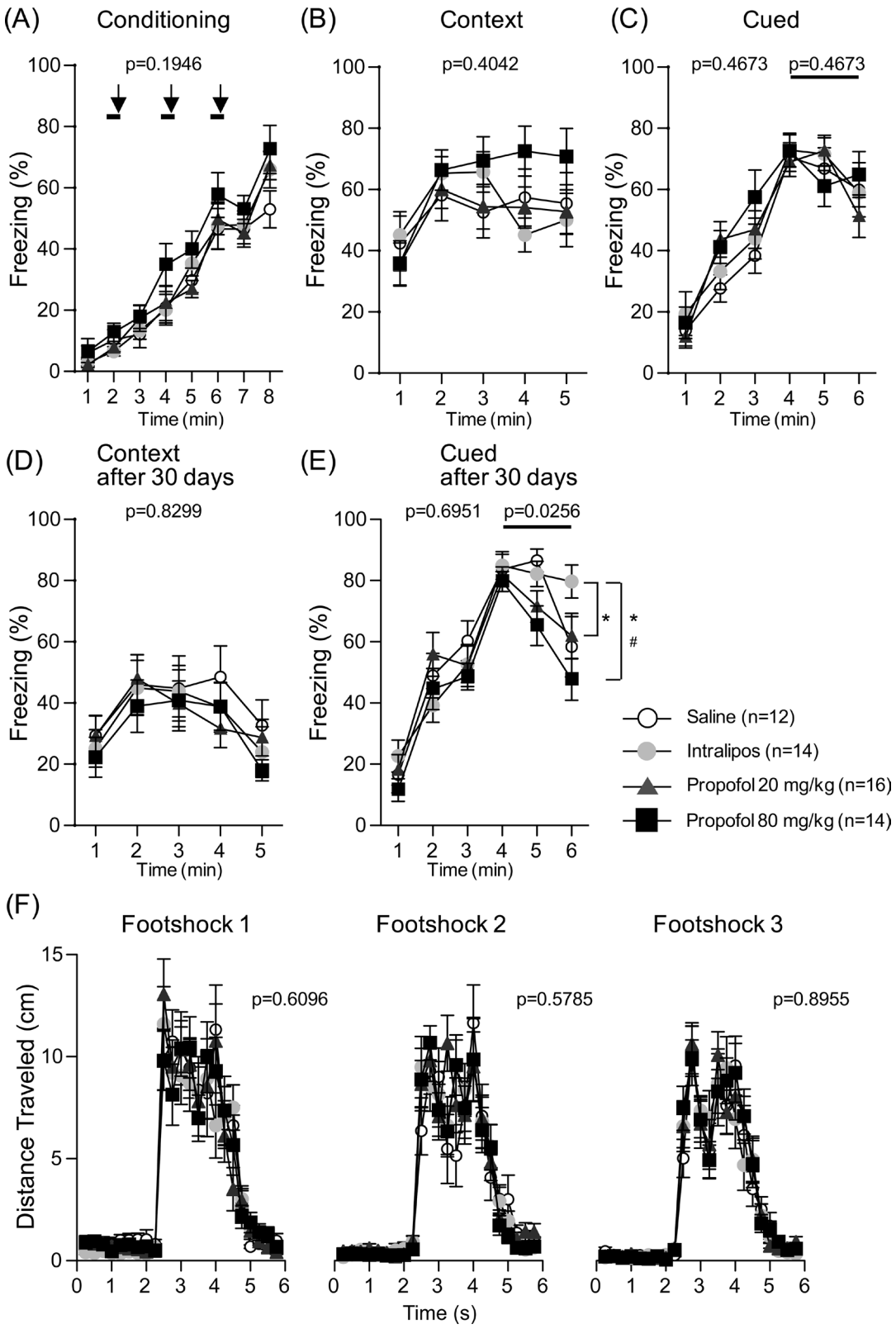

Fig. 2. High-dose propofol treatment impaired long-term memory in the cued fear conditioning test. The percentage of freezing time in the conditioning (A), context testing (B, D), and cued testing with altered context (C, E) conditions. Distance traveled following exposure to the three foot shocks during the conditioning phase was recorded (F). Mice in the propofol groups showed decreased levels of freezing compared to other groups during a cued test performed 30 days after conditioning. Data are presented as means ± SEM for the indicated numbers of animals. The *P* values indicate the treatment effect in the one-way repeated measures analysis of variance. In the cued test performed after 30 days, the asterisk indicates a nominally significant difference in 4-6 min for comparisons between treatment groups ($P<0.05$) and the number sign indicates significance after Bonferroni correction ($P<0.05/6$).

