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Research report

Mice with exonic *RELN* deletion identified from a patient with schizophrenia have impaired visual discrimination learning and reversal learning in touchscreen operant tasks

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ABSTRACT

The Reelin gene (*RELN*) encodes a large extracellular protein, which has multiple roles in brain development and adult brain function. It activates a series of neuronal signal transduction pathways in the adult brain that function in synaptic plasticity, dendritic morphology, and cognitive function. To further investigate the roles of *Reln* in brain function, we generated a mouse line using the C57BL/6 J strain with the specific *Reln* deletion identified from a Japanese patient with schizophrenia (*Reln*-del mice). These mice exhibited abnormal sociality, but the pathophysiological significance of the *Reln* deletion for higher brain functions, such as learning and behavioral flexibility remains unclear. In this study, cognitive function in *Reln*-del mice was assessed using touchscreen-based visual discrimination (VD) and reversal learning (RL) tasks. *Reln*-del mice showed normal learning in the simple VD task, but the learning was delayed in the complex VD task as compared to their wild-type (WT) littermates. In the RL task, sessions were divided into early perseverative phase (sessions with <50% correct) and later learning phase (sessions with ≥50% correct). *Reln*-del mice showed normal perseveration but impaired relearning ability in both simple RL and complex RL task as compared to WT mice. These results suggest that *Reln*-del mice have impaired learning ability, but the behavioral flexibility is unaffected. Overall, the observed behavioral abnormalities in *Reln*-del mice suggest that this mouse model is a useful preclinical tool for investigating the neurobiological mechanism underlying cognitive impairments in schizophrenia and a therapeutic strategy.

1. Introduction

The Reelin gene (*RELN*) encodes a large extracellular protein, which has multiple roles in brain development and adult brain function [1]. During embryonic development, Reelin is mainly expressed in Cajal-Retzius cells of the cerebral cortex, and functions as a major regulator of neuronal migration and formation of cellular layers [2,3]. At postnatal stages, Reelin is gradually widespread throughout the

List of abbreviations: RELN, Reelin gene; VD, visual discrimination; RL, reversal learning; WT, wild-type; ANOVA, analysis of variance; Dab-1, Disabled-1; ITI, inter-trials interval; OFC, orbitofrontal cortex; DS, dorsal striatum.

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neocortical layers and promotes the development of dendritic spines [4]. In the adult brain, it is prominently produced by GABAergic interneurons, and is involved in synaptic plasticity, dendritic morphology, and cognitive function [5–7]. Reelin activates a series of neuronal signal transduction pathways in the adult brain following the tyrosine phosphorylation of intracellular adapter protein Disabled-1 (Dab-1) through binding to its two known receptors, apolipoprotein E receptor 2 and very-low-density lipoprotein receptor [8,9]. This signaling pathway results in increased long-term potentiation of synapses, which was proposed to correlate with learning and memory [10,11].

Clinical genetic studies revealed that *RELN* is associated with several neuropsychiatric disorders, including schizophrenia and autism spectrum disorder [12,13]. Of note, several rare variants of *RELN* have been identified as risk factors for schizophrenia such as *de novo* or rare missense variants and exonic deletion of *RELN* [14–16]. In our previous study, we found a novel exonic deletion in *RELN* in a Japanese patient with schizophrenia using genome-wide high-resolution copy number variation analysis [17]. We also examined the boundaries of the exonic deletion of *RELN* in this patient and confirmed that this deletion included exons 52–58, which correspond to amino residues 2759–3148 within Reelin repeats 7 and 8 [18]. To further assess its function, we generated a mouse line using the C57BL/6J strain with the specific *Reln* deletion identified from the Japanese patient (*Reln*-del mice). The biochemical analysis demonstrated that the expression of Reelin was significantly lower in heterozygous *Reln*-del mice than that in their wild-type (WT) littermates, and barely detected in homozygous *Reln*-del mice. Furthermore, phenotypes, such as cerebellar atrophy, dysplasia of the cerebral layers, and abrogated protein levels of cerebral Reelin, were noted in the homozygous *Reln*-del mice. In a behavioral assay, heterozygous *Reln*-del mice exhibited abnormal sociality in the three-chamber social interaction test, whereas changes in cognitive function (e.g., novel object recognition test) were not observed [19].

Over the past decade, the automated touchscreen-based behavioral testing method has become a well-known translational assay that can be used to assess the cognitive function of preclinical animal models of neuropsychiatric disorders [20]. In our previous report, this method was confirmed to be more sensitive at detecting cognitive abnormality in mice than the other respondent behavioral tasks such as the novel object recognition test [21]. In the present study, we analyzed visual discriminative learning/memory and behavioral flexibility in *Reln*-del mice using the touchscreen-based visual discrimination (VD) and reversal learning (RL) tasks.

