Endocannabinoid signaling from 2-arachidonoylglycerol to CB_1 cannabinoid receptor facilitates reward-based learning of motor sequence

Abbreviated title: Endocannabinoid system promotes sequence learning

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Abbreviations

2-AG: 2-arachidonoylglycerol

CB_1-KO: CB_1-knockout

DGLα: diacylglycerol lipase-α

DGLα-KO: DGLα-knockout

DMSO: dimethyl sulfoxide

LTD: long-term depression

PBS: phosphate buffered saline

WT: wild-type

Abstract

The endocannabinoid system modulates synaptic transmission, controls neuronal excitability, and is involved in various brain functions including learning and memory. 2-arachidonoylglycerol, a major endocannabinoid produced by diacylglycerol lipase-α (DGLα), is released from postsynaptic neurons, retrogradely activates presynaptic CB_1 cannabinoid receptors, and induces short-term or long-term synaptic plasticity. To examine whether and how the endocannabinoid system contributes to reward-based learning of a motor sequence, we subjected male CB_1-knockout (KO) and DGLα-KO mice to three types of operant lever-press tasks. First, we trained mice to press one of three levers labeled A, B, and C for a food reward (one-lever task). Second, we trained mice to press the three levers in the order of A, B, and C (three-lever task). Third, the order of the levers was reversed to C, B, and A (reverse three-lever task). We found that CB_1-KO mice and DGLα-KO mice exhibited essentially the same deficits in the operant lever-press tasks. In the one-lever task, both strains of knockout mice showed a slower rate of learning to press a lever for food. In the three-lever task, both strains of knockout mice showed a slower rate of learning of the motor sequence. In the reverse three-lever task, both strains of knockout mice needed more lever presses for reversal learning. These results suggest that the endocannabinoid system facilitates reward-based learning of a motor sequence by conferring the flexibility with which animals can switch between strategies.

Key words:

endocannabinoid system, lever press, reinforcement learning, reversal learning

Introduction

The endocannabinoid system has been shown to be involved in many aspects of mammalian physiological and pathological functions (Ligresti et al., 2016). The most intensively-studied endocannabinoids are anandamide (Devane et al., 1992) and 2-arachidonoylglycerol (2-AG) (Mechoulam et al., 1995). 2-AG is produced by two types (α, β) of diacylglycerol lipases (Bisogno et al., 2003), the α type (DGLα) being more important in the brain (Gao et al., 2010; Tanimura et al., 2010). In contrast, the main pathway for anandamide synthesis is not fully understood (Augustin and Lovinger, 2018). Receptors of endocannabinoids consist of two subtypes namely the CB_1 and CB_2 cannabinoid receptors (Matsuda et al., 1990; Munro et al., 1993). CB_1 receptors are richly and widely expressed throughout the brain. CB_2 receptors are also expressed in the brain, albeit to a lesser extent than CB_1 receptors (Roche and Finn, 2010), and are mainly expressed in the immune system of the periphery. Specifically, CB_1 receptors are strongly expressed in brain regions involved in learning and memory such as the hippocampus, cerebellum and basal ganglia (Mechoulam and Parker, 2013).

The endocannabinoid system is involved in several forms of activity-dependent modulation of excitatory and inhibitory synaptic transmissions. It is generally accepted that 2-AG is released from postsynaptic neurons in an activity-dependent manner, activates presynaptic CB_1 receptors, and induces short-term or long-term synaptic plasticity in various brain regions including the cortex, hippocampus, amygdala, cerebellum, and basal ganglia (Kano et al., 2009; Augustin and Lovinger, 2018). Some studies also suggested the involvement of anandamide in synaptic plasticity (Augustin and Lovinger, 2018). Behavioral studies on CB_1-knockout (KO) mice have shown that CB_1 receptors are critically involved in several forms of learning including spatial learning (Varvel and Lichtman, 2002), fear

conditioning (Marsicano et al., 2002), eye-blink conditioning (Kishimoto and Kano, 2006), and habit formation (Hilario et al., 2007), which are primarily dependent on the hippocampus, amygdala, cerebellum, and basal ganglia, respectively.

Reward-based motor learning involves the cortico-basal ganglia circuit (Schultz, 2016). Anatomical studies show that CB_1 receptors are highly expressed in the basal ganglia and localized to presynaptic terminals of excitatory corticostriatal projection neurons and inhibitory medium spiny neurons (MSNs) (Kano et al., 2009). Electrophysiological studies show that CB_1 receptors are required for multiple forms of striatal synaptic plasticity (Lovinger, 2010). For example, repetitive stimulation of cortical afferents at high frequencies induces long-term depression (LTD) at excitatory synapses onto striatal MSNs, and this LTD is prevented by pharmacological blockade of CB_1 receptors (Lovinger and Mathur, 2012). Behavioral studies show that CB_1-KO mice exhibit altered reward-based behavior including feeding behavior (Bellocchio et al., 2010), place preference (Castane et al., 2002), operant lever-pressing (Crombag et al., 2010), nose-poking (Holter et al., 2005), wheel-running (Dubreucq et al., 2010), and wheel-running with nose-pose (Muguruza et al., 2019). However, reward-based behavioral task for motor sequence has not been examined in CB_1-KO mice. Although many behavioral tests have been performed in CB_1-KO mice, there are limited number of behavioral studies using DGLα-KO mice (Shonesy et al., 2018). Moreover, it remains to be determined which endocannabinoid, 2-AG or anandamide, is responsible for each CB_1-dependent brain function.

In the present study, we aimed to elucidate possible roles of 2-AG to CB_1 endocannabinoid signaling in reward-based learning of motor sequence. We used CB_1-KO and DGLα-KO mice and examined their performances during the three-lever operant task (Yoneda et al., 2017), a form of reward-based motor learning task useful for studying different aspects of motor learning, including sequence learning and

reversal learning. We demonstrate that CB_1-KO and DGLα-KO mice show similar behavioral phenotypes characterized by significant impairments in action-outcome learning, sequence learning, and reversal learning. These results indicate that the 2-AG to CB_1 endocannabinoid signaling is critically involved in multiple processes of reinforcement-based motor learning.

Experimental Procedures

Animals

Experimental procedures were approved by the Animal Welfare Committee of Kanazawa University and Tokushima Bunri University. We obtained behavioral data from CB_1-KO (Sugaya et al., 2016), DGLα-KO (Tanimura et al., 2010), and wild-type (WT) littermates of the C57BL/6NCr strain. For the experiments with SR141716A, we used normal C57BL/6NCr mice. We used only male mice for all experiments in this study. After being group-housed in a colony room, 6-week-old mice were transferred to the experimental area, and housed individually in plastic cages with four compartments at 23 ± 2˚C on a 12/12 h light–dark cycle (light off at 1:00 p.m.). Food and water were available *ad libitum*. Before starting operant task on 8-week-old mice, the mice were allowed to habituate to the testing area for one week and to the experimenter for another one week. To maintain motivation to lever press for food reward, we adjusted the food intake each day. The number of food pellets given each day (AIN-76A, 10 mg, Research Diets, Inc., New Brunswick, NJ, U.S.A.), including the pellets delivered as a reward during the operant task and the pellets given in the home cage after the operant task, was set to 200 (2 g), 250 (2.5 g), 300 (3 g), or 350 (3.5 g) depending on body weight. However, if the calculated number of pellets given in the home cage was less than 50, 50 pellets were given in the home cage. Operant task training was conducted

five times a week (Monday-Friday), and after the training on Friday the mice were allowed free access to food until Sunday evening. Body weight was measured every day, and the food intake was finely controlled so that each group of mice could show a similar change of body weight over several weeks (Fig. 2).

Pharmacological blockade of CB_1 receptors.

Normal male C57BL/6NCr mice were used in pharmacological experiments. For pharmacological blockade of CB_1 receptors, the CB_1 antagonist SR141716A (N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride) (3 mg/kg, i.p.) was dissolved in solution (1% dimethyl sulfoxide (DMSO) and 4% Cremophor in phosphate buffered saline (PBS)) and administered to mice 20 min before the operant task. The dose and timing were the same as used in a previous study (Kishimoto and Kano, 2006). For control mice, the PBS solution containing 1% DMSO and 4% Cremophor was administered.

Apparatus

The operant tasks were conducted in operant chambers (OP-3101K, O'HARA & Co. Ltd., Japan). Each of these chambers was placed in a sound-attenuating box. Three levers protruded into the chamber, and the right (A), center (B), and left (C) levers were positioned 2, 4, and 2 cm above the floor, respectively (Fig. 1). The B-lever was set 2 cm higher than the other two so that mice could press the B-lever with a forelimb by standing up on the hind legs. There was no light above each lever, or any other internal cues for lever pressing. The software for the operant task with multiple levers (O'HARA & Co., Ltd.) controlled the operant tasks and collected the data. Food pellets (AIN-76A), used for positive reinforcement, were delivered into a feeding trough located beneath the B-lever. In the operant chamber, water was available *ad libitum*.

Operant tasks

Operant tasks were performed during the dark phase of the cycle. One 60-min training session was conducted each day and five times a week (Monday-Friday), except for the experiments with SR141716A (seven times a week). The three-lever task was preceded by the one-lever task as shaping. In the one-lever task, reinforcement (one 10 mg food pellet) was delivered when the mouse pressed one of active levers (fixed ratio 1, FR1). According to the number of active levers, we set three learning levels. The number of active levers was initially set to three (the first level). At this level, pressing any of the three levers (A-, B-, and C-lever) was rewarded. When the mouse pressed the same lever (e.g. A-lever) more than 100 times per session in two consecutive sessions, the most frequently pressed lever (e.g. A-lever) was inactivated to decrease the number of active levers to two (the second level). At this level, pressing any of the two active levers (e.g. B- and C-lever), but not the inactive lever (e.g. A-lever), was rewarded. When the mouse pressed either of the two active levers more than 100 times per session in two consecutive sessions, the more frequently pressed lever (e.g. B-lever) was inactivated to decrease the number of active levers to one (the third level). At this level, pressing the active lever (e.g. C-lever), but not the inactive levers (e.g. A- and B-lever), was rewarded. The one-lever task was completed when the mouse pressed the last active lever (e.g. C-lever) more than 100 times per session in two consecutive sessions.

The mouse was then trained to press three levers in the order of A, B, and C for a food reward (three-lever task). In this task, we set a time restriction. If either the A-B interval (the time interval between the A-lever press and the B-lever press) or B-C interval was longer than T (Fig. 1C), no food pellet was delivered. The time T was initially set to 99.9 s (the longest T value accepted by the software), switched to 3 s,

and then further decreased in a step-by-step fashion depending on the performance. The time T was changed when the mouse attained more than 100 reinforcements per session in two sessions. For CB_1-KO and their WT littermates, T was decreased from 3 to 1 s in 0.5 s steps, and from 1 to 0.5 s in 0.1 s steps. For DGLα-KO and their WT littermates, T was decreased from 3 to 1 s in 1 s steps.

