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Assessment of impulsivity in adolescent mice: A new training procedure for a 3-choice serial reaction time task

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ABSTRACT

Immaturity in impulse control among adolescents could result in substance abuse, criminal involvement, and suicide. The brains of adolescents and adults are anatomically, neurophysiologically, and pharmacologically different. Therefore, preclinical models of adolescent impulsivity are required to screen drugs for adolescents and elucidate the neural mechanisms underlying age-related differences in impulsivity. The conventional 3- or 5-choice serial reaction time task, which is a widely used task to assess impulsivity in adult rodents, cannot be used for young mice because of two technical problems: impaired growth caused by food restriction and the very long training duration. To overcome these problems, we altered the conventional training process, optimizing the degree of food restriction for young animals and shortening the training duration. We found that almost all basal performance levels were similar between the novel and conventional procedures. We also confirmed the pharmacological validity of our results: the 5-hydroxytryptamine 2C (5-HT_{2C}) receptor agonist Ro60-0175 (0.6 mg/kg, subcutaneous) reduced the occurrence of premature responses, whereas the 5-HT_{2C} receptor antagonist SB242084 (0.5 mg/kg intraperitoneal) increased their occurrence, consistent with results of previous studies using conventional procedures. Furthermore, we detected age-related differences in impulsivity using the novel procedure: adolescent mice were found to be more impulsive than adult mice, congruent with the results of human studies. Thus, the new procedure enables the assessment of impulsivity in adolescent mice and facilitates a better understanding of the neurophysiological/pharmacological properties of adolescents.

1. Introduction

Many studies have shown that adolescents are more impulsive than adults [1–4]. As is well known, a higher impulsivity is a risk factor for criminal involvement, substance abuse, and suicide [5–8]. Thus, increased impulsivity in adolescents could lead to various problems, such as risky driving and substance abuse [9–11]. In addition, deficits in impulse control are often observed in psychiatric disorders that mainly occur in adolescents, such as attention-deficit/hyperactivity disorder [12] and schizophrenia [13].

To address these issues, it is important to screen potential anti-impulsivity drugs and understand the neural mechanisms of age-related differences in impulsivity. To this end, preclinical models using rodents are useful, especially mouse models, as many transgenic mouse models have already been developed.

The 5-choice serial reaction time task (5-CSRTT) [14] and 3-choice serial reaction time task (3-CSRTT) [15] have been used widely to assess impulsivity in adult rodents. In these tasks, a light is briefly flashed through one of the 5 or 3 holes and animals are required to make a nose-poke response in the lit hole (*i.e.*, the correct response) to get a food pellet. Nose-poke responses before the presentation of the light stimulus are termed premature responses and are considered an impulsive action. Responses, including premature responses, other than the correct response result in a time-out period. Such tasks have helped scientists to find various pro/anti-impulsivity drugs [16–19] and elucidate the neural mechanisms of impulsive behavior in adult animals [20,21].

However, these findings from these tasks cannot be simply extrapolated to adolescents for two reasons. First, the side effects of several medications, including suicidal tendency associated with use of antidepressants, are age-dependent [22,23]. Second, neurophysiological evidence suggests that the prefrontal cortex, which plays a pivotal role in impulse control, does not fully develop until the age of 25 [24].

Therefore, formulating a task that can assess impulsivity in adolescent mice is imperative. Conventional protocols of 5-CSRTT/3-CSRTT cannot be used in young mice for two reasons: first, the usual

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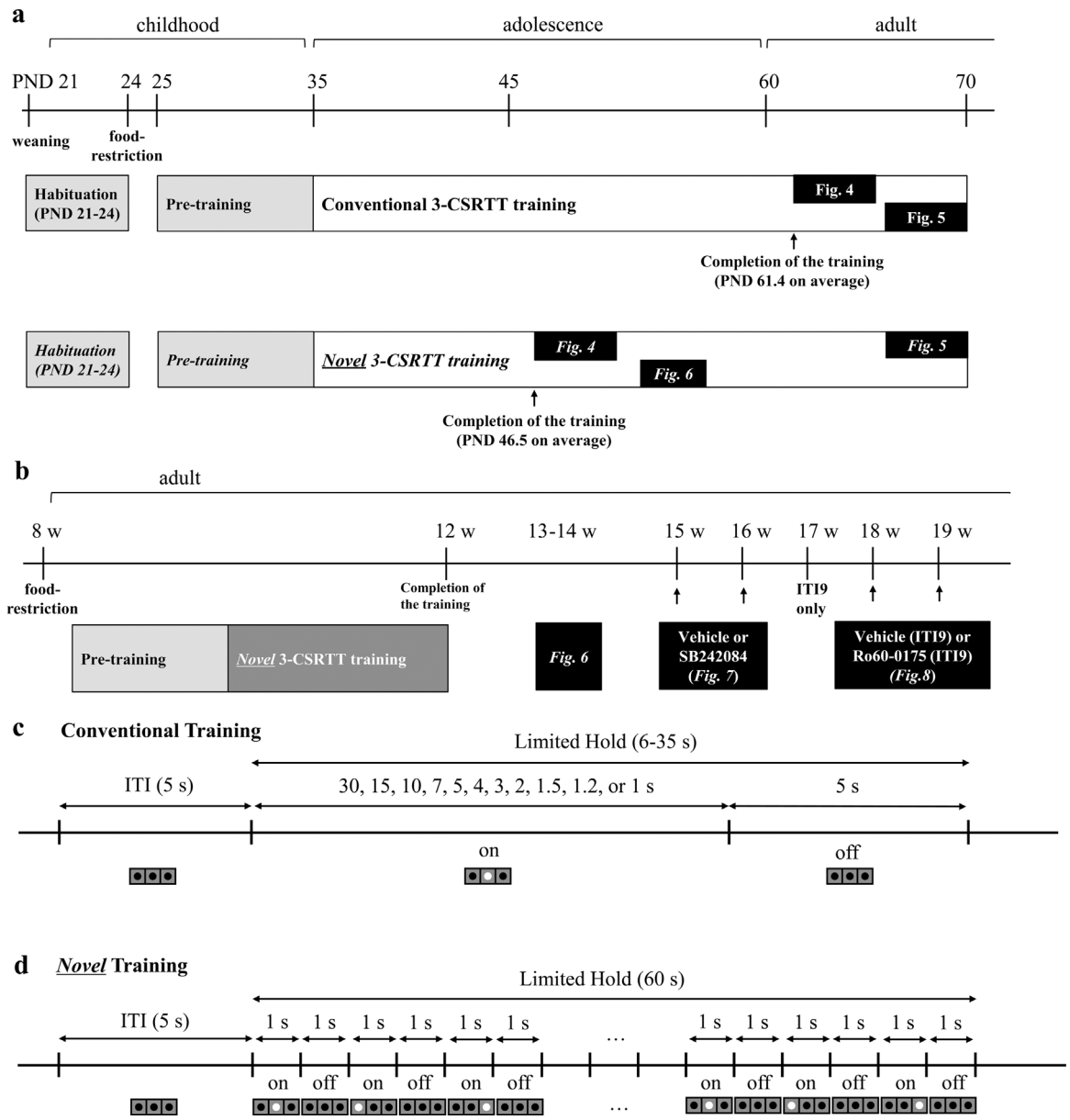