2. Materials and methods

2.1. Animals

Reln-del mice were generated by the CRISPR/Cas9 method in a C57BL/6 J genetic background as described previously [19]. Heterozygous *Reln*-del mice were generated by intercrossing *Reln*-del males and C57BL/6J females. Littermate WT C57BL/6 J mice were used as controls, and 7- to 8-week-old WT (male $n = 6$, female $n = 5$) and *Reln*-del (male $n = 6$, female $n = 6$) mice were used in experiments. Mice were housed in groups of a maximum of 5 per cage and maintained under standard conditions (23 ± 1 °C, 50 ± 5% humidity) with a 12-h light/dark cycle (09:00–21:00 h light period). All animals were examined during the light phase. Food and water were available ad libitum. All animal experiments were approved and performed in accordance with the guidelines of the Animal Care and Use Committee of Nagoya University Graduate School of Medicine.

2.2. Apparatus for touchscreen-based VD and RL tasks

The touchscreen-based VD and RL tasks were performed using the touchscreen chamber system (Phenosys, Berlin, Germany; Brain Science Idea, Osaka, Japan) as our previous report [21].

2.3. Experimental schedules for touchscreen-based VD and RL tasks

The experimental schedules used were described in detail in previous studies [21–23]. Briefly, the touchscreen-based behavioral tasks were started with the introduction of food and water restriction to provide sufficient motivation for mice to perform the tasks. Access to food and water was restricted for 2 h (17:00–19:00) each day at least 1 week before pretraining. Food and water restriction were maintained throughout the touchscreen tasks until the end of experiment, and the body weight of mice was maintained at 85–90% of unrestricted animals. The pretraining consists of 5 stages: habituation, initial touch, must touch, must initiate, and punish correct. After mice learned how to operate the touchscreen (≥75% on two consecutive sessions), they were then subjected to the VD task and RL task (Fig. 1A, B). Performance in the RL task was analyzed according to the previous studies [24,25].

2.4. Statistical analyses

All data are expressed as means ± SEM. Statistical analyses were performed with GraphPad Prism 6.0 (GraphPad Software, Inc., CA, USA). The significance of differences was assessed using the Student's *t*-test or log-rank test for comparisons of two groups. In multiple comparisons, the significance of differences was evaluated using an analysis of variance (ANOVA) with repeated measures. The Tukey–Kramer test was used for a post-hoc analysis when *F* ratios were significant.

3. RESULTS

3.1. No difference in performance between WT and Reln-del mice in the pretraining and simple VD task

The VD task was performed to evaluate the discriminative learning and memory [23], whereas the RL task was performed to assess choice shifting and choice relearning [26]. There was no difference in the number of total trials to reach the pretraining criterion between WT and *Reln*-del mice, suggesting normal visuospatial and motor functions ($t(21) = 1.465$, $p = 0.1576$; Fig. 2A, B). The animals were subsequently subjected to the simple VD task, in which mice were required to touch a stimulus to obtain a reward from a pair of visual stimuli (marble and fan) (Fig. 2C). There were no significant differences in the percentage of mice reaching the learning criterion by daily training ($p = 0.2268$; Fig. 2D), total number of sessions ($t(21) = 1.329$, $p = 0.1981$; Fig. 2E), total trials ($t(21) = 1.424$, $p = 0.1691$; Fig. 2F), total normal trials ($t(21) = 1.329$, $p = 0.1981$; Fig. 2G), or total correction trials ($t(21) = 1.413$, $p = 0.1723$; Fig. 2H) to reach the task criterion between the genotypes. Collectively, this suggested that visual discrimination learning of *Reln*-del mice in the simple VD task was not impaired.

3.2. Impaired performance of Reln-del mice in the simple RL task

In order to analyze the behavioral flexibility in *Reln*-del mice, the animals were then subjected to the RL task in which the same pair of visual stimuli was presented but the reward contingencies were reversed (Fig. 3A). In the simple RL task, there were no significant differences in the percentage of mice reaching the learning criterion by daily training ($p = 0.0714$; Fig. 3B), total number of sessions ($t(21) = 1.864$, $p = 0.0764$; Fig. 3C), total trials ($t(21) = 1.726$, $p = 0.0991$; Fig. 3D), total normal trials ($t(21) = 1.864$, $p = 0.0764$; Fig. 3E) and total correction trials ($t(21) = 1.471$, $p = 0.1561$; Fig. 3F) between WT and *Reln*-del mice. Analysis of early perseverative (sessions with <50% correct) and later learning phases (sessions with ≥50% correct) [24,25] revealed that in the early perseverative phase, no significant differences were observed in the total number of sessions ($t(21) = 0.8031$, $p = 0.4309$; Fig. 3G), trials ($t(21) = 0.7278$, $p = 0.4748$; Fig. 3H), normal trials ($t(21) = 0.8031$, $p = 0.4309$; Fig. 3I) and correction trials ($t(21) = 0.6515$, $p = 0.5218$; Fig. 3J) between two genotypes. In the