After the completion of the three-lever task, the order was reversed (reverse three-lever task). The time T was initially set to 99.9 s, switched to 3 s, and then further decreased to 1 s in 0.5 s steps (CB_1-KO and their WT littermates) or 1 s steps (DGLα-KO and their WT littermates) depending on the performance (more than 100 reinforcements per session once for CB_1-KO and their WT littermates or twice for DGLα-KO and their WT littermates).

Data analysis of operant tasks

In each session, we obtained the numbers of A-lever presses (A), B-lever presses (B), C-lever presses (C), the number of lever presses (A+B+C), and reinforcements (R). In the one-lever task, we also calculated the disparity ratio (((A + B + C)/Max - 1)/2) and the inactive lever press ratio (I/(A + B + C)), where Max is the maximum value among A-C and I is the number of inactive lever presses. In the three-lever task and its reverse variation, we analyzed the success rate (R × 3/(A + B + C)), and the number of the following lever press patterns: ABC, ABCABC, CBA, CBACBA. We also calculated the CBA/(ABC+CBA) ratio, the ABCABC/lever-press ratio, and the CBACBA/lever-press ratio, where “lever-press” is the number of lever presses (A+B+C), in each session or in five sessions in total. Data are expressed as mean ± standard error of mean (SEM).

Locomotor activity tests

For open-field test, mice were placed into the center of a square open-field apparatus (40 x 40 cm; Eiko Science, Tokushima, Japan). Movements of the animals were analyzed by an automatic monitoring system (TopScan, CleverSys, Inc., Reston, VA) for 20 min. Horizontal motor activity was evaluated by the distance that the animals traveled. Vertical motor activity was evaluated by the number of rearing events (standing upright on the hind legs).

Spontaneous physical activities in the home-cage environment were analyzed by using essentially the same methods as described in previous studies (Hiasa et al., 2013; Kishimoto et al., 2015). Mice were recorded for 3 h. A camcorder (Panasonic, NV-GS300) was mounted on a tripod that was angled perpendicular to the cage to provide a side view of the cage. The camera footage was transferred to and saved on a computer with the mAgicTV software (I-O DATA). The video data were analyzed using the CleverSys HomeCageScan system (CleverSys Inc., Reston, VA). Spontaneous behaviors such as distance traveled, walking, and rearing were evaluated.

Experimental design and statistical analysis

In the experiments with SR141716A, the performance of the one-lever task was compared between SR141716A-treated mice (n = 8) and control mice (n = 8). The performances during the three types of lever-press tasks (one-lever, three-lever, and reverse three-lever tasks) were compared between CB_1-KO mice (n = 10) and their WT littermates (n = 11), or between DGLα-KO mice (n = 11) and their WT littermates (n = 14). The locomotor activity tests were compared between CB_1-KO mice (n = 10) and their WT littermates (n = 10) and between DGLα-KO mice (n = 10) and their WT littermates (n = 10). Heterozygous mating ($CB_1^{+/-}$ x $CB_1^{+/-}$ or $DGL\alpha^{+/-}$ x $DGL\alpha^{+/-}$) was used to generate knockout and WT littermates, and only male mice were used for the experiments. Behavioral tests were conducted by the experimenters who were blind to

the genotypes of the animals. A previous study showed that a similar group size was sufficient to obtain statistically significant effects of orally administered theobromine by using the same behavioral test ($1 - \beta > 0.8$) (Yoneda et al., 2017).

Statistical significance was evaluated using two-way repeated measures ANOVA, Student's t test, Welch's t test, and Mann-Whitney U test. Differences were regarded as statistically significant when $p < 0.05$. Statistical analysis was performed using the Statcel software and SPSS.

Results

Body weights of CB_1-KO and DGLα-KO mice

It has been reported that mice lacking CB_1 receptors (Cota et al., 2003) or DGLα (Powell et al., 2015) exhibit decreased body weight as compared with WT littermates. In general agreement with the previous reports, 8-week-old CB_1-KO and DGLα-KO mice had a significantly lower body weight than WT mice (Fig. 2A). However, the knockout mice and their WT littermates showed a similar weight gain after the start of the operant tasks (Fig. 2B-D).

Locomotor activity of CB_1-KO and DGLα-KO mice

First, to evaluate the roles of DGLα and CB_1 receptors in the locomotor activity, we analyzed the locomotor behaviors of CB_1-KO and DGLα-KO mice in the novel open-field environment. Both CB_1-KO and DGLα-KO mice exhibited normal horizontal activity, equivalent to that of WT mice (Fig. 3A). However, the vertical activity was significantly lower in both CB_1-KO and DGLα-KO mice, compared to WT mice (Fig. 3B). Next, we analyzed the spontaneous behaviors of CB_1-KO and DGLα-KO mice in the home-cage environment. Using the HomeCageScan software, three separate parameters of animal movement were measured: distance traveled,

walking, and rearing. There were no significant differences in these values between WT and CB_1-KO / DGLα-KO mice (Fig. 3C, D, E).

CB_1-KO mice in the one-lever task

In the one-lever task, the increase in learning level was slower and more variable in CB_1-KO mice than in WT mice (Fig. 4A). The CB_1-KO mice required significantly more sessions to complete the one-lever task (Fig. 4B). Specifically, CB_1-KO mice needed more sessions to complete the first level, in which three levers were active, than WT mice. CB_1-KO mice also needed more sessions to complete the second level (two active levers). In contrast, the number of sessions required to complete the last level (one active lever) was not different between these two groups.

For the completion of the first level, mice had to press the same lever more than 100 times per session, which depended on how often the mice pressed the levers (the number of lever presses) and how exclusively they pressed the same lever (disparity ratio). The disparity ratio was used as an index of the imbalance among the three levers the mice pressed and ranged from 0 to 1. A score of 0 was assigned if the mouse pressed only one lever and a score of 1 was assigned if it pressed the three levers equally. During the first seven sessions, the number of lever presses (A + B + C) increased in both mouse groups. However, the number of lever presses during 4^{th}-7^{th} sessions was significantly lower in CB_1-KO mice (Fig. 4C, top). In contrast, no significant difference was found in the disparity ratio between the two groups (Fig. 4C, bottom). These results indicate that the difference in the number of sessions required for completing the first level (three active levers) between CB_1-KO and WT mice can be accounted for by the difference in the number of lever presses.

For the completion of the second level, mice needed to press either of the two active levers more than 100 times. We analyzed the number of lever presses (Fig. 4D,

top), the disparity ratio (Fig. 4D, bottom), and the inactive lever press ratio (Fig. 4E, top) for the first two sessions after inactivation of one lever. There were no significant differences in these values between the two groups. We also analyzed the inactive lever press ratio in the first two sessions after inactivation of the second lever (the last level), and found no difference between the two groups (Fig. 4E, bottom).

CB_1-KO mice in the three-lever task

For the three-lever task, we analyzed the number of lever presses, the number of ABC patterns, the number of reinforcements, the success rate, the number of ABCABC patterns, and the ABCABC/lever-press ratio in the first five sessions (Fig. 5A). In these sessions, time restriction was mild ($T \geq 3$ s) and the number of ABC patterns and the number of reinforcements were almost the same. There was no significant interaction effect of session and genotype for all the values shown in Fig. 5A, except for the ABCABC/lever-press ratio. A significant main effect of session was observed for all values except the number of lever presses. These values increased during the first five sessions. A significant main effect of genotype was observed for the ABCABC/lever-press ratio during 3^{rd}-5^{th} sessions. Figure 5B shows the results for the first five sessions in total. The ABCABC/lever-press ratio was smaller in the CB_1-KO mice than in the WT mice. These results show that CB_1-KO mice can learn the order of levers, but need more lever-presses for learning. In addition, we found that analyzing the number of ABCABC patterns is helpful to detect even a slight difference in learning.

When the time T was shortened from 1 s to 0.5 s by 0.1 s steps, the number of mice with good performance (the number of reinforcements (R) > 100) decreased gradually in both mouse groups, being 10 and 10 (T = 1 - 0.8 s), 10 and 9 (T = 0.7 s), 9 and 9 (T = 0.6 s), and 6 and 8 (T = 0.5 s) for WT and CB_1-KO mice, respectively.

Moreover, at shorter T the reinforcement/ABC ratio was even higher in CB_1-KO mice than in WT mice ($p < 0.05$, data not shown), indicating that the ability of quick lever pressing is not impaired in CB_1-KO mice.

CB_1-KO mice in the reverse three-lever task

The performance in the reverse three-lever task (CBA) was compared between CB_1-KO and WT mice (Fig. 6). The number of lever presses, the number of ABC patterns, the number of CBA patterns, the CBA/(ABC+CBA) ratio, the number of reinforcements, the success rate, the number of CBACBA patterns, and the CBACBA/lever-press ratio were analyzed for the first five sessions ($T \geq 2$ s). There was no significant interaction effect of session and genotype. A significant main effect of session was observed for all the values shown in Fig. 6A. The number of lever presses and the number of ABC patterns decreased during the first five sessions, whereas the other values increased. A significant main effect of genotype was observed for the number of lever presses. CB_1-KO mice pressed levers more frequently. Figure 6B shows the results for the first five sessions in total. The number of lever presses was larger in the CB_1-KO mice than in the WT mice. Figure 6C shows the number of sessions and the number of lever presses required for reversal learning (CBA>ABC). The number of lever presses was larger in the CB_1-KO mice than in the WT mice. These results show that CB_1-KO mice need more lever-presses for reversal learning of the motor sequence.

DGLα-KO mice in the one-lever, three-lever, and reverse three-lever task

Similar experiments were performed on DGLα-KO mice and their WT littermates. We found that the behavioral phenotypes of DGLα-KO mice in the one-lever task (Fig. 7), the three-lever task (Fig. 8), and the reverse three-lever task (Fig. 9) were essentially similar to those of CB_1-KO mice.

In the one-lever task, DGLα-KO mice exhibited a slower increase in the learning level, and required more sessions to complete the one-lever task. The number of sessions required at the first and third levels were higher in DGLα-KO mice than in WT mice (Fig. 7B). During the first seven sessions, both groups exhibited an increase in the number of lever presses and a decrease in the disparity ratio (Fig. 7C). The number of lever presses was lower in DGLα-KO mice than in WT mice, whereas there was no difference in the disparity ratio between the two groups. For the first two sessions after the inactivation of one lever (the second level), there were no significant differences in the number of lever presses (Fig. 7D, top), the disparity ratio (Fig. 7D, bottom), and the inactive lever press ratio (Fig. 7E, top) between the two groups. In the first two sessions after the second inactivation (the last level), the inactive lever press ratio was not significantly different between the two groups (Fig. 7E, bottom).