Fig. 1. Schematic representation of the experimental design. (a) Thirty-seven mice for the adolescent 3-CSRTT experiment underwent the habituation (PNDs 21–24) and pre-training procedures. Among these mice, 23 underwent the novel training procedure and 14 underwent the conventional training procedure. When the adolescent mice achieved a stable performance (see 2.3.3 *Common components of the conventional and novel 3-CSRTT training procedures*), training was considered complete. Please note that the time frames of the trainings shown in the figure are based on the number of days (conventional 37.4 ± 9.9, novel 22.5 ± 7.2 days, mean ± 1SD) and not the number of sessions (see Fig. 3). In rodents, "childhood" loosely refers to PND 21–35, "adolescence" refers to PND 36–59, and "adulthood" refers to PND 60–90 [43,44]. PND, postnatal days. The black bars reflect the experimental time points for each figure. (b) Twelve adult mice underwent the habituation, pre-training, and novel training procedures. When the adolescent mice achieved a stable performance (see 2.3.3 training was considered complete. The black bars reflect the experimental time points for each figure. (c) In the conventional procedure, the stimulus duration was gradually decreased (30, 15, 10, 7, 5, 4, 3, 2, 1.5, 1.2, and 1 s) when the mouse attained the criteria for progression, as described in Methods Section 2.3.4 (d) In the novel training procedure, one of the holes was turned on for 1 s and then turned off for 1 s. The cycle was repeated for up to 60 s in each trial. When a mouse correctly made a nosepoke response into the lit hole, the trial ended and a reward pellet was delivered. Responses to non-illuminated holes during the limited hold had no consequence (see 2.3.5).

food restriction procedure in 5-CSRTT/3-CSRTT could disrupt the normal growth of mice during adolescence. Second, conventional protocols of 5-CSRTT/3-CSRTT do not enable us to conduct experiments in adolescent mice because 6–9 weeks are required to complete training [25,26] and mice reach adolescence approximately 5 weeks after weaning (Fig. 1a).

To solve these problems, we established a novel training procedure for the 3-CSRTT in mice with two revisions compared with the previous training procedure. First, we optimized the degree of food restriction for younger mice, allowing them to grow normally and be motivated enough to perform tasks. Second, we decreased the number of sessions required to complete the training. We introduced unpunished training sessions (*i.e.* without a time-out period), with many chances to detect a brief light stimulus during the usual training procedure. We confirmed that the basal performance levels were similar between the novel and conventional procedures. Further, we examined whether our methods could detect age-related differences in impulsivity in mice, as is deducible in the case of humans. We also confirmed the validity of the

new training method in assessing impulsivity by examining and comparing the effects of the 5-hydroxytryptamine 2C (5-HT_{2C}) receptor antagonist SB242084 and the 5-HT_{2C} receptor agonist Ro60-0175, which have been used in the 5-CSRTT [26].

2. Materials and methods

2.1. Subjects

The mice used for experiments were the offspring of timed-pregnant C57BL/6N mice supplied from Nippon SLC Co. Ltd (Hamamatsu, Japan). Animals were housed at 25 ± 2 °C and a relative humidity of 40–50%. Lights of the animal rooms were turned on from 19:00 to 07:00 h. All tests were performed during the dark period. Sex was determined on postnatal day (PND) 10 and only male mice were used. The pups were weaned at PND 21 (Fig. 1a). Thirty-two mice were used to determine the body weight of mice under free-feeding conditions, and these were housed in groups of 3 or 5. Thirty-seven mice were individually housed immediately after weaning and were used to assess the time required for the completion of training in the novel procedure (n = 23) or the conventional procedure (n = 14). Twelve mice were used to examine the effects of drugs on the performance of mice in the 3-CSRTT. They were group-housed until the age of 8 weeks and then individually housed to receive training for the 3-CSRTT (Fig. 1b). Food and water were provided *ad libitum* until the training period began. All procedures were in accordance with the guidelines for the Care and Use of Laboratory Animals of the Animal Research Committee of the Hokkaido University.

2.2. Apparatus

Aluminum operant chambers measuring W22 × D26 × H18 cm^3 (Med Associates Inc., St. Albans, VT, USA) were used for the 3-CSRTT. The curved rear wall of each chamber contained nine holes. Each hole had an infrared photocell beam for the detection of nose-poke responses, and a 0.28-W LED light was located behind each hole. Every other hole was sealed so that only the three centrally positioned ports were accessible. A food magazine was located on the opposite wall of the chamber, and a house light (28 W bulb) was located at the top of this wall. The food magazine had a 0.28-W LED light located on its ceiling. The apparatus was controlled by a computer program written in the MED-PC language (Med Associates Inc., St. Albans, VT, USA).

2.3. 3-CSRTT procedures for adolescent mice

2.3.1. 3-CSRTT habituation procedure for adolescent mice

The same habituation procedures were used in the conventional and novel 3-CSRTTs. On PND 21, the mice were weaned and individually housed, with free access to water, usual food (CE-2; CLEA JAPAN Inc., Tokyo, Japan), and 5 g of reward pellets (20 mg each, dustless precision pellets; Bio-Serv, Frenchtown, NJ, USA). The mice underwent 1-min of handling on PNDs 21, 22, 23, and 24. Twenty-four hours before the first training session on PND 25, available food was restricted to 1.0 g (*i.e.*, CE-2). The experimental design and timeline have been described in Fig. 1a.

2.3.2. The 3-CSRTT pre-training procedure for adolescent mice

The same pre-training procedure consisting of 4 phases was used in the conventional and novel 3-CSRTTs. In the first phase, a reward pellet was always delivered to the food magazine 1 min after the mouse entered the magazine during a 45-min session. Once the mouse ate 25 or more reward pellets in a session, it was moved to the next phase. In the second phase, the mouse was required to nose poke in any of the 3 holes during the stimulus duration of 60 s to obtain a reward pellet. In the first session of the second phase, a reward pellet was placed in each hole to facilitate nose-poke response in the holes. A new trial was initiated automatically, and each session lasted until 50 trials had been completed. When a mouse ate 15 or more reward pellets in a session, in the case of the second or any subsequent session, it was moved to the next phase. In the third phase, the mouse was required to enter the magazine to start the next trial. Each session lasted for either 60 min or until 50 trials had been completed, whichever occurred first. In the fourth phase of pre-training, one of the three hole lights were illuminated for 30 s in a pseudo-random order (RANDD command in MED-PC language). Responses to non-illuminated holes had no consequence. Each session lasted for either 60 min or until 100 trials had been completed, whichever occurred first. When the mouse fulfilled the following criteria: accuracy of 50% or higher and 15 or higher correct responses, or 30 or more correct responses [*i.e.*, (accuracy ≥ 50% ∧ correct ≥ 15) ∨ (correct ≥ 30)], it was moved to the conventional or novel training procedures of the 3-CSRTT.