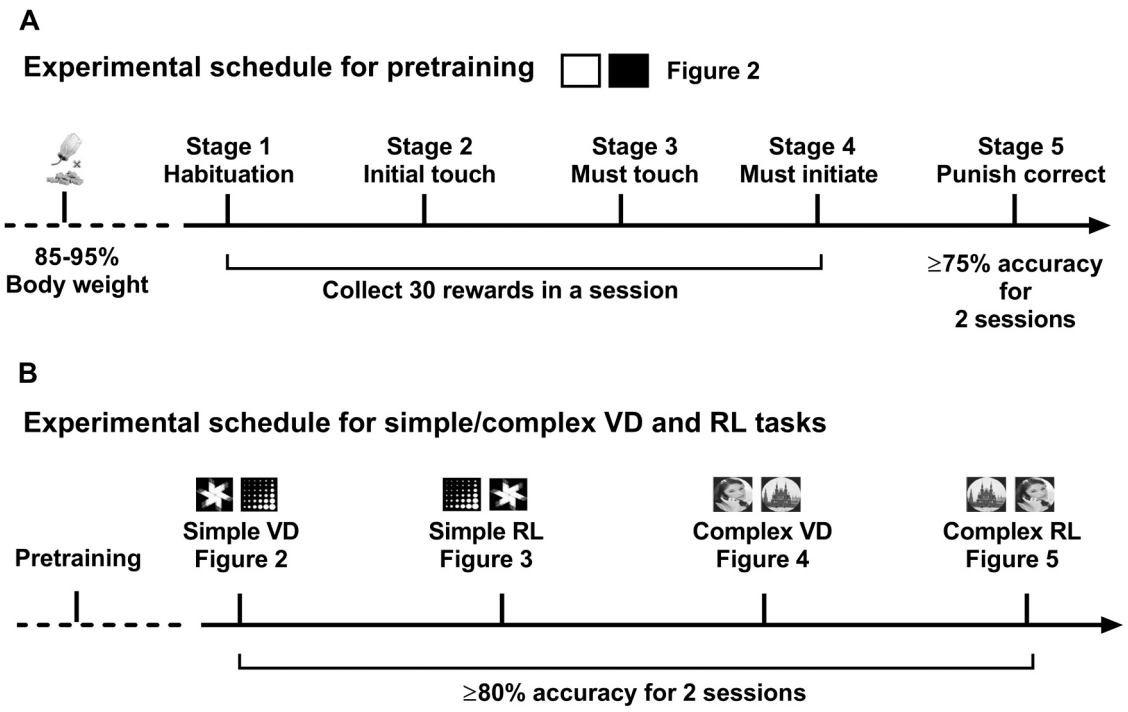

Fig. 1. Experimental schedules for touchscreen-based behavioral tests. (A) Experimental schedule for pretraining. (B) Experimental schedule for simple and complex VD and RL tasks.

later learning phase, however, *Reln*-del mice showed impaired performance in the number of sessions (t (21) = 2.129, $^{*}p < 0.05$; Fig. 3G), trials (t (21) = 2.165, $^{*}p < 0.05$; Fig. 3H), normal trials (t (21) = 2.129, $^{*}p < 0.05$; Fig. 3I) and correction trials (t (21) = 2.088, $^{*}p < 0.05$; Fig. 3J). Thus, *Reln*-del mice showed no impairment in early perseverative phase, but they showed impaired relearning capability in later learning phase, suggesting that behavioral flexibility was unaffected but later learning was impaired by the deletion of *Reln* in the simple RL task.

3.3. Impaired performance of Reln-del mice in the complex VD task

Next, we increased the difficulty of tasks by changing the pairs of visual stimuli to more complicated ones (from marble & fan to face & castle; Fig. 4A) leaving other paradigms unchanged. In the complex VD task, *Reln*-del mice were significantly slower to reach the learning criterion by daily training than WT mice ($p < 0.01$; Fig. 4B). Indeed, *Reln*-del mice needed more sessions (t (21) = 2.655, $p < 0.05$, Fig. 4C), trials (t (21) = 2.788, $p < 0.05$, Fig. 4D), normal trials (t (21) = 2.655, $p < 0.05$, Fig. 4E), and correction trials (t (21) = 2.803, $p < 0.05$, Fig. 4F) to reach the complex VD task criterion (greater than 80% accuracy on two consecutive days) than WT mice. Taken together, visual discrimination learning was significantly impaired in *Reln*-del mice when the visual stimuli became more complicated.