In the three-lever task (Fig. 8), there was no significant interaction effect of session and genotype during the first five sessions ($T \geq 2$ s). The number of lever presses decreased, whereas the number of ABC patterns, the number of reinforcements, the success rate, the number of ABCABC patterns, and the ABCABC/lever-press ratio increased. A significant main effect of genotype was observed for the number of ABCABC patterns and the ABCABC/lever-press ratio. These values were lower in the DGLα-KO mice than in the WT mice. These results clearly show that DGLα-KO mice are impaired in the three-lever task.

In the reverse three-lever task (Fig. 9), there was no significant interaction effect of session and genotype for the first five sessions ($T \geq 2$ s). The number of lever presses and the number of ABC patterns decreased, whereas the number of CBA patterns, the CBA/(ABC+CBA) ratio, the number of reinforcements, the success rate, the number of CBACBA patterns, and the CBACBA/lever-press ratio increased. When the performance of DGLα-KO mice during the first five sessions in total was compared

to that of WT mice, the number of lever presses and the number of ABC patterns were higher, whereas the CBA/(ABC+CBA) ratio and the CBACBA/lever-press ratio were lower in the DGLα-KO mice (Fig. 9B). The number of sessions and the number of lever presses required for reversal learning were larger in the DGLα-KO mice (Fig. 9C). These results clearly demonstrate that DGLα-KO mice are impaired in the reversal learning of the motor sequence.

Effects of pharmacological blockade of CB_1 receptors on the one-lever task performance

The results obtained so far strongly suggest that endocannabinoid signaling is involved in reward-based motor learning. However, the observed lower performance might be influenced by potential compensatory or developmental mechanisms of a constitutive lack of CB_1 receptors or DGLα. Therefore, we treated WT mice with the CB_1 receptor antagonist SR141716A, and analyzed their performance during the one-lever task.

The SR141716A-treated mice exhibited a slower increase in the learning level, and required more sessions to complete the one-lever task. The number of sessions required at the first level was higher in SR141716A-treated mice than in control mice (Fig. 10B). During the first seven sessions, both groups exhibited an increase in the number of lever presses and a decrease in the disparity ratio (Fig. 10C). The number of lever presses during 4^{th}-7^{th} sessions was lower in SR141716A-treated mice than in control mice, whereas there was no difference in the disparity ratio between the two groups. For the first two sessions after the inactivation of one lever (the second level), the disparity ratio was lower in SR141716A-treated mice than in control mice (Fig. 10D, bottom), whereas the number of lever presses (Fig. 10D, top) and the inactive lever press ratio (Fig. 7E, top) were not different between the two groups. In the first two sessions after the second inactivation (the last level), the inactive lever press ratio

was also not different between two groups (Fig. 10E, bottom). These results indicate that SR141716A-treated mice and CB_1-KO mice exhibit essentially the same impairment in the one-lever task.

Discussion

In the present study, we examined how the DGLα-CB_1 endocannabinoid system contributes to reward-based learning of a motor sequence by subjecting CB_1-KO mice and DGLα-KO mice sequentially to the one-lever, three-lever (ABC), and reverse three-lever (CBA) tasks. We found that CB_1-KO mice and DGLα-KO mice exhibited similar deficits in these tasks. In the one-lever task, both CB_1-KO mice and DGLα-KO mice showed a delayed increase in the number of lever presses, suggesting a slower rate of learning of the causal link between the action (lever press) and the outcome (food). In the three-lever task (ABC), both strains of knockout mice showed a delayed increase in the ABCABC/lever-press ratio, suggesting a slower rate of sequence learning. In the reverse three-lever task (CBA), both strains of knockout mice needed more lever presses for the shift from the ABC to CBA patterns, showing a slower rate of reversal learning. These findings, taken together, suggest that the 2-AG-CB_1 endocannabinoid signaling facilitates several aspects of reward-based motor learning.

Our data, which showed that CB_1-KO and DGLα-KO mice had a lower body weight than WT mice, are consistent with results previously reported for CB_1-KO mice (Cota et al., 2003; Ravinet Trillou et al., 2004) and DGLα-KO mice (Powell et al., 2015). In the previous studies, the mean body weight of CB_1-KO mice (Cota et al., 2003) and that of DGLα-KO mice (Powell et al., 2015) at eight weeks of age were approximately 91% and 80%, respectively, of that of WT mice. In the present study, the mean body weights of CB_1-KO mice and DGLα-KO mice at eight weeks of age

were 84% and 85%, respectively (Fig. 2A). In addition, our data showed that CB_1-KO and DGLα-KO mice gained weight normally after the operant tasks started, suggesting that feeding behavior is normal in these knockout mice during the behavioral examination.

Several studies indicated that the locomotor activity in CB_1-KO or DGLα-KO mice was lower than that in WT mice (Zimmer et al., 1999; Sugaya et al., 2013), although other studies did not find such a phenomenon (Bilkei-Gorzo et al., 2005; Powell et al., 2015). We confirmed that both CB_1-KO and DGLα-KO mice exhibited nearly normal locomotor activity, but showed a significantly decreased vertical activity in the open-field environment. Our results support the idea that the endocannabinoid system facilitates the exploratory behavior via CB_1 receptor activation (Jacob et al., 2009; Haring et al., 2011; Kishimoto et al., 2015). Therefore, it seems likely that decreased vertical locomotor activity affects the reward-based learning performance, especially in the tasks that require standing up for lever press. If this is the case, lower performance is expected to be observed even in the early phase of the task (1^{st}-3^{rd} sessions, for example). Our data show, however, that the number of lever presses is not different in the early phase of the one-lever task between WT and CB_1-KO mice, and even larger in the three-lever and reverse three-lever task in CB_1-KO mice. Therefore, we conclude that the possibility that low task performance is caused by the decrease in vertical locomotor activity is unlikely.

Several studies reported that CB_1-KO mice press levers less frequently than WT mice in the operant lever press task (Baskfield et al., 2004; Sanchis-Segura et al., 2004; Guegan et al., 2013). The authors suggested that poor motivation for food, motor suppression, and changes in learning and memory may cause the reduced lever press in CB_1-KO mice. In the present study, we observed that CB_1-KO mice pressed levers less frequently in the one-lever task (Fig. 4), which is consistent with the previous studies

suggesting the possibility of poor motivation, motor suppression, or changes in learning and memory. In the three-lever task (Fig. 5) and reverse three-lever task (Fig. 6), however, CB_1-KO mice pressed levers more frequently than WT mice. The experiments with shorter T in the three-lever task also indicated that the ability of quick lever pressing was not impaired in CB_1-KO mice. Therefore, our results cannot be explained simply by poor motivation or motor suppression, at least in the three-lever and reverse three-lever tasks. The most likely explanation of our results is that CB_1-KO mice have learning impairments.

It is important to know whether CB_1-KO and DGLα-KO mice have the same phenotype or have some difference. If the learning impairment is more severe in CB_1-KO mice than in DGLα-KO mice, an involvement of anandamide or some other endocannabinoids is expected. If it is more severe in DGLα-KO mice, an involvement of CB_2 receptors or some other cannabinoid receptors is expected. Although they are different strains and a slightly different protocol was used for the two strains, we tried to compare WT from both strains. We checked the number of sessions required to complete one-lever task, the number of lever presses, the ABCABC/lever-press ratio, the CBACBA/lever-press ratio, the number of lever presses required for reversal learning in three-lever and reverse three-lever tasks. We found no significant difference between WT from both strains. Then, we compared CB_1-KO and DGLα-KO mice and found no significant difference between the two strains of knockout mice. However, we still cannot entirely exclude the possibility of subtle difference between CB_1-KO and DGLα-KO mice, because we used a slightly different protocol.

The mechanisms underlying the phenotypes of the CB_1-KO and DGLα-KO mice remain to be elucidated. One possibility is that the lack of endocannabinoid signaling affects brain development and results in morphological or functional abnormality. Indeed, previous studies have demonstrated that CB_1-KO mice exhibit a

reduction in both apical dendritic length and branch points of neurons within layer II/III of the prefrontal cortex (Hill et al., 2011; Lee et al., 2014). Since the prefrontal cortex is involved in cognitive flexibility (Park and Moghaddam, 2017), it is possible that the behavioral phenotypes of CB_1-KO mice might be partially attributable to the morphological changes in the prefrontal cortex. However, our data from the experiments with SR141716A confirmed that SR141716A-treated mice and CB_1-KO mice exhibit a similar behavioral phenotype in the one-lever task, indicating that the behavioral phenotype of CB_1-KO mice in the one-lever task is independent of morphological change. Another possibility is that endocannabinoid-mediated synaptic plasticity is involved in reward-based motor learning. The basal ganglia are involved in reward-based motor learning (Haber, 2016), and they abundantly express CB_1 receptors. In the striatum, endocannabinoid-mediated synaptic plasticity has been reported at excitatory glutamatergic synapses and inhibitory GABAergic synapses (Goodman and Packard, 2015; Augustin and Lovinger, 2018; Xu et al., 2018). Thus, it is possible that the behavioral phenotypes of CB_1-KO and DGLα-KO mice are caused by the deficit of endocannabinoid-mediated synaptic plasticity in the striatum or some other brain areas of the cortico-basal ganglia loops. It is also possible that the endocannabinoid system influences not only the glutamatergic and GABAergic systems, but also the dopaminergic system (El Khoury et al., 2012; Wenzel and Cheer, 2014). Motor learning depends on the cortico-basal ganglia circuit. The endocannabinoid system functions in both the basal ganglia and the cortex. In which brain region, the basal ganglia or the cortex, this endocannabinoid system is more critical for motor learning still remains to be determined.

This study demonstrated that the 2-AG to CB_1 endocannabinoid signaling is involved in motor learning. However, the interpretation of these results in terms of when, where, and how endocannabinoids contribute to motor learning, and specifically

the role of the 2-AG to CB_1 signaling, is limited by the nature of constitutive knockout mice. Further studies using conditional knockout mice would be necessary to elucidate precise mechanisms of action.

The endocannabinoid system is involved in various brain functions. 2-AG, a major endocannabinoid produced by DGLα, is released from postsynaptic neurons, activates presynaptic CB_1 cannabinoid receptors, and induces synaptic plasticity. In the present study, we aimed to elucidate possible roles of the endocannabinoid system in reward-based motor learning. We used CB_1-KO and DGLα-KO mice and examined their performances during three types of operant lever-press tasks. Our data show that CB_1-KO and DGLα-KO mice have similar behavioral phenotypes, which are characterized by significant impairments in action-outcome learning, sequence learning, and reversal learning. These results indicate that the 2-AG to CB_1 endocannabinoid signaling is critically involved in multiple processes of reward-based motor learning.

