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Title	Behavioral characteristics of 5-HT2C receptor knockout mice : Locomotor activity, anxiety-, and fear memory-related behaviors
Author(s)	Nebuka, Mao; Ohmura, Yu; Izawa, Shuntaro et al.
Citation	Behavioural brain research, 379, 112394 https://doi.org/10.1016/j.bbr.2019.112394
Issue Date	2020-02-03
Doc URL	https://hdl.handle.net/2115/86505
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Type	journal article
File Information	Behav Brain Res 379 112394.pdf

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Behavioral Characteristics of 5-HT_{2C} Receptor Knockout Mice: Locomotor activity, Anxiety-, and Fear Memory-related Behaviors

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Abstract (249/250 words)

Pharmacological studies have suggested that the serotonin 5-HT_{2C} receptor is involved in locomotor activity, anxiety, and fear memory. However, the results of locomotor activity and anxiety in 5-HT_{2C} receptor knockout mice have been mixed, and the effects of 5-HT_{2C} receptor knockout on contextual fear memory have not yet been addressed. In the present study, we reconcile these inconsistent results by analyzing behavioral data in detail and by examining the effects of 5-HT_{2C} receptor knockout on contextual fear memory. We demonstrated that the higher locomotor activity in 5-HT_{2C} receptor knockout mice was observed only in the late phase of the test, indicating that the analyses in the previous study using the total locomotor activity would lead to variable results. Moreover, by analyzing mouse behavior in detail, we found that 5-HT_{2C} receptor knockout mice displayed a hesitating attitude by staying in the central area as well as risk assessment behavior in the elevated plus-maze test. However, the time spent in the open arms was longer in 5-HT_{2C} receptor knockout mice than in wild-type littermates when a zero-maze test lacking the central area was used. In the contextual fear conditioning test, 5-HT_{2C} receptor knockout mice showed rapid within-session extinction of fear, but not between-session extinction, compared with wild-type littermates. However, this remains inconclusive because the facilitation of extinction might be confounded with higher locomotor activity in 5-HT_{2C} receptor knockout mice. Taken together,

the present results provide reasonable explanations about previous inconsistent findings and partially filled the gaps between pharmacological and genetic findings.

Keywords: Htr2c; 5-HT_{1C}; motor activity; freezing behavior

1. Introduction

Seven families of serotonin 5-HT receptors comprising a total of 14 subtypes have been identified, and each subtype has distinct functions [1]. Of these subtypes, the serotonin 5-HT_{2C} receptor has attracted considerable attention in brain research because the 5-HT_{2C} receptor is highly expressed in several brain regions involved in emotional functions [2]. To examine the roles of the 5-HT_{2C} receptor in emotional functions, several pharmacological tools have been developed [3-5].

Pharmacological studies have suggested that the blockade of serotonin 5-HT_{2C} receptor increases locomotor activity and attenuates anxiety in rodents, though the stimulating effects on locomotor activity seem to be task- or species-dependent [6-9]. For example, a 5-HT_{2C} receptor antagonist increased locomotor activity in mice [9] while the antagonist alone did not significantly increase the locomotor activity in rats, though there was a trend [6]. Although researchers have developed relatively selective 5-HT_{2C} receptor antagonists, we still need to consider the possibility that off-target effects of the pharmacological agents contribute to the effects. To complement pharmacological findings, researchers have used gene knockout (KO) mice and attempted to clarify the roles of the 5-HT_{2C} receptor in emotional functions [10, 11].

However, the results of locomotor activity and anxiety in 5-HT_{2C} receptor KO mice have been mixed. Hill et al. [11] demonstrated that 5-HT_{2C} receptor KO mice were hyperactive but

exhibited normal anxiety levels, while Heisler et al. [10] showed that 5-HT_{2C} receptor KO mice display normal locomotor activity but lower anxiety levels compared with wild-type littermates. Note that they used similar but slightly different procedures or tests: Hill et al. used the averaged locomotor activity over five days of testing during the first and last 30 min of the session while Heisler et al. used the total locomotor activity of a one-shot open field test for 30 min; Hill et al. used an elevated plus-maze test while Heisler et al. used a zero-maze test. Thus, one purpose of the present study was to reconcile these inconsistent results by analyzing behavioral data in detail.