3.4. Impaired performance of Reln-del mice in the complex RL task

Similarly, the RL task was performed using complex stimuli when the mice reached the complex VD criterion. In the complex RL task (Fig. 5A), *Reln*-del mice were significantly slower to reach the learning criterion by daily training than WT mice ($p < 0.01$; Fig. 5B). The mutation mice required approximately twice the total number of sessions (t (21) = 2.939, $^{**}p < 0.01$; Fig. 5C), trials (t (21) = 0.2661, $^{*}p < 0.05$; Fig. 5D), normal trials (t (21) = 2.939, $^{**}p < 0.01$; Fig. 5E), and correction trials (t (21) = 3.535, $^{*}p < 0.05$; Fig. 5F) to reach the criterion than WT mice. Analysis of early perseverative and later learning phases revealed that in the early perseverative phase, no significant difference in the number of sessions (t (21) = 1.642, $p = 0.1155$; Fig. 5G), trials (t (21) = 1.45, $p = 0.1618$; Fig. 5H), normal trials (t (21) = 1.642, $p = 0.1155$; Fig. 5I) and correction trials (t (21) = 1.309, $p = 0.2045$; Fig. 5J) between two genotypes. However, in later learning phase, *Reln*-del mice showed impaired performance in the number of sessions (t (21) = 2.996, $^{**}p < 0.01$; Fig. 5G), trials (t (21) = 2.776, $^{*}p < 0.05$; Fig. 5H), normal trials (t (21) = 2.996, $^{**}p < 0.01$; Fig. 5I) and correction trials (t (21) = 2.475, $^{*}p < 0.05$; Fig. 5J). Taken together, *Reln*-del mice showed impaired performance in complex RL task, which maybe due to their impaired relearning capability in later learning phase. No significant difference in early perseverative phase suggests that behavioral flexibility was unaffected by the deletion of *Reln*. These results suggested that *Reln*-del mice showed continuation of VD impairment in the complex RL task.

4. Discussion

We recently reported that *Reln*-del mice exhibit abnormal brain structures and social dysfunction [19]. In addition, the in vitro analysis of cortical neurons from *Reln*-del mice revealed lower Reelin expression, weaker Reelin signaling, lower spine density, and less complexity of neuronal morphology than in neurons from WT mice [27]. In the present study, we evaluated the ability of learning and behavioral flexibility in *Reln*-del mice using touchscreen-based behavioral tasks.

Touchscreen-based behavioral tasks have been developed for rodents to provide a better translational approach across species to further understand the cognitive impairments observed in neuropsychiatric disorders and to evaluate potential pharmacological interventions [20]. Mice carrying human disease-related genetic mutations, such as deletion of *DISC1* and 22q11.2, exhibit cognitive impairments in the touchscreen-based behavioral task [21,28]. According to clinical findings, one of the characteristics of the schizophrenia patient with the exonic deletion of *RELN* was impaired cognitive function [18], which is consistent with our present studies of *Reln*-del mice.

Heterozygous *Reln*-del mice were previously reported to exhibit abnormal sociality in the three-chamber social interaction test, whereas changes in cognitive function (e.g., novel object recognition test) were not observed [19]. The present study also suggests that the experimental