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Figure Legends

Figure 1. Experimental set-up for the three-lever operant task. **A**: An illustration of an operant box containing three levers, a water bottle, and a feeding trough, and its connection to a diet feeder and a personal computer. **B**: A photograph of the operant test panel containing three levers (A, B, and C) and a feeding trough. **C**: A schematic illustration of lever signal, showing when and which lever is pressed (p) and released (r). Different lever produces different size of signal. Horizontal and vertical axes indicate time and signal size, respectively. A-B interval is the time between pressing A-lever and B-lever, and B-C interval is the time between pressing B-lever and C-lever.

Figure 2. Body weights of CB_1-KO and DGLα-KO mice. **A:** Comparisons of body weights between WT (left open columns, n = 10) and CB_1-KO mice (left closed column, n = 11) (t-test, $p < 0.05$), and between WT (right open columns, n = 14) and DGLα-KO mice (right closed column, n = 11) ($p < 0.05$) at eight weeks of age. **B:** Comparisons of weight gain between WT and CB_1-KO mice (from 8 to 17 weeks, $p = 0.51$), and between WT and DGLα-KO mice (from 8 to 13 weeks, $p = 0.17$). **C, D**: Time courses of weight gain for WT (open circles) and CB_1-KO mice (**C**) (two-way ANOVA, interaction $p = 0.65$, session $p < 0.001$, genotype $p < 0.01$) or DGLα-KO mice (**D**) (interaction $p = 0.24$, session $p < 0.001$, genotype $p < 0.05$). *$p < 0.05$, **$p < 0.01$.

Figure 3. Locomotor activities of CB_1-KO and DGLα-KO mice in the open-field (**A-B**) and home-cage environments (**C-E**). Averaged data obtained from 10 WT (left open columns) and 10 CB_1-KO mice (left closed columns), or 10 WT (right open columns) and 10 DGLα-KO mice (right closed columns) were compared. **A**:

Comparisons of horizontal activity between WT and CB_1-KO mice (t-test, $p = 0.65$), and between WT and DGLα-KO mice ($p = 0.58$). **B**: Comparisons of vertical activity between WT and CB_1-KO mice ($p < 0.05$), and between WT and DGLα-KO mice ($p < 0.05$). **C:** Comparisons of distance traveled between WT and CB_1-KO mice ($p = 0.36$), and between WT and DGLα-KO mice ($p = 0.54$). **D**: Comparisons of walking between WT and CB_1-KO mice ($p = 0.65$), and between WT and DGLα-KO mice ($p = 0.39$). **E**: Comparisons of rearing between WT and CB_1-KO mice ($p = 0.09$), and between WT and DGLα-KO mice ($p = 0.10$). *$p < 0.05$.

Figure 4. Comparison of the performance during the one-lever task between WT and CB_1-KO mice. Individual (**A**) and averaged (**B**-**E**) data obtained from 11 WT (open circles and columns) and 10 CB_1-KO mice (closed circles and columns). **A**: The learning level is plotted against session number. **B**: The number of sessions required at the first (Mann-Whitney test, $p < 0.01$), second ($p < 0.05$), and third ($p = 0.92$) levels, and the total number of sessions ($p < 0.001$, effect size = 2.29, $1 - \beta = 0.998$). **C**: The number of lever presses (two-way ANOVA, interaction $p < 0.05$; for 1^{st}-3^{rd} sessions, interaction $p = 0.47$, session $p < 0.001$, genotype $p = 0.47$; for 4^{th}-7^{th} sessions, interaction $p = 0.98$, session $p < 0.05$, genotype $p < 0.001$) and disparity ratio (interaction $p = 0.76$, session $p < 0.05$, genotype $p = 0.90$) plotted as a function of session number during the first seven sessions. **D**: The number of lever presses (interaction $p = 0.65$, session $p = 0.09$, genotype $p = 0.21$) and disparity ratio (interaction $p = 0.09$, session $p < 0.05$, genotype $p = 0.36$) in the first and second sessions after inactivation of one lever (the second level). **E**: Inactive lever press ratio in the first and second sessions after inactivation of one lever (the second level) (top, interaction $p = 0.98$, session $p < 0.001$, genotype $p = 0.11$), or after the second inactivation (the third level) (bottom, interaction $p = 0.36$, session $p < 0.001$, genotype

p = 0.41). *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 5. Comparison of the performance during the three-lever task between WT and CB_1-KO mice. Averaged data for each session (**A**) or for the first five sessions (**B**) obtained from 11 WT (open circles and columns) and 10 CB_1-KO mice (closed circles and columns) during the first five sessions. **A**: The number of lever presses (two-way ANOVA, interaction p = 0.48, session p = 0.25, genotype p = 0.15), the number of ABC patterns (interaction p = 0.11, session p < 0.001, genotype p = 0.94), the number of reinforcements (interaction p = 0.20, session p <0.001, genotype p = 0.94), the success rate (interaction p = 0.12, session p < 0.001, genotype p = 0.46), the number of ABCABC patterns (interaction p = 0.11, session p < 0.001, genotype p = 0.18), and the ABCABC/lever-press ratio (interaction p < 0.05; for 3^{rd}-5^{th} sessions, interaction p = 0.30, session p < 0.05, genotype p < 0.05) are plotted against session number. **B**: Each mouse's results in the first five sessions were summated and the total numbers (or their ratio values) were averaged and compared between WT and CB_1-KO mice (t-test, lever press p = 0.15, ABC p = 0.94, ABCABC p = 0.18, ABCABC/lever-press p < 0.05, effect size = 1.02, $1 - \beta = 0.60$). Each averaged value was normalized to that of WT mice. *p < 0.05.

Figure 6. Comparison of the performance during the reverse three-lever task between WT and CB_1-KO mice. Averaged data for each session (**A**) or for the first several sessions (**B-C**) obtained from 11 WT (open circles and columns) and 10 CB_1-KO mice (closed circles and columns). **A**: The number of lever presses (two-way ANOVA, interaction p = 0.16, session p < 0.001, genotype p < 0.05), the number of ABC patterns (interaction p = 0.29, session p < 0.001, genotype p = 0.08), the number of CBA patterns (interaction p = 0.54, session p < 0.05, genotype p = 0.44), the

CBA/(ABC+CBA) ratio (interaction $p = 0.73$, session $p < 0.001$, genotype $p = 0.54$), the number of reinforcements (interaction $p = 0.52$, session $p < 0.05$, genotype $p = 0.48$), the success rate (interaction $p = 0.33$, session $p < 0.001$, genotype $p = 0.18$), the number of CBACBA patterns (interaction $p = 0.12$, session $p < 0.001$, genotype $p = 0.17$), and the CBACBA/lever-press ratio (interaction $p = 0.11$, session $p < 0.001$, genotype $p = 0.13$) are plotted against session number. **B**: Each mouse's results in the first five sessions were summated and the total numbers (or their ratio values) were averaged and compared between WT and CB_1-KO mice (t-test, lever press $p < 0.05$, ABC $p = 0.08$, CBA $p = 0.44$, CBA/(ABC+CBA) $p = 0.25$, CBACBA $p = 0.17$, CBACBA/lever-press $p = 0.10$). Each averaged value was normalized to that of WT mice. **C**: The number of sessions and the number of lever presses required for reversal learning (shift from ABC to CBA (CBA > ABC) pattern) were also calculated in each mouse, averaged and compared between WT and CB_1-KO mice (session, Mann-Whitney test, $p = 0.33$; lever press, t-test, $p < 0.05$, effect size = 0.91, $1 - \beta = 0.51$). Each averaged value was normalized to that of WT mice. *$p < 0.05$.

Figure 7. Comparison of the performance during the one-lever task between WT and DGLα-KO mice. Individual (**A**) and averaged (**B**-**E**) data obtained from 14 WT (open circles and columns) and 11 DGLα-KO mice (closed circles and columns). **A**: The learning level is plotted against session number. **B**: The number of sessions required at the first (Mann-Whitney test, $p < 0.05$), second ($p = 0.24$), and third ($p < 0.05$) levels, and the total number of sessions ($p < 0.05$, effect size = 0.98, $1 - \beta = 0.62$). **C**: The number of lever presses (two-way ANOVA, interaction $p = 0.13$, session $p < 0.001$, genotype $p < 0.05$) and disparity ratio (interaction $p = 0.75$, session $p < 0.001$, genotype $p = 0.27$) are plotted against session number during the first seven sessions. **D**: The number of lever presses (interaction $p < 0.01$; the first session, genotype $p =$

0.07; the second session, genotype p = 0.06) and disparity ratio (interaction p = 0.27, session p = 0.35, genotype p = 0.54) in the first and second sessions after inactivation of one lever (the second level). **E**: Inactive lever press ratio in the first and second sessions after inactivation of one lever (the second level) (top, interaction p = 0.25, session p < 0.001, genotype p = 0.42), or after the second inactivation (the third level) (bottom, interaction p = 0.99, session p < 0.001, genotype p = 0.10). *p < 0.05.

Figure 8. Comparison of the performance during the three-lever task between WT and DGLα-KO mice. Averaged data for each session (**A**) or for the first five sessions (**B**) obtained from 14 WT (open circles and columns) and 11 DGLα-KO mice (closed circles and columns) during the first five sessions. **A**: The number of lever presses (two-way ANOVA, interaction p = 0.81, session p < 0.01, genotype p = 0.95), the number of ABC patterns (interaction p = 0.50, session p < 0.001, genotype p = 0.20), the number of reinforcements (interaction p = 0.44, session p < 0.001, genotype p = 0.19), the success rate (interaction p = 0.07, session p < 0.001, genotype p = 0.15), the number of ABCABC patterns (interaction p = 0.07, session p < 0.001, genotype p < 0.05), and the ABCABC/lever-press ratio (interaction p = 0.08, session p <0.001, genotype p < 0.05) are plotted against session number. **B**: Each mouse's results in the first five sessions were summated and the total numbers (or their ratio values) were averaged and compared between WT and DGLα-KO mice (t-test, lever press p = 0.95, ABC p = 0.20, ABCABC p < 0.05, ABCABC/lever-press p < 0.05, effect size = 0.99, 1 – β = 0.65). Each averaged value was normalized to that of WT mice. *p < 0.05.