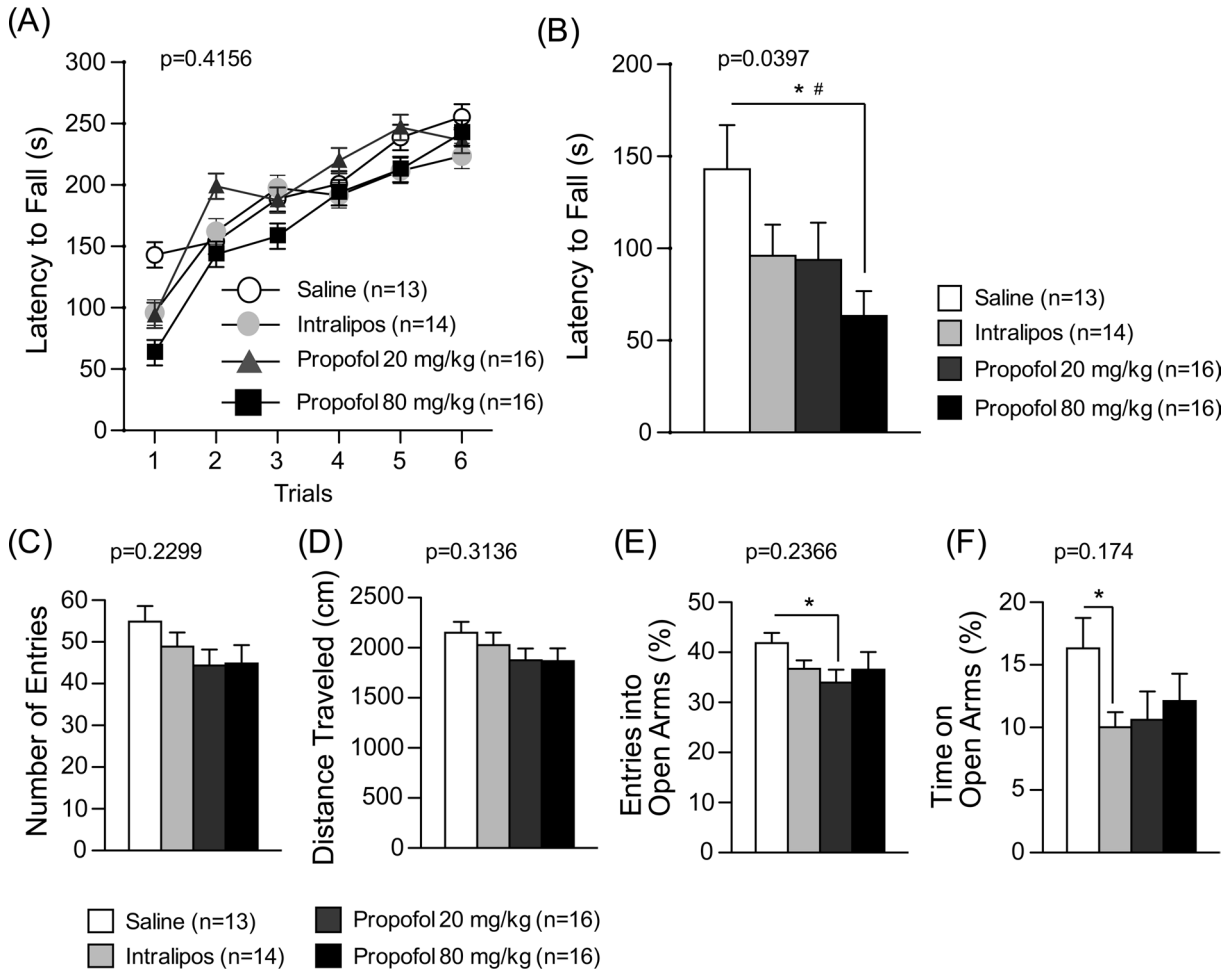

Fig. 3. The results of the other behavioral tests. In the rotarod test, the latency to fall from the accelerating rotarod was measured in three trials per day (A). In the first trial, propofol-treated mice showed shorter latency to fall (B). The elevated plus maze test: number of arm entries (C), distance traveled (D), percentage of entries into open arms (E), and percentage of time in open arms (F) are shown. Data are presented as means ± SEM for the indicated numbers of animals. The *P* values indicate a treatment effect in the one-way analysis of variance (ANOVA) or repeated measures ANOVA. The asterisk indicates a nominally significant difference for comparisons between treatment groups ($P<0.05$) and the number sign indicates significance after Bonferroni correction ($P<0.05/6$).

in the cued but not the contextual memory task. This result suggests that chronic propofol treatment influences the function of the amygdala. The $GABA_A$ receptor agonist, sevoflurane, blocks episodic memory in humans by reducing connectivity between the amygdala and hippocampus [42]. Propofol is a $GABA_A$ receptor agonist and may act similarly to sevoflurane.

Propofol has a characteristic milky white appearance. It is prepared using a lipid emulsion because it is highly hydrophobic. This milky reagent is composed of soya oil, glycerol, and egg lecithin. As investigating propofol effects, these reagents injected into mice even these reagents may also affect mice behavior. However, in the previous propofol studies, the control vehicles were not standardized, which may affect the results. To assess the effects separately, we compared the effects of treatments with four solutions: saline, intralipos (only milky regents), and low-dose and high-dose propofol. In the elevated plus maze test, the intralipos and propofol groups tended to be more anxious than the saline group (Fig. 3F). Furthermore, in the rotarod test, the latency to fall from the apparatus was shorter in the intralipos- and propofol-treated mice compared to the saline-treated mice in the first trial (Fig. 3A and 3B). In previous reports, the phytoestrogen content of soy oil and egg lecithin were shown to have anxiolytic effects in rats [14, 15]. Though it is unknown which component causes these behavioral changes, our results suggested that intralipos, but not propofol, affects motor coordination and anxiety.

Although the mechanisms that underlie the behavioral responses induced by repeated propofol treatment require further investigation, the distinctive behavioral alterations observed in mice in this study provide basic data on the safe use of propofol, in order to avoid side effects following multiple exposure. This will encourage the proper use of propofol.

Animal Studies

All behavioral testing procedures were approved by the Institutional Animal Care and Use Committee of University of Toyama.

Data Repository

Raw data on the behavioral test and information about each mouse are accessible on the public database "Mouse Phenotype Database" (http://www.mouse-phenotype.org/).

Conflict of Interest

The authors declare that there are no conflicts of interest for this article.

Authors' Contributions

KF, HO, YN, YK, and MA performed the experiments. KF and KT were responsible for experimental design and analysis. KF, ES, MD, and KT prepared the manuscript.

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