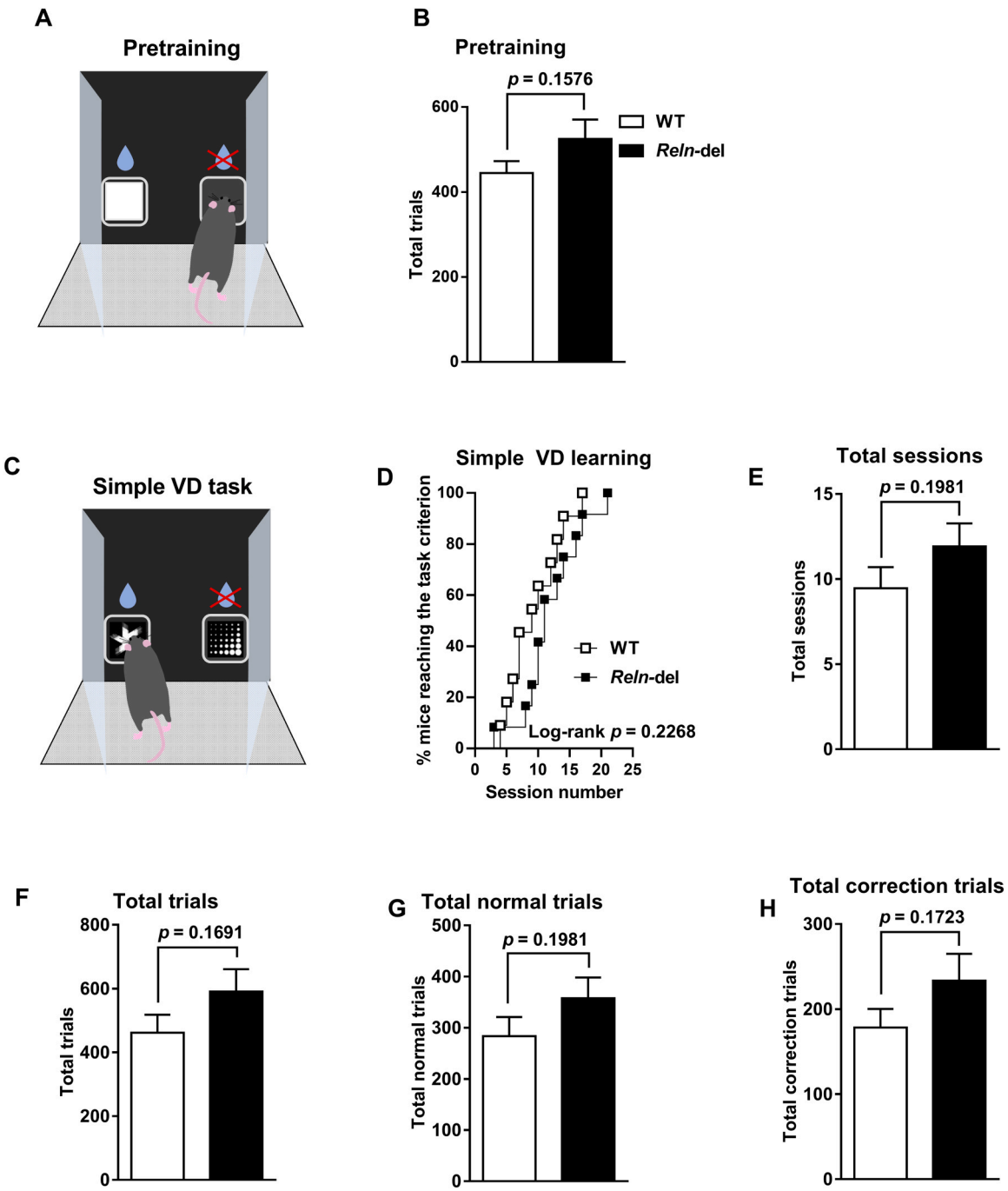

Fig. 2. No difference in performance between WT and *Reln*-del mice in the pretraining and simple VD task. (A) Introduction of incorrect responses in pretraining. (B) Total number of trials to reach the criterion in the pretraining session. (C) In the simple VD task, two stimuli (fan and marble) were presented simultaneously. The fan was shown as the correct response associated with a reward and the marble was an incorrect response with no reward. (D) Simple VD learning: percentage of mice to reach the learning criterion by daily training. Total number of (E) sessions, (F) trials, (G) normal trials, and (H) correction trials to reach the discrimination criterion. Values indicate the mean ± SEM. WT (male $n = 6$, female = 5) and *Reln*-del (male $n = 6$, female $n = 6$) mice.

conditions are important factors for detecting the cognitive dysfunction in mouse models of neuropsychiatric disorders, consistent with previous reports stating that computerized operant behavioral tests have a high reproducibility and low variability, and are more sensitive to cognitive abnormality in mice [20,21,29]. In addition, the touchscreen-based behavioral tasks are actually useful for examinates cognition of patients with neuropsychiatric disorders [30,31].

In our study, the battery tasks started with 5 stages of pretraining before the VD task to shape screen-touch behavior. *Reln*-del mice exhibited no impaired performance in any of these 5 stages during pretraining, suggesting that their visuospatial and motor functions are normal. Subsequently, in the simple VD task, mice were required to learn one of two stimuli (marble and fan) associated with the reward. No significant differences were observed between the genotypes in the performance of the simple VD task, although *Reln*-del mice learned slightly slower than their WT littermates. This was also observed in the following simple RL task in which the previously incorrect stimulus became the correct stimulus and vice versa. When reversal sessions were divided according to whether performance was <50% correct (early perseverative phase) or ≥50% correct (later learning phase), and the