Figure 9. Comparison of the performance during the reverse three-lever task between WT and DGLα-KO mice. Averaged data for each session (**A**) or for the first several sessions (**B-C**) obtained from 14 WT (open circles and columns) and 11 DGLα-KO

mice (closed circles and columns). **A**: The number of lever presses (two-way ANOVA, interaction $p = 0.11$, session $p < 0.001$, genotype $p < 0.01$), the number of ABC patterns (interaction $p = 0.07$, session $p < 0.001$, genotype $p < 0.01$), the number of CBA patterns (interaction $p = 0.98$, session $p < 0.01$, genotype $p = 0.13$), the CBA/(ABC+CBA) ratio (interaction $p = 0.13$, session $p < 0.001$, genotype $p < 0.05$), the number of reinforcements (interaction $p = 0.94$, session $p < 0.05$, genotype $p = 0.16$), the success rate (interaction $p = 0.22$, session $p < 0.001$, genotype $p < 0.05$), the number of CBACBA patterns (interaction $p = 0.35$, session $p < 0.001$, genotype $p = 0.053$), and the CBACBA/lever-press ratio (interaction $p = 0.30$, session $p < 0.001$, genotype $p = 0.07$) are plotted against session number. **B**: Each mouse's results in the first five sessions were summated and the total numbers (or their ratio values) were averaged and compared between WT and DGLα-KO mice (t-test, lever press $p < 0.01$, ABC $p < 0.01$, CBA $p = 0.13$, CBA/(ABC+CBA) $p < 0.01$, CBACBA $p = 0.053$, CBACBA/lever-press $p < 0.05$). Each averaged value was normalized to that of WT mice. **C**: The number of sessions and the number of lever presses required for reversal learning (shift from ABC to CBA (CBA > ABC) pattern) were also calculated in each mouse, averaged and compared between WT and DGLα-KO mice (session, Mann-Whitney test, $p < 0.001$; lever press, t-test, $p < 0.01$, effect size = 1.47, $1 - \beta = 0.94$). Each averaged value was normalized to that of WT mice. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Figure 10. Comparison of the performance during the one-lever task between vehicle-treated control and SR141716A-treated mice. Individual (**A**) and averaged (**B**-**E**) data obtained from 8 control (open circles and columns) and 8 SR141716A-treated mice (closed circles and columns). **A**: The learning level is plotted against session number. **B**: The number of sessions required at the first (Mann-Whitney

U test, $p < 0.01$), second ($p = 0.32$), and third ($p = 0.32$) levels, and the total number of sessions ($p < 0.01$). **C**: The number of lever presses (two-way ANOVA, interaction $p < 0.01$; for 1^{st}-3^{rd} sessions, interaction $p < 0.01$; for 4^{th}-7^{th} sessions, interaction $p = 0.27$, session $p < 0.001$, group $p < 0.001$) and disparity ratio (interaction $p = 0.32$, session $p < 0.05$, group $p = 0.24$) plotted as a function of session number during the first seven sessions. **D**: The number of lever presses (interaction $p = 0.75$, session $p < 0.05$, group $p = 0.78$) and disparity ratio (interaction $p = 0.38$, session $p < 0.01$, group $p < 0.001$) in the first and second sessions after inactivation of one lever (the second level). **E**: Inactive lever press ratio in the first and second sessions after inactivation of one lever (the second level) (top, interaction $p = 0.15$, session $p < 0.001$, group $p = 0.30$), or after the second inactivation (the third level) (bottom, interaction $p = 0.27$, session $p < 0.001$, group $p = 0.48$). $^{**}p < 0.01$, $^{***}p < 0.001$.

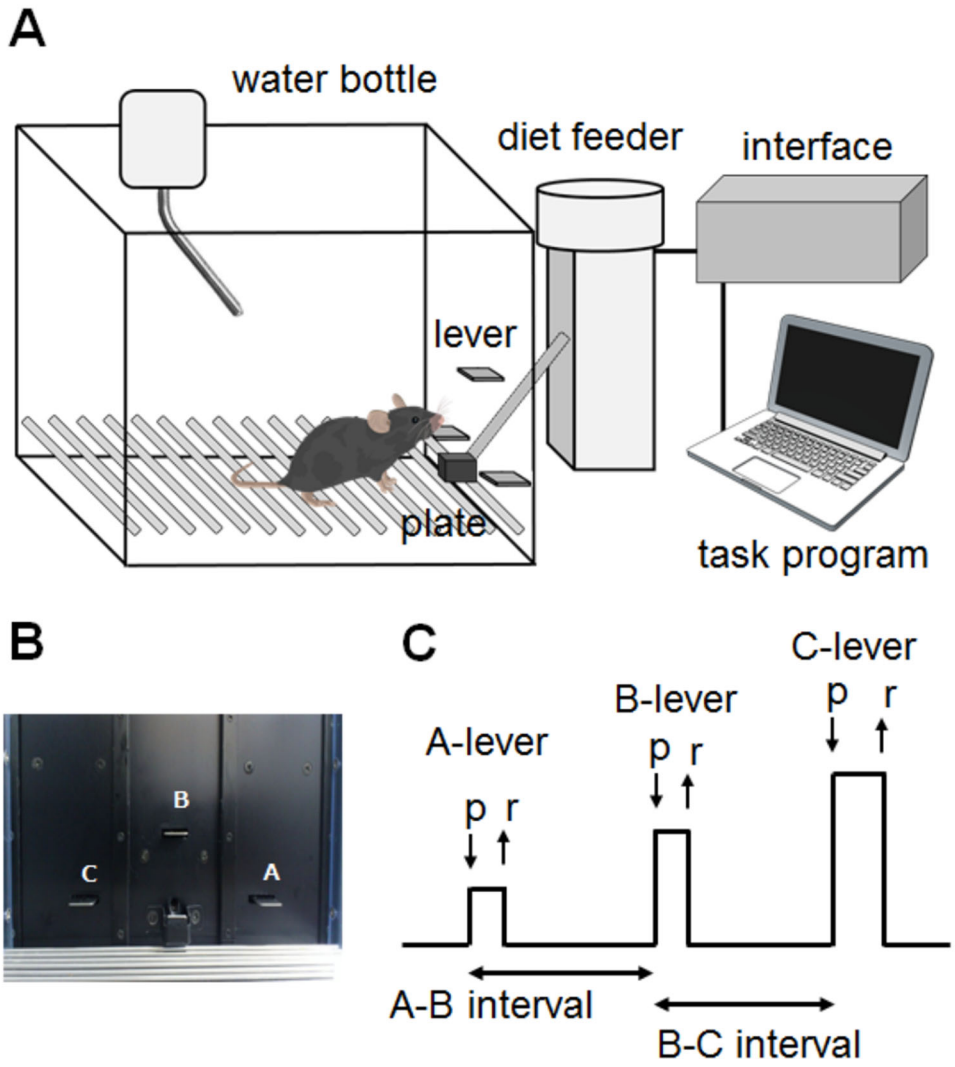
A
water bottle
diet feeder
interface
lever
plate
task program
B
C
B
C
A
C-lever
B-lever
A-lever
p r
p r
p r
A-B interval
B-C interval

Figure 1.

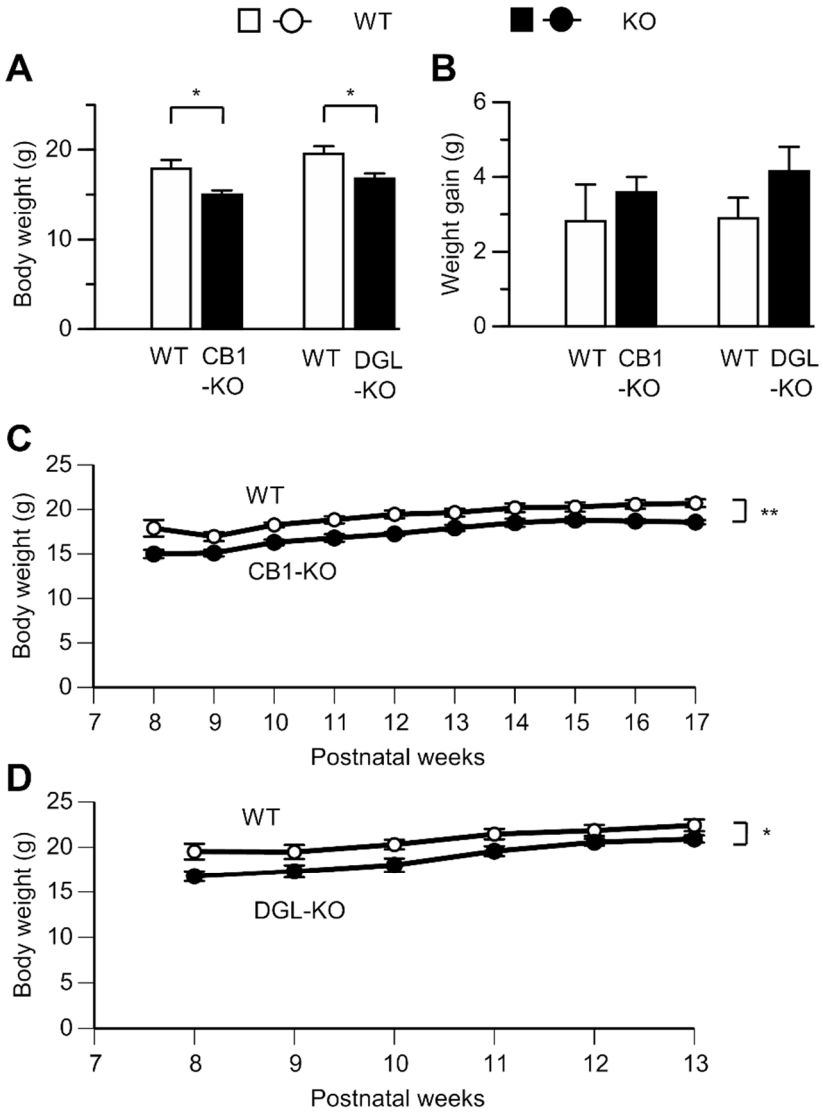
WT
KO
A
Body weight (g)
0
10
20
*
*
WT
CB1
-KO
WT
DGL
-KO
B
Weight gain (g)
0
2
4
6
WT
CB1
-KO
WT
DGL
-KO
C
Body weight (g)
0
5
10
15
20
25
WT
CB1-KO
**
7
8
9
10
11
12
13
14
15
16
17
Postnatal weeks
D
Body weight (g)
0
5
10
15
20
25
WT
DGL-KO
*
7
8
9
10
11
12
13
Postnatal weeks

Figure 2.