2.3.3. Common components of the conventional and novel 3-CSRTT training procedures

Each session lasted for either 60 min or until 100 trials had been completed. The training was considered complete when the mouse reached the target phase (stimulus duration 1 s) and exhibited a stable performance for at least two consecutive sessions. We set the criteria for determining stable performance as follows: the number of correct responses is 20 or more and the accuracy is more than 70%. Training was conducted for one session per day and seven sessions per week, until mice PND 35. Following this, they were trained for five sessions per week (Monday–Friday). Mice were excluded from training when they could not proceed to the next phase within 10 sessions.

2.3.4. Conventional 3-CSRTT training procedure for adolescent mice

The conventional procedure is based on the methods used by a previous study [27]. Briefly, after a fixed inter-trial interval (ITI: 5 s), one of the three hole lights was illuminated for 30 s in a pseudo-random order (Fig. 1c). Responses to non-illuminated holes resulted in 5 s time-out. The stimulus duration was decreased in a stepwise manner as the training progressed (the stimulus duration decreased in a gradually, as follows – 30, 15, 10, 7, 5, 4, 3, 2, 1.5, 1.2, and 1 s). When the mouse attained the criteria of greater than 70% accuracy and no less than 25 correct responses, the stimulus duration was decreased.

2.3.5. Novel 3-CSRTT training procedure for adolescent mice

In the novel training procedure, the mouse under training was allowed to respond to the lit holes for 60 s after the fixed ITI (5 s). One of the three hole lights was turned on for 1 s in a pseudo-random order and then turned off for 1 s; the cycle was repeated for 60 s (Fig. 1d). Once the mouse correctly responded to the illuminated hole, while it was illuminated, a reward pellet was delivered. Responses to non-illuminated holes had no consequence. When the mouse correctly responded to the lit hole 25 times or more in a session for two consecutive days, it was moved to the target phase described above. Details of the computer programs that we used, written in the MED-PC language, have been provided in the Supplementary material.

2.3.6. Dietary control and body weight calculation in adolescent mice

During the 3-CSRTT training period, mice received dietary control as follows: provision of 2.0 g food (3 weeks old), 2.5 g food (4 weeks old), 2.8 g food (5–7 weeks old), and 2.4 g food (8–9 weeks old). Eight hours before the training session, any remaining food was removed. We calculated the putative body weights as follows: the body weight that measured just before the sessions times 100, divided by 92 (*i.e.* BW × 100/92). We used this calculation because the body weights just before the sessions were approximately 92% the day-peak weights

measured 8 h before the daily training sessions (Appendix Table A1).

2.4. 3-CSRTT procedures for adult mice

When the mice were 8 weeks old, they individually housed and administered food-restricted diets (Fig. 1b). Thereafter, their body weights were maintained at 85% of the weight recorded under free-feeding conditions. Usual food (CE-2) was administered after the daily sessions in the mice's home cages. Their food intakes in the home cage were between 1.0 and 2.0 g during the training and experimental periods. Water was freely available. Twenty-four hours before the first training day, food was restricted to 0.4 g of reward pellets. The later procedures were the same as those for adolescent mice, except for the criteria to move to the next training phase. We changed the criteria slightly for adult mice because their weights are higher than those of adolescent mice (Appendix Table A2).

2.5. Behavioral parameters and basal performance calculation in the 3-CSRTT

The following nine behavioral measures in the 3-CSRTT were analyzed:

(a) Percentage of premature responses: [premature responses/(premature + correct + incorrect responses)] × 100, a measure of impulsive action
(b) Accuracy (percentage of correct responses): [correct responses/(correct + incorrect responses)] × 100, a measure of attentional function
(c) The number of initiated trials (counts per session), a measure of motivation for the task
(d) Percentage of omissions [(number of omissions/total initiated trials) × 100], a measure of attentional function and motivation for the task
(e) Percentage of perseverative responses: [perseverative responses/correct responses] × 100, a measure of compulsive behavior
(f) Correct response latency (s), a measure of attentional function, motivation for the task, and motor function
(g) Reward latency (s), a measure of motivation for reward and motor function
(h) Correct responses (counts per session), a measure of attentional function, motivation for the task, and eating capacity
(i) Percentage of responses during time-out periods [number of responses during time-out periods/(incorrect responses + omissions + perseverative responses)] × 100, a measure of motivation for nose-poking in holes

Please note that the number of correct responses is a mixed index of motivation and attentional function. Reduced motivation or smaller eating capacity for the food reward would reduce the number of initiated/completed trials, resulting in a reduction of the number of correct responses because of a reduced chance to make correct responses. Accuracy is a more reliable measure of attentional function because it is a standardized rate (accuracy = [correct responses/(correct + incorrect responses)] × 100) and relatively independent of motivation levels or eating capacity (*e.g.* [28]). Thus, by process of elimination, we could regard the changes in the number of correct responses as changes of motivation/eating capacity when we did not observe changes in accuracy.

To compare the baseline performance between the conventional and novel procedures, the baseline performance was calculated in two different ways: the average of each parameter was calculated in 3 consecutive sessions after the target mouse's performance stabilized (Figs. 1a and 4) or the average of each parameter was calculated in 3 consecutive sessions conducted in the second half of 9 weeks, in 9 week-old mice, to control for age-related effects (Figs. 1a and 5). In the latter calculation, only those mice were included whose performance stabilized before the second half of the 9-week period.

To detect age-related differences by using the novel procedure, the average of each parameter was calculated in 2 consecutive sessions in 7-week-old and 13–14-week-old mice, respectively (Figs. 1a, b, and 6). We used this calculation to exclude the data obtained in the day after any drug treatments or weekends (see 2.3.3) because they could affect performance.

2.6. Experiment 1: training time and behavioral parameters in the novel and conventional 3-CSRTT

The training times (sessions) to complete training in the novel and conventional 3-CSRTTs were compared to examine the validity of the novel 3-CSRTT as a concise method of measuring impulsive action. When the adolescent mice achieved a stable performance (see 2.3.3 *Common components of the conventional and novel 3-CSRTT training procedure*), training was considered complete. We also compared behavioral parameters (see 2.5 *Behavioral parameters and basal performance calculation in the 3-CSRTT*) between the novel and conventional procedures to check whether novel procedures altered baseline performance. Moreover, we compared behavioral parameters (see 2.5) in the novel 3-CSRTT in adolescent mice and adult mice to examine whether we could detect the effects of age on impulsive action, as we are able to do in case of humans. The number of animals used for statistical analyses in each figure differed because some animals were used for other studies (not shown here) and received repeated drug administrations soon after the completion of training. The repeated drug administrations could alter 3-CSRTT performance. Therefore, we excluded the mice that received repeated drug administrations to make accurate comparisons between the procedures.