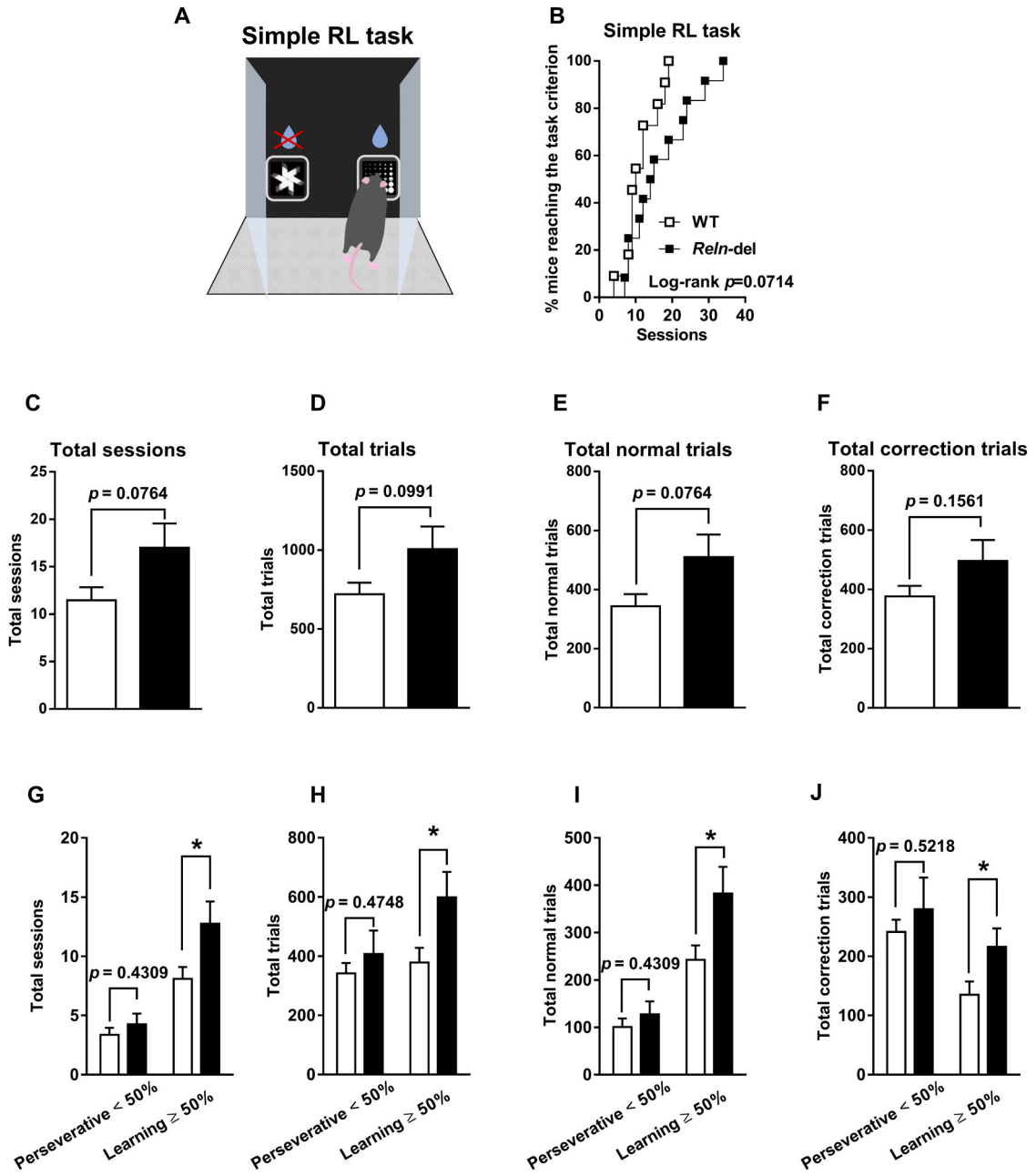

Fig. 3. Impaired performance of *Reln*-del mice in the simple RL task. (A) In the simple RL task, the contingency of the stimulus pair was reversed from the previous VD task. The previous reward stimulus (fan) became an incorrect response, whereas the previous non-rewarded stimulus (marble) became the correct response. (B) Simple RL task: percentage of mice to reach the learning criterion by daily training. Total number of (C) sessions, (D) trials, (E) normal trials, and (F) correction trials to reach the reversal criterion. Analysis of early perseverative (sessions with <50% correct) and later learning phases (sessions with ≥50% correct) in total number of (G) sessions, (H) trials, (I) normal trials and (J) correction trials. Values indicate the mean ± SEM. WT (male $n = 6$, female = 5) and *Reln*-del (male $n = 6$, female = 5) mice. $^*p < 0.05$ vs WT.

total sessions, trials, normal trials and correction trials committed in each phase were calculated according to the previous studies [24,25], a significant impairment was evident in the later learning phase but not early perseverative phase in *Reln*-del mice. Thus, *Reln*-del mice may have some impairments of visual discrimination learning in the simple VD and RL tasks.

We next increased the difficulty of tasks by changing the pairs of stimuli to more complicated ones (from marble-fan pair to human face-castle pair) leaving other paradigms unchanged. Under these experimental conditions, *Reln*-del mice had markedly impaired visual discrimination learning. Appetite for rewards and body weight of mice are important for operant behavior tasks, therefore, we monitored the body weight of two genotypes every session. *Reln* deletion showed no effects on the body weight. In addition, reward consumption was the same in every session for each mouse (20 μL of milk). Thus, it is unlikely that impaired VD learning in *Reln*-del mice was due to the altered appetite for rewards.

Previous reports showed that normal performance in VD task depends on the intact function of the corticostriatal circuitry [21,26], which is essential for learning behaviors in humans, nonhuman primates, and rodents [29,32]. The c-Fos expression increased in dorsal striatum (DS) during choice learning and relearning, suggesting that DS was increasingly activated during these two phases [26]. The in vivo neuronal recordings in DS showed that the DS neuronal activity