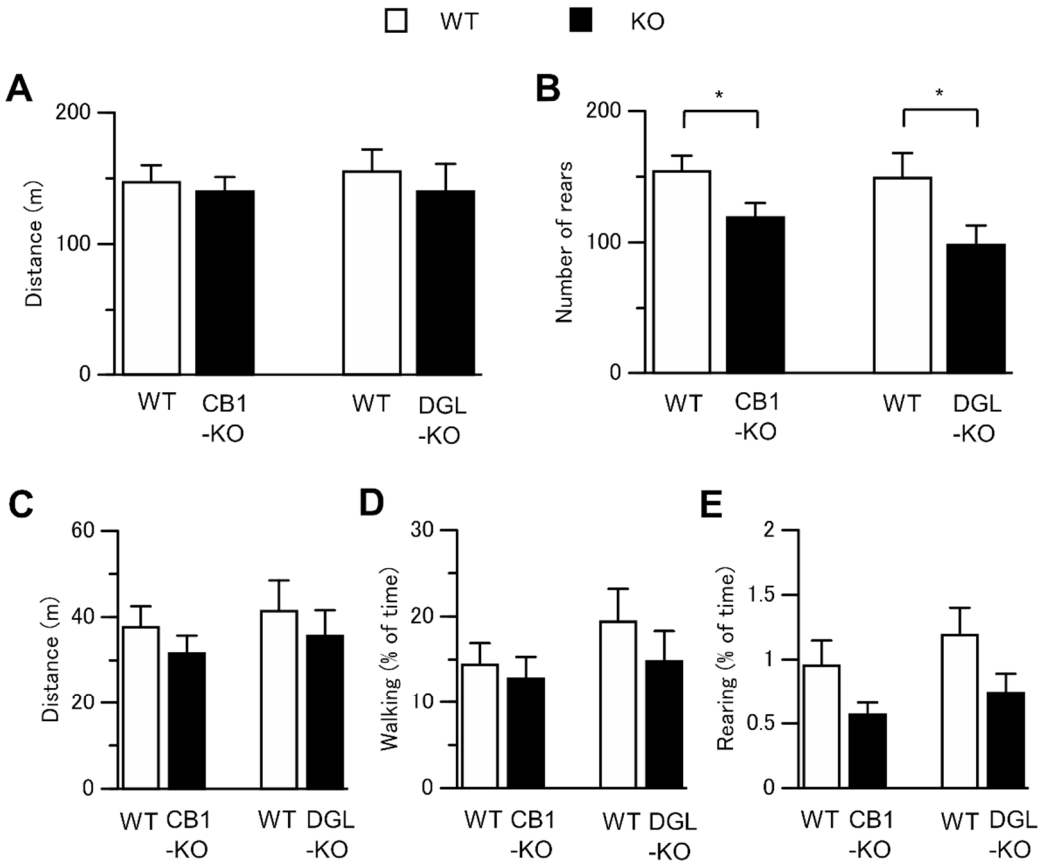
WT
KO
A
Distance (m)
200
100
0
WT
CB1
-KO
WT
DGL
-KO
B
Number of rears
*
*
C
Distance (m)
60
40
20
D
Walking (% of time)
30
10
E
Rearing (% of time)
2
1.5
1
0.5

Figure 3.

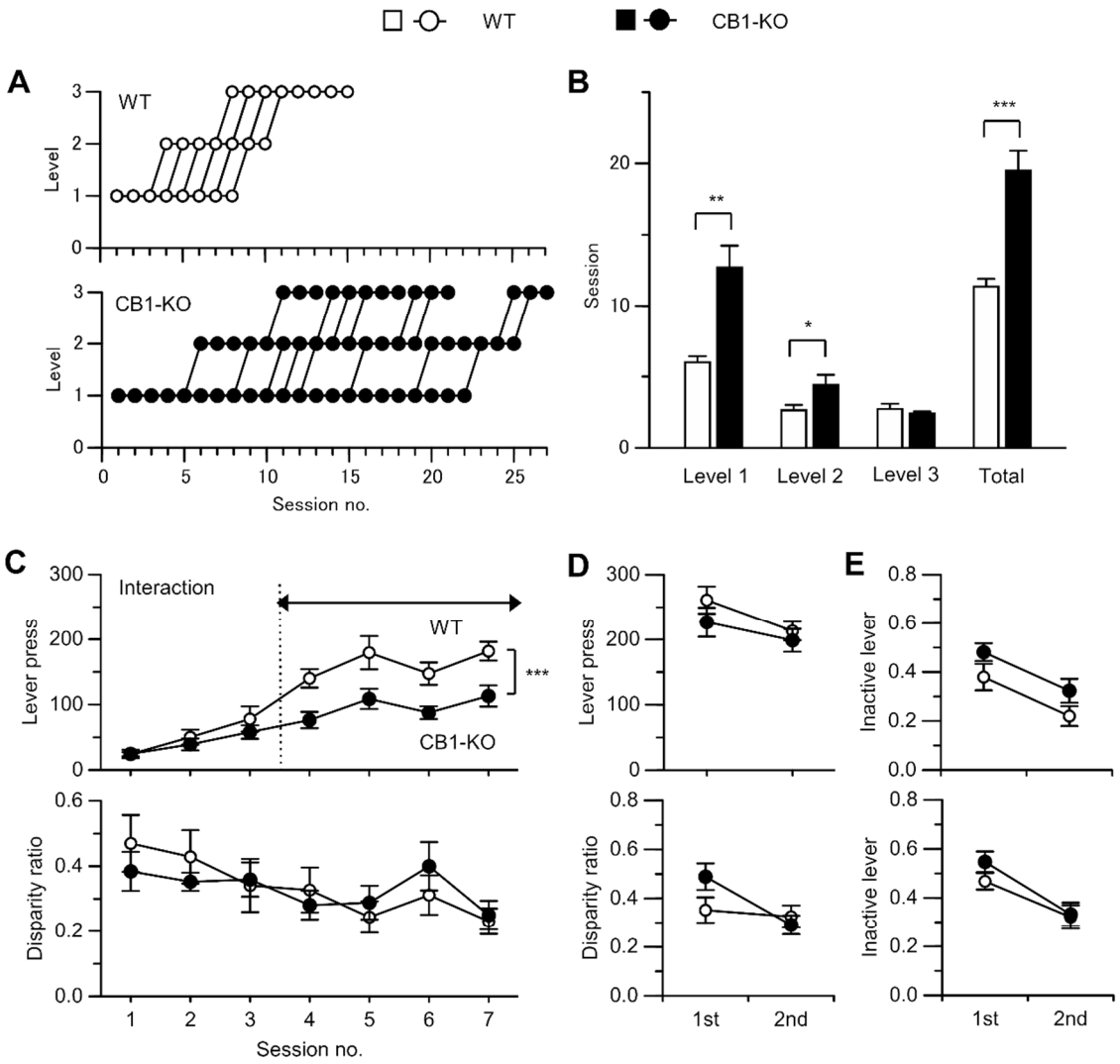
WT
CB1-KO
A
WT
CB1-KO
Level
Session no.
B
Session
Level 1
Level 2
Level 3
Total
**
*
***
C
Interaction
WT
CB1-KO
***
Lever press
Disparity ratio
Session no.
D
Lever press
Disparity ratio
1st
2nd
E
Inactive lever
Inactive lever
1st
2nd

Figure 4.

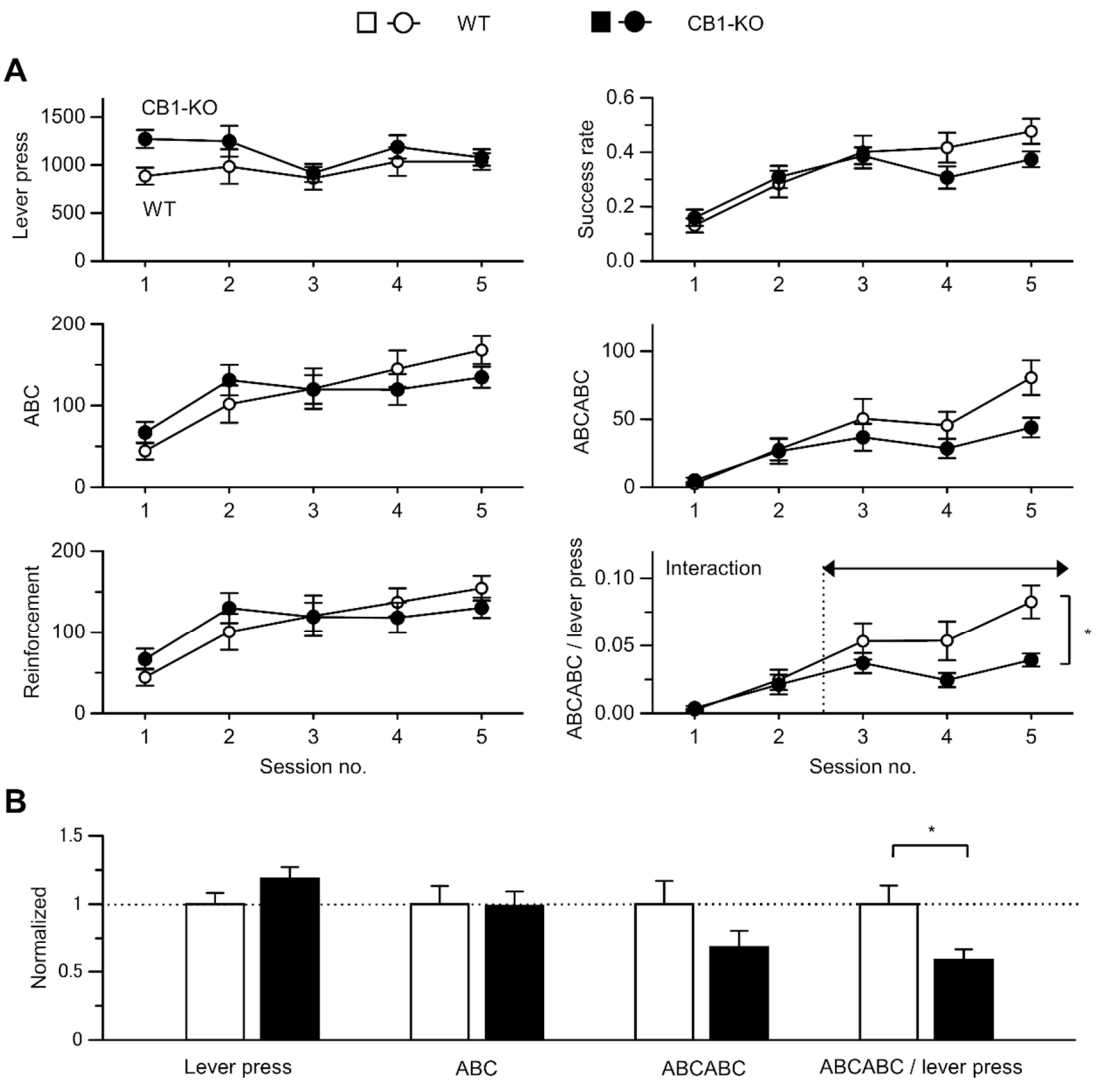
WT
CB1-KO
A
CB1-KO
WT
Lever press
Success rate
ABC
ABCABC
Reinforcement
ABCABC / lever press
Interaction
Session no.
Session no.
B
Normalized
Lever press
ABC
ABCABC
ABCABC / lever press

Figure 5.