2.7. Experiment 2: pharmacological validity of the novel training procedure

Twelve adult mice received SB242084 [0 and 0.5 mg/kg; intraperitoneally (i.p.)], a 5-HT_{2C} antagonist, 30 min before testing. Moreover, the same mice also received Ro60-0175 [0 and 0.6 mg/kg; subcutaneously (s.c.)], a 5-HT_{2C} agonist, 15 min before testing. The order of drug treatments was counterbalanced. Each drug session was conducted with more than a week's interval between the drug administrations. Drug doses were chosen based on previous studies [26,29]. A long ITI (9 s) was used for Ro60-0175 administration to detect the suppressive effects of the drug on premature responses. Long ITI sessions lasted for 70 min. One week before starting the experiment with Ro60-0175 administration, the mice were habituated to a long ITI session at least once. When the number of correct responses reached 20 or more in the long ITI session, we began to conduct the experiment.

2.8. Drugs

Ro60-0175 fumarate was dissolved in 0.9% saline and injected subcutaneously 15 min before testing, at a volume of 5 mL/kg. SB242084 2HCl was dissolved in 0.9% saline containing 5% dimethyl sulfoxide and injected intraperitoneally 30 min before testing at a volume of 10 mL/kg. The drugs were purchased from Tocris Bioscience (Bristol, UK). We have expressed the dosages in terms of the salt of the drug.

2.9. Statistical analysis

We estimated the relative growth rates (RGR) of the body weights for each treatment group by fitting a hierarchical Bayesian model

(HBM) in which the effects of individual differences (*i.e.*, random effects) and the time change of mean RGRs can be separated. The HBM is essentially equivalent to an ANOVA model in terms of time-wise effects, repeated measurements, and individual random effects. A single time-change was described as an exponential growth model for each group. The growth model is formulated as $W_{t+1} = W_t \exp(r_t)$ where W_t is the body weight at week t and r_t is the relative growth rate between t and $t + 1$ ($t \in \{4, 5, 6, 7\}$). The RGR, $r_{i,t}$, can be decomposed into $r_{i,t} = \alpha_t + \beta_{i,t}$ where α_t is the common RGR for all mice in a treatment group while $\beta_{i,t}$ represents the random effects of an individual mouse i. The difference in $\beta_{i,t}$ for each group represented the effects of treatment (*i.e.* free-fed control and 3-CSRTT-trained) to be detected. These differences were evaluated by comparing two posteriors at each observation time. To detect changes of growth rate during the entire experimental period for each treatment group, we defined a cumulative RGR, $\overline{\alpha}_k$, as $\overline{\alpha}_k = \alpha_4 + ... + \alpha_k$ by summing up the posterior distributions of $\{\alpha_t\}$.

All prior distributions for parameters in the model were assumed to be normally distributed except that non-informative uniform priors for variance parameters were specified. To obtain posterior distributions of the Bayesian model, we used JAGS ([30], version 4.3.0 for this study), a Markov chain Monte Carlo sampling software. Details of the model are described in the JAGS code in the Supplementary materials of this paper. The posterior samples were obtained from three independent Markov chains in which 15,000 values were sampled with a 10-iteration interval after a burn-in of 50,000 iterations.

Mean and SD were used for comparison of training time. Mean and standard error of the mean (SEM) were used for behavioral measures. Unpaired *t*-tests were used to compare the basal performance between the conventional and novel procedures and to compare the basal performance in the 3-CSRTT between adolescent and adult mice. If Levene's test was significant, Welch's *t*-test was used instead of Student's *t*-test. Paired *t*-tests were used to determine the effects of the drugs. The alpha level was set at 0.05 for all statistical procedures. All statistical procedures were conducted using SPSS (version 23.0 J; SPSS Inc., Chicago, Illinois, USA).

3. Results

3.1. Growth curves of body weights of adolescent mice during the 3-CSRTT training

The cumulative RGRs are shown in Fig. 2. The overlapping of Bayesian confidence intervals (95%) of $\overline{\alpha}_k$ between free-fed control mice and 3-CSRTT-trained mice increased with age.

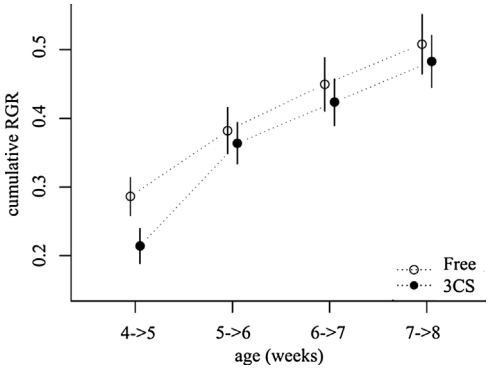

Fig. 2. Cumulative relative growth rate($\overline{\alpha}_k$) in free-fed mice and food-restricted mice that underwent 3-CSRTT training.

Thirty-two mice were group-housed under free-feeding conditions and weighed every week (open circles). Thirty-one mice received the 3-CSRTT training (filled circles). The overlapping of Bayesian confidence intervals (95%) of $\overline{\alpha}_k$ between free-fed control mice and 3-CSRTT-trained mice increased with age. The solid circles are the medians of Bayesian posterior distributions of $\overline{\alpha}_k$ while the vertical segments represent the 95% Bayesian confidence intervals of $\overline{\alpha}_k$.

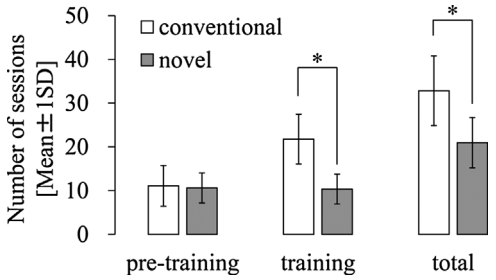

Fig. 3. Number of sessions required to complete the conventional or novel 3-CSRTT training procedures.

Twenty adolescent mice completed the novel 3-CSRTT training, while twelve adolescent mice completed the conventional 3-CSRTT training. Both the training procedures share the same pre-training procedures (shown in the left) and later become different. The middle bars indicate the number of sessions required to complete the conventional or novel training after completing the common pre-training procedures. The bars shown in the right represent the total numbers of sessions needed to complete the whole training (*i.e.*, pre-training + training). White bars represent the mean session numbers of the conventional procedure, dark grey bars represent the mean session numbers of the novel procedure, and the lines represent standard deviations. *$p < 0.05$, trained by the conventional procedure *versus* the novel procedure.