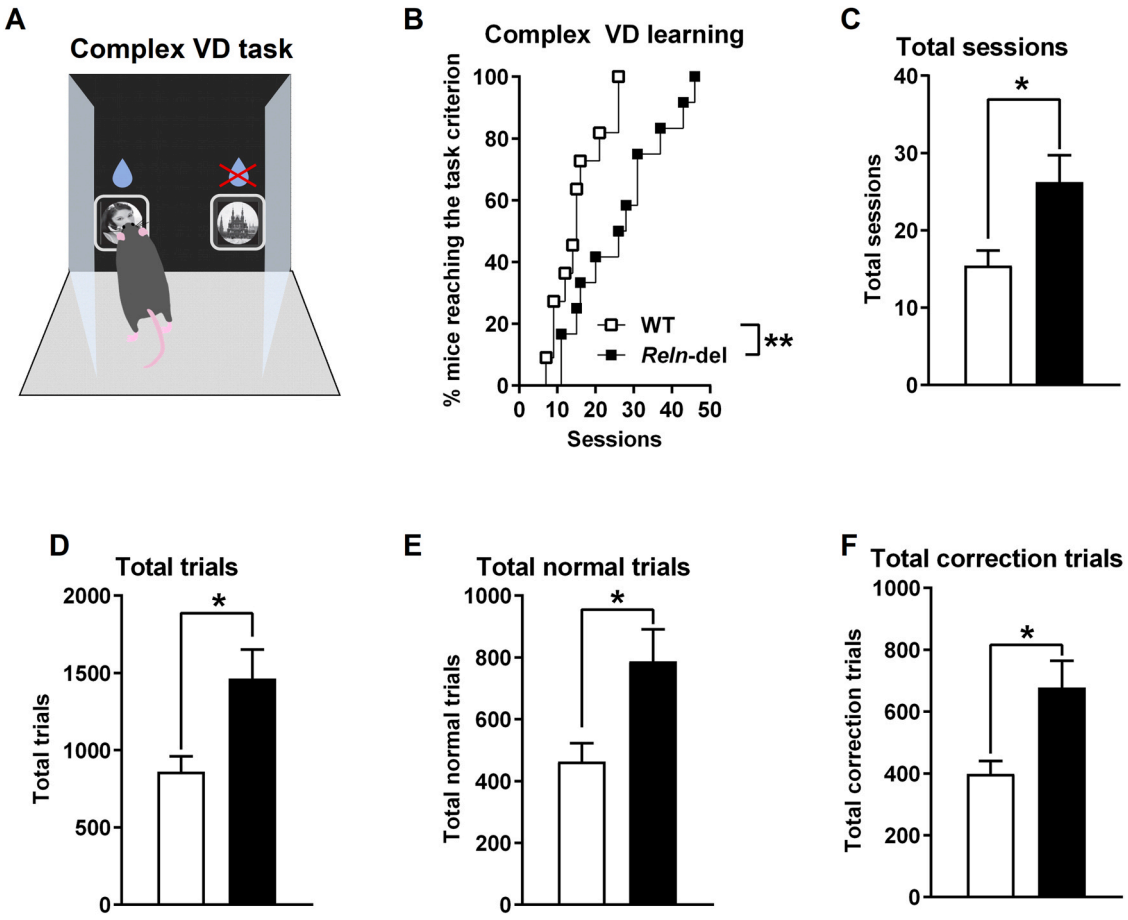

Fig. 4. Impaired performance of *Reln*-del mice in the complex VD task. (A) In the complex VD task, two stimuli (face and castle) were presented simultaneously. The face stimulus was shown as the correct response associated with a reward and the castle stimulus was an incorrect response with no reward. (B) Complex VD learning: percentage of mice to reach the learning criterion by daily training. Total number of (C) sessions, (D) trials, (E) normal trials, and (F) correction trials to reach the complex discrimination criterion. Values indicate the mean ± SEM. WT (male $n = 6$, female $n = 5$) and *Reln*-del (male $n = 6$, female $n = 5$) mice. $^{*}p < 0.05$, $^{**}p < 0.01$ vs WT.

associated with choice learning task [26], while DS lesions showed impairment in choice relearning in mice [33]. Thus, DS is critical for cognitive function [34]. RL task is employed as a measure of perseveration which has been found to be dependent upon orbitofrontal cortex (OFC) function [35–37]. In the RL task, the c-Fos expression increased specifically in the OFC, which also means the OFC was active during choice shifting [26]. Previous reports have repeatedly demonstrated that OFC is critical for behavioral flexibility [38]. Neuropsychological studies showed damage to OFC leads to impairment of behavioral flexibility [37], patients with OFC damage also showed deficient in object RL task [39]. In our study, no significant difference in early perseverative phase suggests that OFC may be unaffected by the deletion of *Reln* [26]. Nevertheless, since *Reln*-del mice showed impairment in learning and relearning phases, it is possible that the cognitive dysfunction in *Reln*-del mice may be associated with the dysfunction in the DS. Further studies are required to clarify specific brain regions and types of cells that are related to cognitive dysfunction in *Reln*-del mice.

In conclusion, this is the first report that associative learning and behavioral flexibility are impaired in *Reln*-del mice. Our study suggests that Reelin plays an important role in cognition, and *Reln*-del mice will enable us to examine the neurobiological mechanisms underlying the cognitive dysfunction caused by the deletion of *Reln* and therapeutic strategies.

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Ethics approval and consent to participate

Animals were handled in accordance with the guidelines established by the Institutional Animal Care and Use Committee of Nagoya University, the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society, and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Consent for publication

Not applicable.