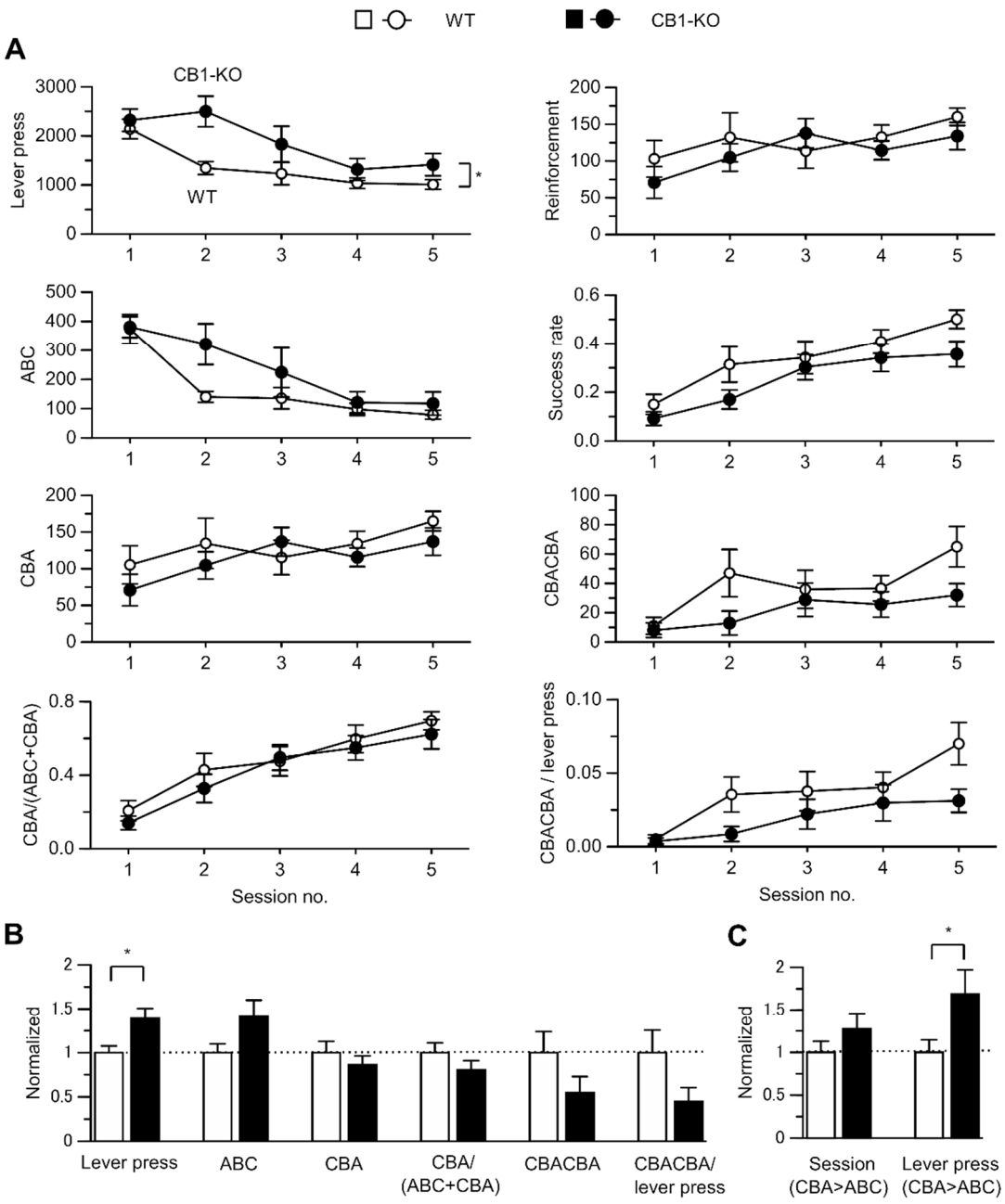
WT
CB1-KO
A
CB1-KO
WT
Lever press
Reinforcement
ABC
Success rate
CBA
CBACBA
CBA/(ABC+CBA)
CBACBA / lever press
Session no.
Session no.
B
Normalized
Lever press
ABC
CBA
CBA/ (ABC+CBA)
CBACBA
CBACBA/ lever press
C
Normalized
Session (CBA>ABC)
Lever press (CBA>ABC)

Figure 6.

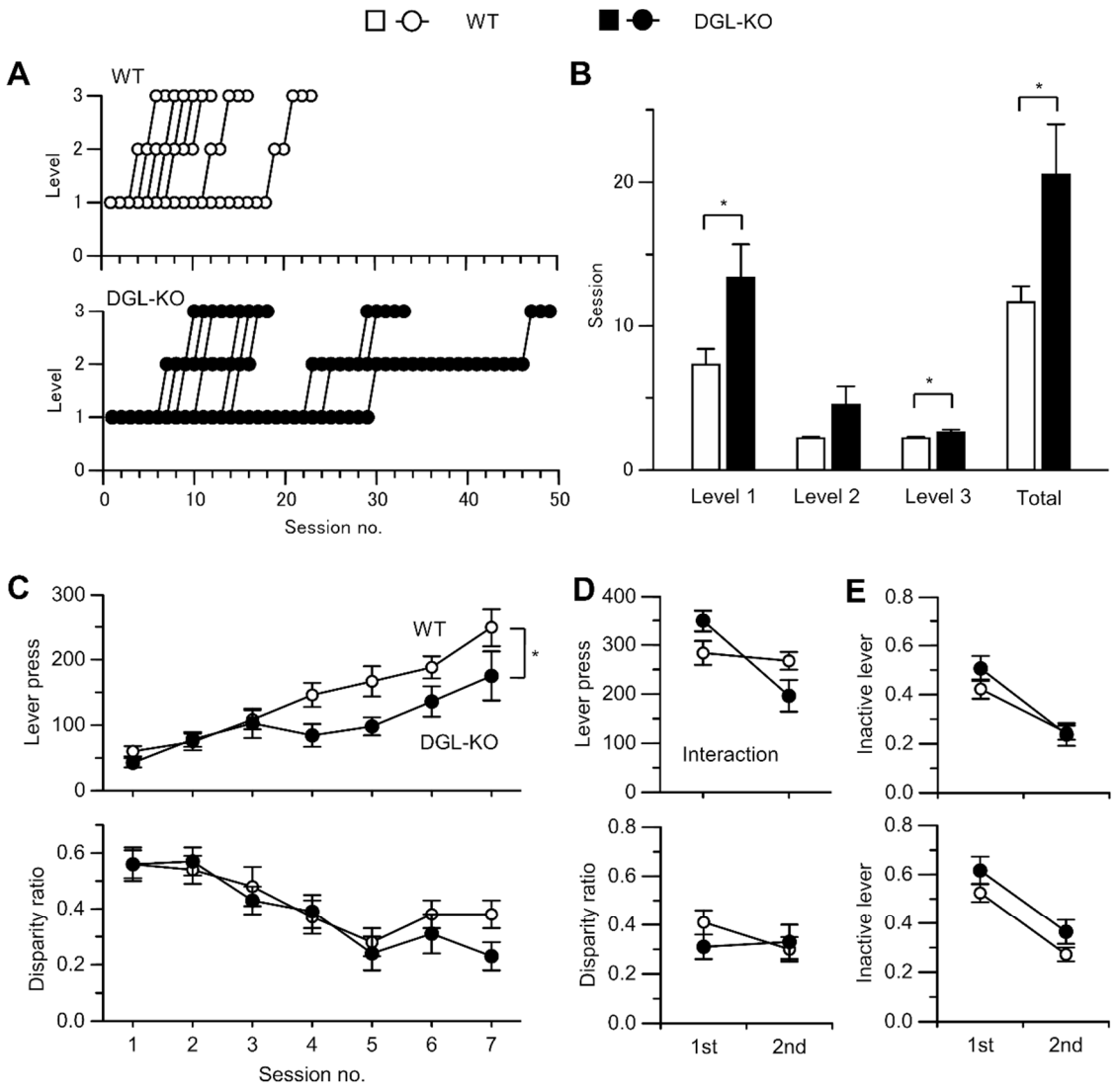
WT
DGL-KO
A
WT
DGL-KO
Level
Session no.
B
Session
Level 1
Level 2
Level 3
Total
C
WT
DGL-KO
Lever press
Disparity ratio
Session no.
D
Lever press
Interaction
Disparity ratio
1st
2nd
E
Inactive lever
Inactive lever
1st
2nd

Figure 7.