3.2. Number of sessions required to complete the novel and conventional 3-CSRTT training in adolescent mice

The number of sessions to complete the same pre-training phases was not different between novel and conventional training procedures (10.6 ± 3.4, 11.1 ± 4.6, respectively (mean ± 1SD)) (Fig. 3). To complete the novel training procedure, 10.4 ± 3.4 sessions were needed, whereas 21.8 ± 5.7 sessions were needed to complete the conventional training procedure ($t_{15.843} = 6.310$, $p < 0.001$, Welch's *t*-test) (Fig. 3). In total, to complete training, 21.0 ± 5.7 sessions were required with the novel training procedure, whereas 32.8 ± 8.0 sessions were required with the conventional 3-CSRTT training procedure ($t_{30} = 4.901$, $p < 0.001$) (Fig. 3). Three mice undergoing the novel procedure and 2 undergoing the conventional procedure failed to fulfil the criteria for progression within 10 sessions and were excluded from all analyses.

3.3. Baseline performance in the conventional and novel 3-CSRTT

Figs. 4–6 show high accuracy (above 80%) while the number of correct responses is relatively low (around 30). These results are due to the higher omission rate demonstrated by adolescent mice (*e.g.* Fig. 4d). The number of completed trials was fewer even though they initiated many trials (*e.g.* Fig. 4c). Please note that accuracy depends on the number of completed trials, but not the number of initiated trials (see 2.5).

Fig. 4a–i show the baseline performance in the 3-CSRTT when controlling for the number of sessions after the completion of training (see Fig. 1a and Methods Section 2.5). The number of initiated trials in the novel procedure was significantly lower than that in the conventional procedure ($t_{26} = 2.98$, $p = 0.006$) (Fig. 4c). In addition, the number of correct responses in the novel procedure was significantly lower than that in the conventional procedure ($t_{26} = 2.698$, $p = 0.012$) (Fig. 4h). Unpaired *t*-tests revealed no significant differences in other parameters, such as the percentage of premature responses ($t_{26} = 1.322$, $p = 0.198$) (Fig. 4a), accuracy ($t_{26} = 0.651$, $p = 0.521$) (Fig. 4b), the percentage of omissions ($t_{26} = 1.272$, $p = 0.215$) (Fig. 4d), the percentage of perseverative responses ($t_{26} = 1.455$,

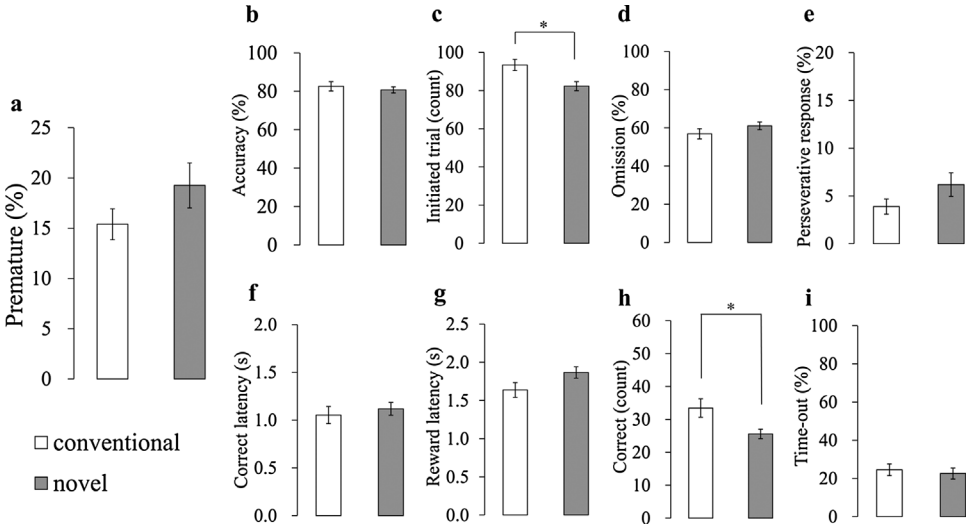

Fig. 4. Baseline performance in the conventional and novel 3-CSRTT when controlling for the number of sessions after training completion.
Adolescent mice received training for either the novel (n = 16) or conventional (n = 12) 3-CSRTTs. White bars represent the mean for the conventional 3-CSRTT training procedure, whereas grey bars represent the mean for the novel 3-CSRTT training procedure (a–i). Unpaired *t*-test was conducted for each parameter. Performance was compared between 3 consecutive sessions after the performance of the mice stabilized. The lines represent the SEM. $*p < 0.05$, the conventional training procedure *versus* the novel training procedure.

$p = 0.158$) (Fig. 4e), correct response latency ($t_{26} = 0.595$, $p = 0.557$) (Fig. 4f), reward latency ($t_{26} = 1.896$, $p = 0.069$) (Fig. 4g), and the percentage of responses during time-out periods ($t_{26} = 0.464$, $p = 0.646$) (Fig. 4i). Four of 20 adolescent mice undergoing the novel procedure were used for other studies, resulting in 16 adolescent mice in this analysis (see 2.6). Meanwhile, all 12 adolescent mice undergoing the conventional procedure were included in this analysis.

Fig. 5a–i show the baseline performance in the 3-CSRTT when controlling for the age-related effects (see Fig. 1a and Methods Section 2.5). The differences observed in Fig. 4 disappeared under same age conditions (Fig. 5c and h), except for the percentage of perseverative responses (Fig. 5e) ($t_{21} = 3.038$, $p = 0.006$). Unpaired *t*-tests revealed no significant differences in other parameters, such as, the percentage of premature responses ($t_{20.927} = 1.920$, $p = 0.069$, Welch's *t*-test) (Fig. 5a), accuracy ($t_{21} = 0.789$, $p = 0.439$) (Fig. 5b), the number of initiated trials ($t_{20.736} = 1.411$, $p = 0.173$, Welch's *t*-test) (Fig. 5c), the percentage of omissions ($t_{21} = 0.364$, $p = 0.720$) (Fig. 5d), correct response latency ($t_{21} = 0.149$, $p = 0.883$) (Fig. 5f), reward latency ($t_{21} = 1.199$, $p = 0.244$) (Fig. 5g), the number of correct responses ($t_{21} = 0.536$, $p = 0.598$) (Fig. 5h), and the percentage of responses during time-out periods ($t_{21} = 0.466$, $p = 0.646$) (Fig. 5i). Two of 16 mice undergoing the novel procedure were used for other studies, resulting in 14 mice in this analysis (see 2.6). Meanwhile, only mice undergoing the conventional procedure whose performance stabilized before the second half of the 9-week period were included in this analysis, resulting in 9 mice in this analysis.