CRediT authorship contribution statement

Jingzhu Liao, **Geyao Dong**, **Bolati Wulaer** performed experiments and analyzed data. **Jingzhu Liao**, **Geyao Dong**, **Bolati Wulaer**, **Masahito Sawahata**, **Hiroyuki Mizoguchi**, **Daisuke Mori**, **Norio**

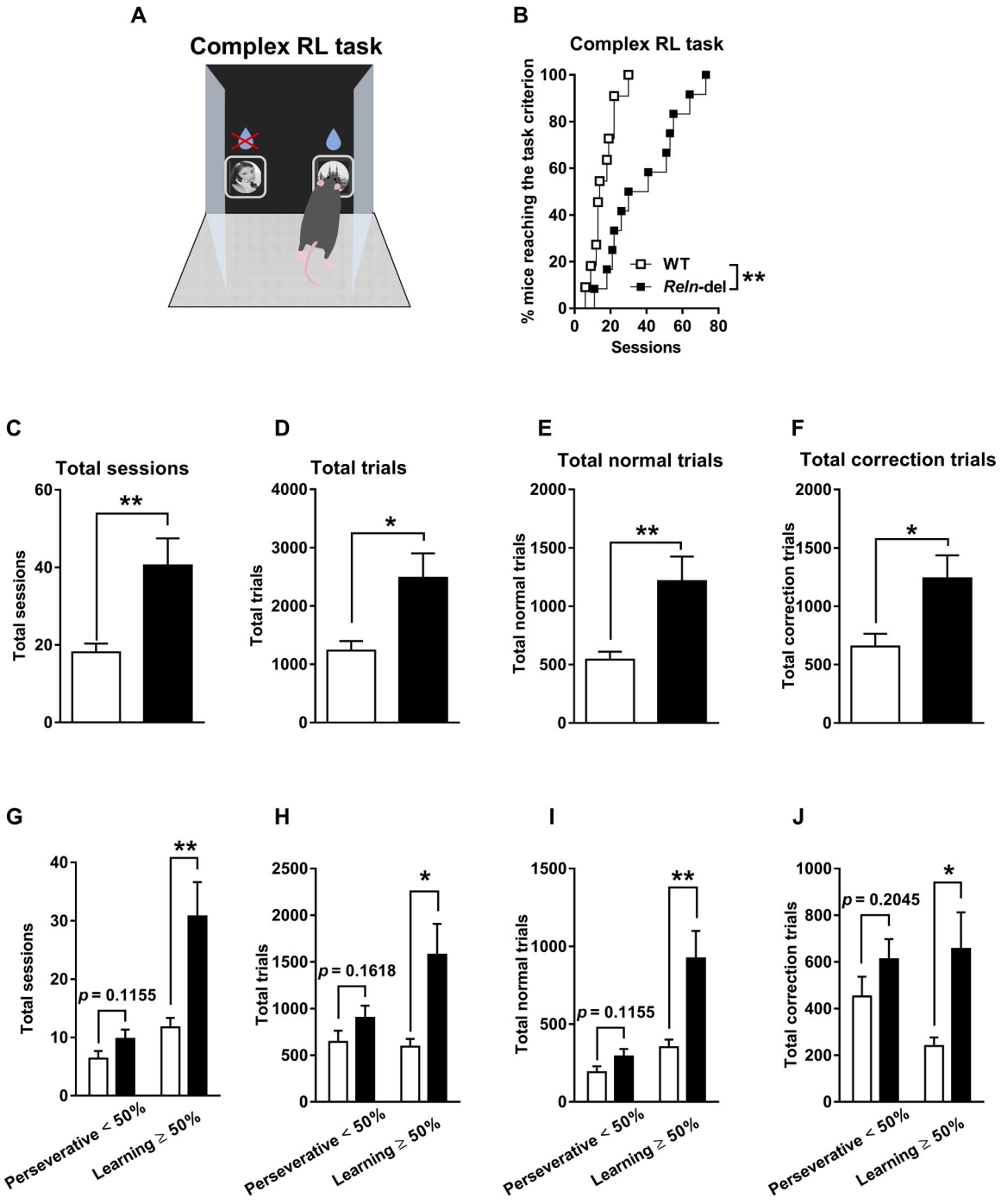

Fig. 5. Impaired performance of *Reln*-del mice in the complex RL task. (A) In the complex RL task, the contingency of the stimulus pair was reversed from the previous VD task. The previous reward stimulus (face) became an incorrect response, whereas the previous non-rewarded stimulus (castle) became the correct response. (B) Complex RL task: percentage of mice to reach the learning criterion by daily training. Total number of (C) sessions, (D) trials, (E) normal trials, and (F) correction trials to reach the reversal criterion. Analysis of early perseverative and later learning phases in total number of (G) sessions, (H) trials, (I) normal trials and (J) correction trials. Values indicate the mean ± SEM. WT (male $n = 6$, female $n = 5$) and *Reln*-del (male $n = 6$, female $n = 5$) mice. $^{*}p < 0.05$, $^{**}p < 0.01$ vs WT.

Ozaki, **Toshitaka Nabeshima**, **Taku Nagai**, and **Kiyofumi Yamada**, designed the study and wrote the manuscript. **Hiroyuki Mizoguchi**, **Taku Nagai**, and **Kiyofumi Yamada** provided the necessary experimental apparatuses. All authors reviewed, edited, and approved the final manuscript.

Declaration of Competing Interest

The authors have no conflicts of interest to disclose.

Availability of data and materials

All data used in this study are available from the corresponding author on reasonable request.

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