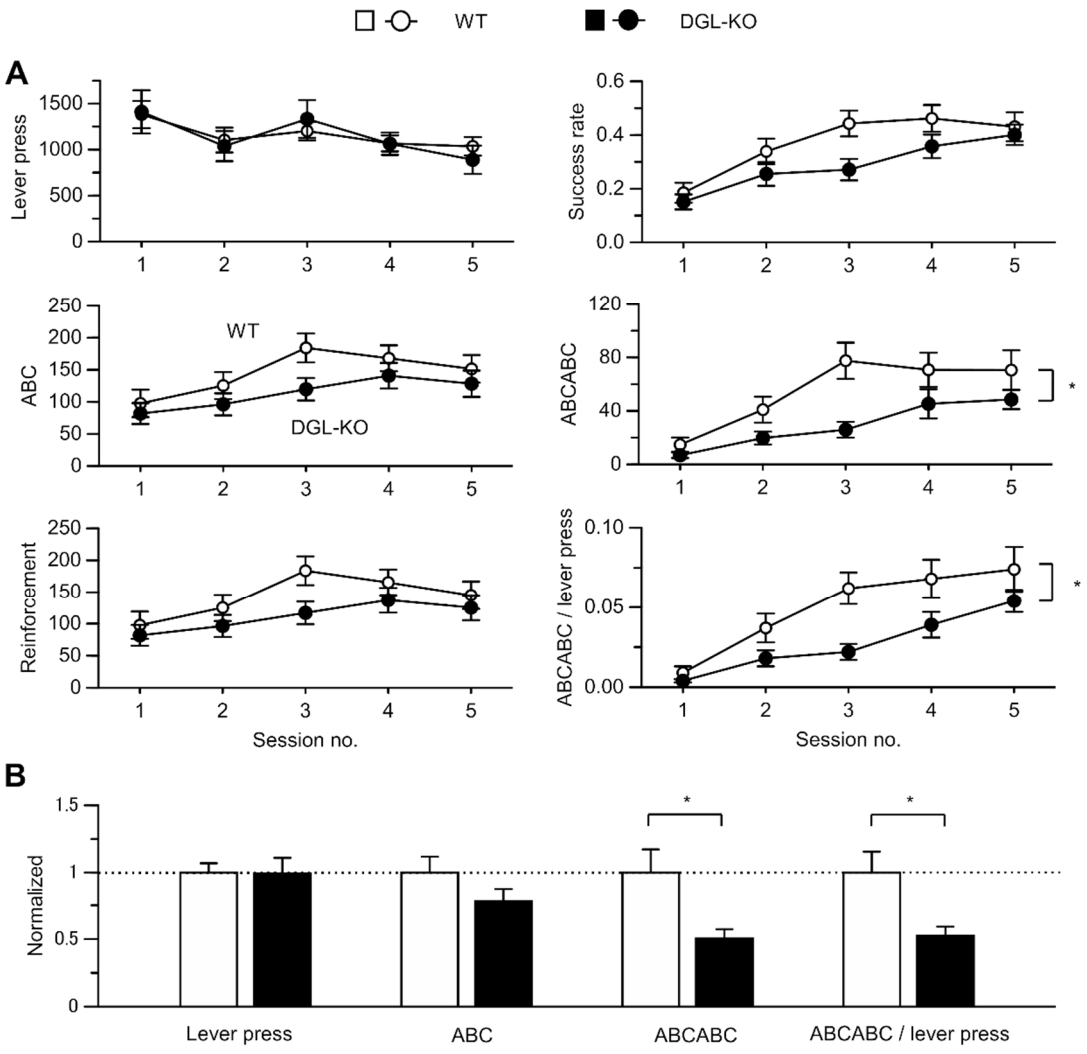
WT
DGL-KO
A
Lever press
Success rate
ABC
WT
DGL-KO
ABCABC
Reinforcement
ABCABC / lever press
Session no.
Session no.
B
Normalized
Lever press
ABC
ABCABC
ABCABC / lever press

Figure 8.

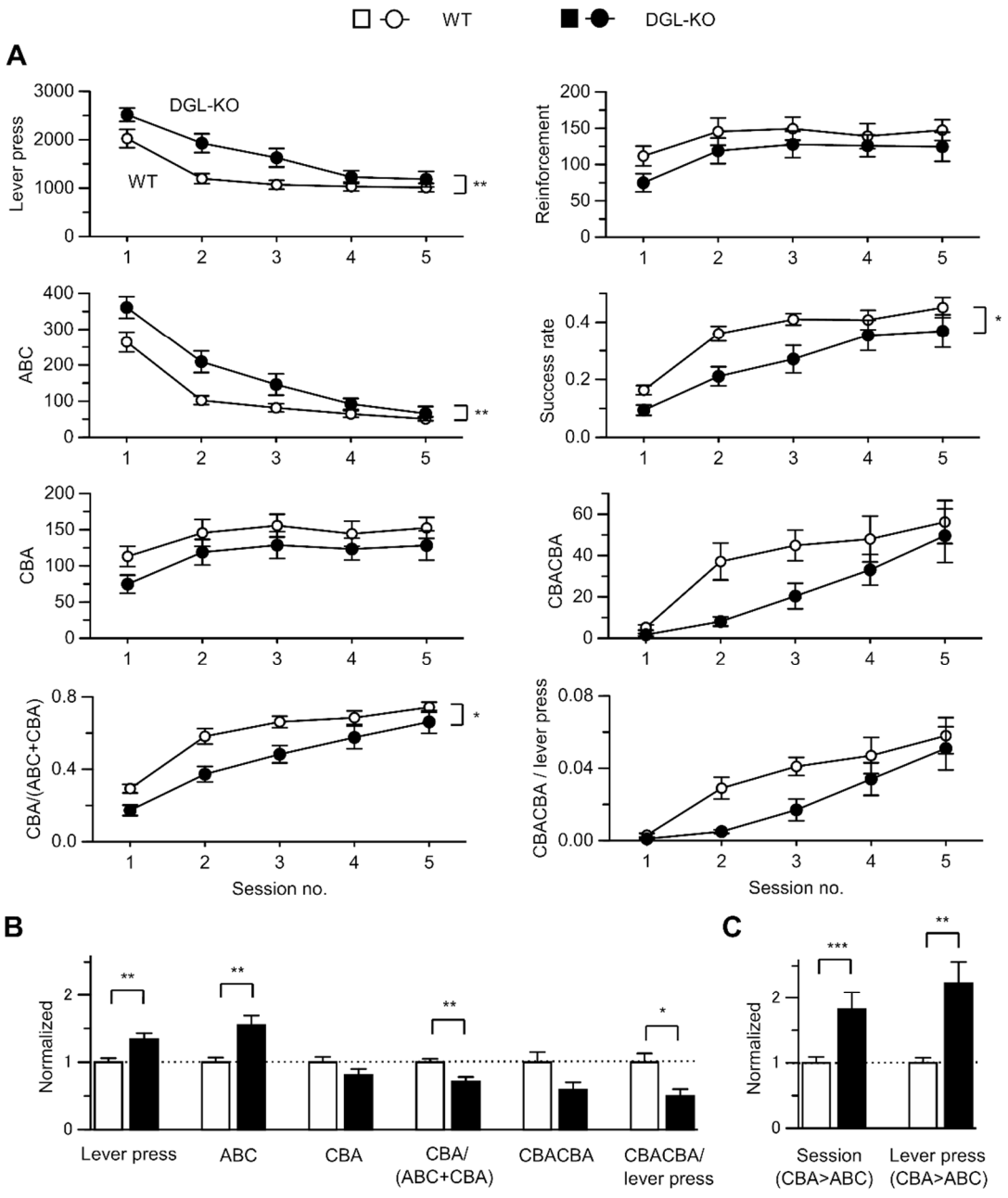
WT
DGL-KO
A
Lever press
DGL-KO
WT
Reinforcement
ABC
Success rate
CBA
CBACBA
CBA/(ABC+CBA)
CBACBA / lever press
Session no.
Session no.
B
Normalized
Lever press
ABC
CBA
CBA/ (ABC+CBA)
CBACBA
CBACBA/ lever press
C
Normalized
Session (CBA>ABC)
Lever press (CBA>ABC)

Figure 9.

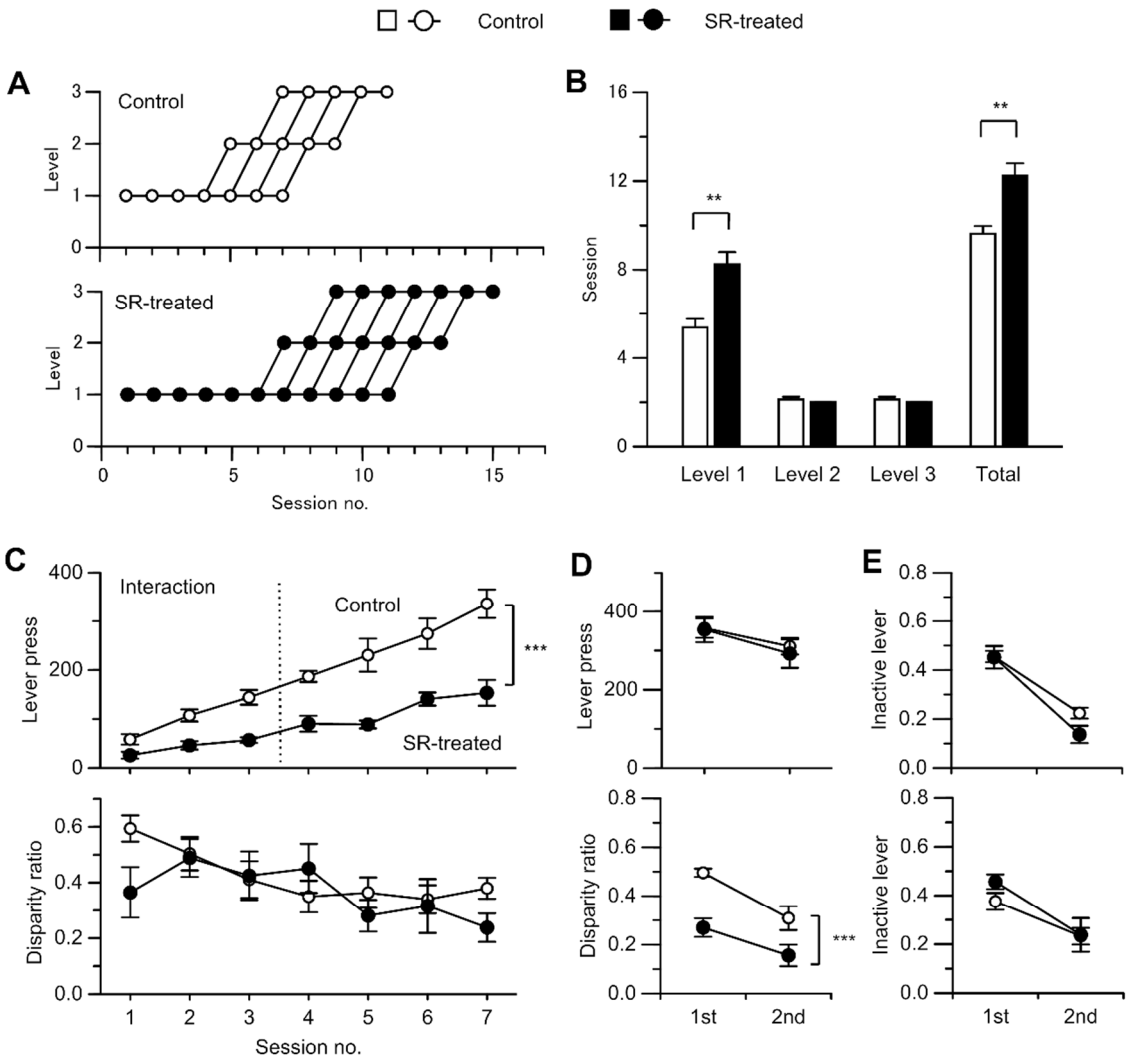
Control
SR-treated
A
Control
SR-treated
Level
Session no.
B
Session
Level 1
Level 2
Level 3
Total
**
C
Interaction
Lever press
Disparity ratio
Session no.
***
D
E
Inactive lever
1st
2nd

Figure 10.