Fig. 6a–i show the baseline performance in the 3-CSRTT in adolescent mice and adult mice (see also Fig. 1a and b). Please note that all the mice used for analysis in Fig. 6 were trained according to the novel procedure. Unpaired *t*-tests revealed that the percentage of premature responses in adolescents was significantly higher than that in adults ($t_{18} = 3.178$, $p = 0.005$) (Fig. 6a). No significant age-related differences were observed in other parameters, such as accuracy ($t_{18} = 1.490$, $p = 0.154$) (Fig. 6b), the number of initiated trials ($t_{18} = 0.249$, $p = 0.806$) (Fig. 6c), the percentage of omissions ($t_{9.742} = 0.568$, $p = 0.583$, Welch's *t*-test) (Fig. 6d), the percentage of perseverative responses ($t_{9.284} = 1.563$, $p = 0.151$, Welch's *t*-test) (Fig. 6e), correct response latency ($t_{18} = 0.634$, $p = 0.534$) (Fig. 6f), reward latency ($t_{18} = 0.737$, $p = 0.470$) (Fig. 6g), the number of correct responses ($t_{8.334} = 0.862$, $p = 0.413$, Welch's *t*-test) (Fig. 6h), or the percentage of responses during time-out periods ($t_{18} = 1.430$, $p = 0.170$) (Fig. 6i). Two of the 14 adolescent mice were used for other studies, resulting in 12 adolescent mice in this analysis (see 2.6). Meanwhile, only adult mice with a 2.0 g/day daily food intake in the home cages (see also 2.4) were included in this analysis to make the feeding conditions uniform, resulting in 8 adult mice in this analysis.

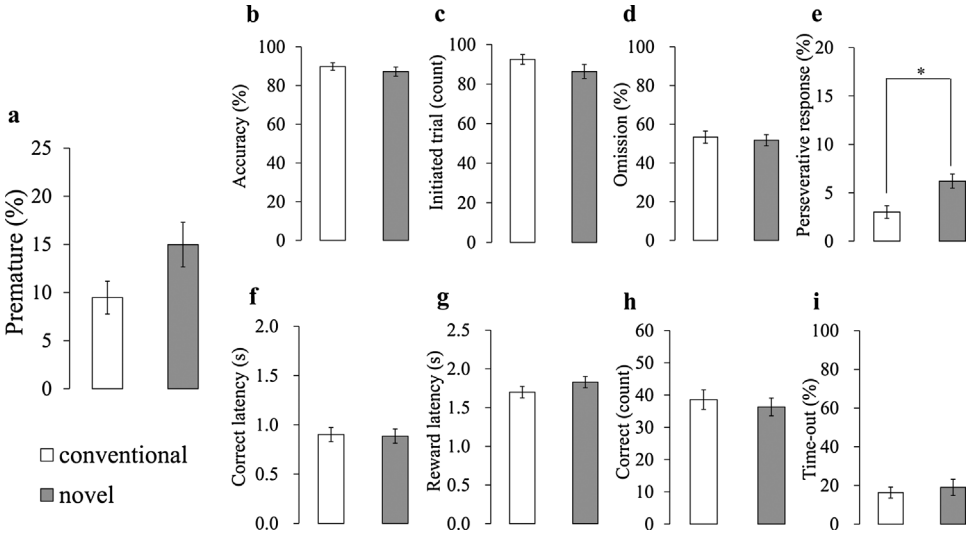

Fig. 5. Baseline performance in the conventional and novel 3-CSRTTs when controlling for age-related effects.
Adolescent mice received training for the 3-CSRTT in the conventional procedure (n = 9) or in the novel procedure (n = 14). White bars represent the mean for the conventional 3-CSRTT training procedure, whereas grey bars represent the mean of the novel 3-CSRTT training procedure (a–i). Unpaired *t*-test was conducted for the parameters. Performance during 3 consecutive sessions was compared in the second half of 9 weeks, in 9-week-old mice. The lines represent the SEM. $*p < 0.05$, the conventional training procedure *versus* the novel training procedure.

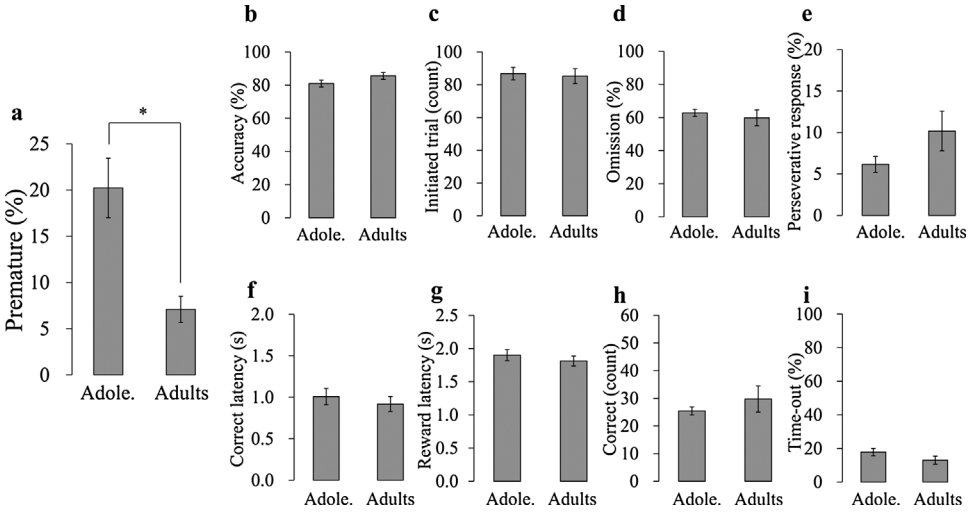

Fig. 6. Age-related differences in impulsivity and other parameters in the novel 3-CSRTT.
Adolescent mice and adult mice received training for the 3-CSRTT in the novel procedure (a–i). An unpaired *t*-test was conducted for the parameters. The performance of 7-week-old (n = 12) and 13–14-week-old (n = 8) mice was compared. Bars represent the mean, and the lines represent the SEM. $^{*}p < 0.05$, adolescent mice *versus* adult mice. Adole., adolescents (7 weeks old); Adults (13–14 weeks old).

3.4. Pharmacological validity of the novel training procedure

Adult mice were used to test the validity of the novel training procedure using pharmacological methods (Figs. 7a–i and 8a–i) (see also Fig. 1b). Paired *t*-test revealed a significant pro-impulsive effect of SB242084, a 5-HT_{2C} receptor antagonist ($t_{11} = 3.131$, $p = 0.010$) (Fig. 7a). In addition, SB242084 reduced the correct response latency ($t_{11} = 2.502$, $p = 0.029$) (Fig. 7f) and reward latency ($t_{11} = 2.245$, $p = 0.046$) (Fig. 7g). No significant effects of the drug were observed in the other parameters: accuracy ($t_{11} = 1.293$, $p = 0.222$) (Fig. 7b), the number of initiated trials ($t_{11} = 1.000$, $p = 0.339$) (Fig. 7c), the percentage of omissions ($t_{11} = 2.030$, $p = 0.067$) (Fig. 7d), the percentage of perseverative responses ($t_{11} = 0.106$, $p = 0.917$) (Fig. 7e), the number of correct responses ($t_{11} = 1.076$, $p = 0.305$) (Fig. 7h), and the percentage of responses during time-out periods ($t_{11} = 0.356$, $p = 0.729$) (Fig. 7i).

Moreover, the paired *t*-test revealed a significant anti-impulsive effect of Ro60-0175, a 5-HT_{2C} receptor agonist ($t_{11} = 2.635$, $p = 0.023$) (Fig. 8a). No significant effects were observed in the other parameters: accuracy ($t_{11} = 1.271$, $p = 0.230$) (Fig. 8b), the number of initiated trials ($t_{11} = 0.653$, $p = 0.527$) (Fig. 8c), the percentage of omissions ($t_{11} = 1.705$, $p = 0.116$) (Fig. 8d), the percentage of perseverative responses ($t_{11} = 0.516$, $p = 0.616$) (Fig. 8e), correct response latency ($t_{11} = 0.181$, $p = 0.860$) (Fig. 8f), reward latency ($t_{11} = 0.142$, $p = 0.890$) (Fig. 8g), the number of correct responses ($t_{11} = 0.580$, $p = 0.574$) (Fig. 8h), and the percentage of responses during time-out periods ($t_{11} = 1.576$, $p = 0.143$) (Fig. 8i).

4. Discussion

The adopted regime of food restriction during the training protocol resulted in a nearly normal growth pattern (Fig. 2). Although the cumulative RGRs at 4–5 weeks of age were lower in trained mice than in free-fed control mice, the difference was significantly reduced at later ages. In our young mice trained by the conventional procedure, the training duration was approximately the same as that in previous studies using adult mice [26,31]. These results indicate that the food regime generated sufficient motivation for the young mice to learn the tasks (Fig. 3), without growth impairment (Fig. 2).

The novel procedure is unique in that it offers many more opportunities for the mice to detect a brief light stimulus in one trial (see 2.3.5 and programs in the Supplementary material) compared to the conventional procedure. In the novel 3-CSRTT procedure, the mice were trained to respond successfully to a 1-s stimulus within approximately 3 weeks (Fig. 3), which is faster than that in conventional procedures [26,31]. Furthermore, the dropout rate for the novel procedure (3/23 = 13.0%) (see 3.2) was similar to that for the conventional procedure in the present (2/14 = 14.3%) (see 3.2) and previous

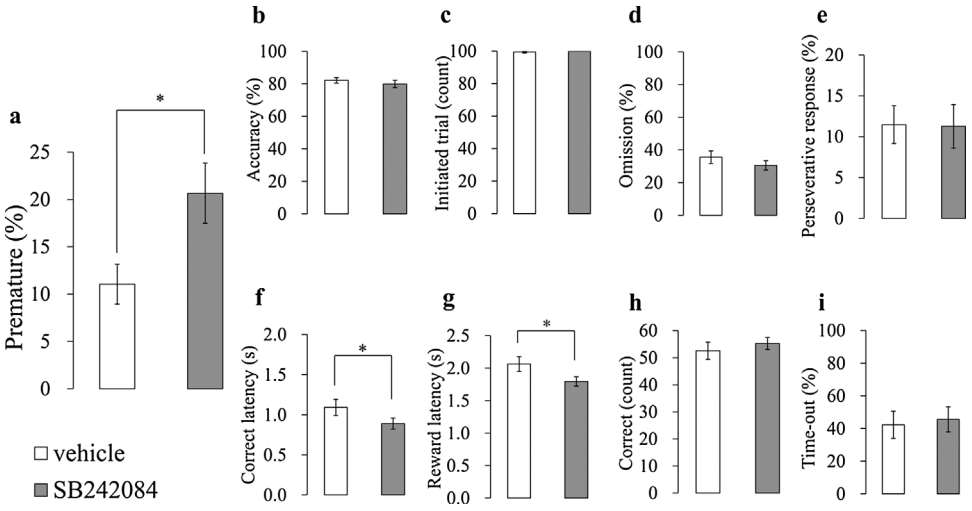

Fig. 7. Effects of SB242084, a 5-HT_{2C} receptor antagonist, on behavioral parameters in the novel 3-CSRTT.
We injected SB242084 (0 and 0.5 mg/kg, i.p.) to twelve adult mice 30 min before the testing sessions. Bars represent the mean, and the lines represent the SEM. $^{*}p < 0.05$, vehicle *versus* SB242084 (0.5 mg/kg).

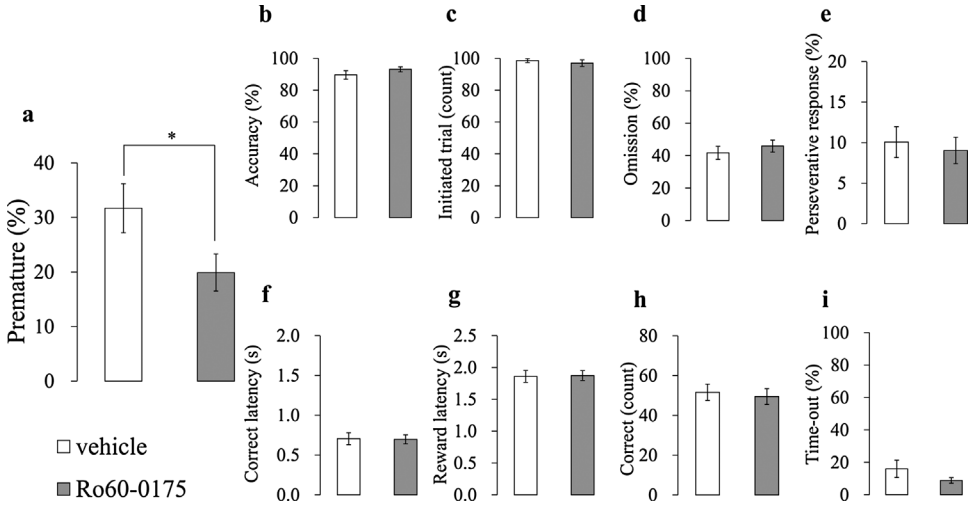

Fig. 8. Effects of Ro60-0175, a 5-HT_{2C} receptor antagonist, on behavioral parameters in the novel 3-CSRTT.
We injected Ro60-0175 (0 and 0.6 mg/kg, s.c.) to twelve adult mice 15 min before the testing sessions. For these tests, the inter-trial interval was set at 9 s to increase the basal level of premature responses. The bars represent the mean, and the lines represent the SEM. *$p < 0.05$, vehicle *versus* Ro60-0175 (0.6 mg/kg).

studies (7/48 = 14.6%) [32].

The performance values obtained after the completion of training using the novel procedure were similar to those obtained for the conventional procedure; however, some differences were also observed. The number of initiated trials and the number of correct responses, which are measures of motivation (see 2.5), were significantly lower in the novel procedure when controlling for the number of experienced sessions after the completion of training. However, when controlling for age-related effect, the differences between novel and conventional procedures disappeared, indicating that the observed differences were caused by the differences of eating capacity due to the differences of age, although, not the differences in the procedure. The percentage of perseverative responses, a measure of compulsive behavior, was higher in the novel procedure in both the analyses (Figs. 4e and 5e), although the difference was very small (3–4%). This might be because the novel procedure encouraged mice to nose-poke in holes (see 2.3.5). Other parameters were not significantly different between the novel and conventional procedures, indicating that the performance levels for the novel procedure were comparable to those for the conventional procedure.

This is the first study to detect the age-dependent difference in impulsive action in mice (Fig. 6a). Furthermore, the percentage of responses during the time-out period was not affected by age (Fig. 6i), indicating that the increase in the percentage of premature responses in adolescents was not the result of an overall increase in nose-poking. It is well known in case of humans that adolescents are more impulsive than adults [1–4], possibly because of immature development of the prefrontal cortex [24].

Furthermore, a pro-impulsive effect of SB242084, a 5-HT_{2C} receptor antagonist, and an anti-impulsive effect of Ro60-0175, a 5-HT_{2C} receptor agonist [26], were replicated in the present study (Figs. 7 and 8). These results demonstrated the pharmacological validity of the novel procedure in measuring impulsivity. Thus, it is likely that the novel procedure will be able to detect pro-/anti-impulsive effects of drugs more readily than the conventional procedure.

These results suggest that the novel procedure evaluates the same behavioral phenomena as the conventional procedures. Therefore, it is now possible to assess impulsivity during adolescence in mice, to screen potential anti-impulsivity drugs for adolescents, and to study the neural mechanisms of impulsive action in adolescents. Our model enables us to utilize transgenic mice and contributes to a better understanding of the neurodevelopmental mechanisms in the adolescence period, during which adolescents experience significant behavioral/neuroanatomical/neurochemical/functional changes [33–36] and are vulnerable to several psychiatric disorders [12,13,37].

Although promising, our method has three limitations. First, we used only male mice. Female mice were too small on PND 24 to start food-restriction. Sex differences in impulsivity are important, especially during adolescence, as some previous studies have shown that the impulsive personality phenotype in adolescence is closely related to sex hormonal factors [38,39]. Second, some brain regions in mice might have already matured by PND 46. A previous study showed that there was a significant difference in performance on the intradimensional/extradimensional set-shifting task between early-adolescent (PND 41) and early-adult rats (PND 68), but not between late-adolescent (PND 53) and adult rats (PND 80), and that some aspects of cortical development seemed to mature by PND 53 [40]. Furthermore, some aspects of the prefrontal cortex seemed to reach maturity by PND 46 [41]. However, we still found that the impulsivity of adolescent mice (7 weeks old) was significantly higher than that of adult mice (13–14 weeks old) (Fig. 6a). We therefore speculate that the function of impulse control matures at a later phase compared to the function related to attentional set shifting. Thus, earlier completion of the training might be required to examine cognitive functions other than impulse control. Third, higher omission rates in adolescent mice (Fig. 5d) might be problematic. Although higher omission rates in adolescent mice were not due to differences in training procedures (Fig. 5d), the decreased number of completed trials might make parameters unreliable. We attenuated this problem by averaging performance scores in 2 or 3 consecutive sessions. Nevertheless, stable performance within a session is preferable in order to detect drug effects using a within-subjects design.

Another new method, which was established very recently [42], might resolve the former two problems. Because it takes only a week to complete the training for the 5-CSRTT, one can start the training at PND 35 or later, that is, when female mice grow enough to implement food restriction. If one uses only male mice, performance at an earlier age (*e.g.* around PND 30) could be tested to examine cognitive functions other than impulse control.The third problem might be resolved by using pellets of 14 mg or less instead of 20 mg pellets.

However, our method still has several advantages over their method. First, our method successfully detected the age-related differences in impulsivity, whereas theirs did not. Second, the performance levels of our method are more similar to those of conventional methods compared to the performance levels of their method. Third, our method is relatively easy to perform because only our programs are new, whereas their method requires special equipment and will need additional approval from the Animal Research Committee of the institute because the mice stay in the operant box for a week in their method. For

example, our institute usually prohibits leaving mice in experimental rooms for > 48 h. Fourth, 6 or more mice can be trained in a same box on a given day in our method, whereas only one subject can be trained in the same box on a given day in their method. Because our method requires 3 weeks while their method requires 1 week, the number of mice that can be trained during a period using our method is larger than that using their method (6 mice/3 weeks *vs*. 3 mice/3 weeks in a box).

Taken together, the method developed by Remmelink and colleagues would be more suitable to examine sex differences in adolescents or cognitive functions other than impulse control, whereas our method is more useful when the primary interest is impulsive action and associated age-related differences. Other factors such as the equipment available, space, number of animals, and institutional rules should also be taken into consideration when choosing between the two methods.

In conclusion, we have successfully overcome the limitations, that is, food restriction-induced growth disruption and long training duration, associated with the conventional 5-CSRTT/3-CSRTT. These are the most widely used operant tasks to study attention and impulsivity in rodents. Our novel 3-CSRTT procedure avoided growth disruptions and significantly shortened the training duration, making it possible to study impulsivity in adolescent mice.

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Appendix A

Table A1
Effect of dietary restriction on mean body weight [in grams, g, and as a percent (%) of day-peak weight] in the mice that received training for the 3-CSRTT.

age (days)	day peak	before training
28	13.1	12.0 (91.9%)
36	17.7	16.3 (91.7%)
43	19.7	18.1 (91.7%)

Table A2
Criteria for each training phase in the novel 3-CSRTT for adolescent and adult mice.

Training phase	Criteria for progression in adolescents	Criteria for progression in adults
pre-training 1	> 25 pellet consumption	> 25 pellet consumption
pre-training 2	> 15 pellet consumption	> 20 pellet consumption
pre-training 3	> 15 pellet consumption	> 20 pellet consumption
pre-training 4	(accuracy ≥ 50% ∧ correct ≥ 15) ∨ (correct ≥ 30)	(accuracy ≥ 50% ∧ correct ≥ 25) ∨ (correct ≥ 40)
training	> 25 correct responses for 2 consecutive days	> 40 correct responses for 2 consecutive days
performance stabilized	> 20 correct responses, > 70% accuracy for 2 consecutive days	> 40 correct responses, > 70% accuracy for 2 consecutive days

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bbr.2018.01.014.

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