We analyzed the temporal change of locomotor activity because rodent locomotor activity in a novel environment decreases over time [12], and both of the previous studies did not provide the data of time-course changes. Furthermore, we analyzed mouse behavior in the central area of the elevated plus-maze because the behavior was not analyzed by Hill et al. [11], and the zero-maze does not have a central area (Fig. 1); however, both mazes have open arms and closed arms surrounded by walls, and mouse behavior in these arms is used to assess anxiety levels.

Insert Figure 1 About Here

Moreover, the roles of the 5-HT_{2C} receptor in contextual fear remain unclear, while conditioned fear with an explicit cue was not altered in 5-HT_{2C} receptor KO mice [11]. A recent study showed that a pharmacological blockade of the serotonin 5-HT_{2C} receptor reduced freezing behavior, a measure of fear memory, in a contextual fear conditioning test [13], though a previous study failed to find this effect [14]. In addition, some previous studies have indicated that a pharmacological blockade of the serotonin 5-HT_{2C} receptor affects learning processes [15, 16]: it might facilitate learning by reducing perseveration while enhancing impulsivity. To complement these pharmacological findings, we used 5-HT_{2C} receptor KO mice and examined the effects of 5-HT_{2C} receptor KO on the acquisition, retrieval, and extinction of contextual fear memory.

Freezing behavior in the fear-conditioning test is a measure of fear memory, but researchers have suspected that it could be confounded with changes in locomotor activity or anxiety levels [17, 18]. Indeed, a previous study demonstrated that the freezing rate in the fear-conditioning test is correlated with anxiety levels in the elevated plus-maze test [19]. Because it is possible that 5-HT_{2C} receptor KO mice exhibit higher locomotor activity and/or lower anxiety levels as stated above, we need to consider these potential confounding factors in order to interpret the differences in freezing behavior between genotypes. To this end, we conducted a factor analysis on several behavioral parameters.

2. Materials and methods

2.1. Subjects

Adult male 5-HT_{2C} receptor KO mice (RRID:IMSR_JAX:015821) [20, 21] or wild-type littermates (12–16 weeks old) were used. These mice were backcrossed to the C57BL/6N strain for more than six generations. C57BL/6N mice were supplied from Nippon SLC Co. Ltd (Hamamatsu, Japan). Animals were group-housed at 25°C ± 2°C and relative humidity of 40%–50%. Food and water were provided *ad libitum*. The lights of the animal rooms were turned on from 19:00 to 07:00 h. All tests were performed during the dark period. All procedures followed the guidelines for the Care and Use of Laboratory Animals from the Animal Research Committee of the Hokkaido University. Mice received one or several behavioral tests as summarized in Supplementary Table S1. The number of animals was almost equalized in each group.

2.2. Open field test

The acrylic box (30 × 30 × 30 cm^3) was covered by rough-surfaced polypropylene sheets. The illumination of the room was set to 400 lux. The behavior of each mouse was monitored by a CCD camera over a 30-min testing period and was automatically analyzed using a software package (LimeLight, Actimetrics, USA). The total distance traveled and the number of total crossings (defined by crossings of the lines made by the division of the chamber into

5 cm × 5 cm squares) were used as measures of locomotor activity. Furthermore, the distances traveled in the central arena (10 cm × 10 cm square) and the marginal area were separately analyzed to assess anxiety-like behavior. In addition, freezing, self-grooming, rearing with support to a wall (escape behavior), and rearing without support (explorative behavior) were counted by hand for the first 5 min to help interpretations of the above main measures.

2.3. Elevated plus-maze test

The elevated plus-maze test was performed, as described previously [22]. In brief, the apparatus was made of wood and consisted of two open arms (25 × 5 cm) and two closed arms (25 × 5 cm) that extended from the central area (5 × 5 cm). The closed arms were surrounded by 20-cm-high sidewalls. The maze was elevated 40 cm above the floor, and the illumination of the room was set to 400 lux (Fig. 1a). The behavior of each mouse was monitored by a CCD camera during a 5-min testing period and was automatically analyzed using a software package (LimeLight). The total distance traveled and the total crossing (the number of exits to/from each arm) were used as measures of locomotor activity. The time spent in the open arms was used as a measure of anxiety-related behavior because mice typically avoid the open arms [23]. We also counted the number of risk assessment behaviors in the boundary between the central area and the open arms. Risk assessment behavior was defined as a stretched-attend posture with the forward elongation of the head and shoulders

toward the open arm, followed by retraction to the original posture. The number of risk assessment behaviors and the amount of time spent in the central area were used as measures of hesitating attitude [24]. In addition, freezing, self-grooming, and head dipping were counted by hand to help interpretations of the above main measures. Head dipping was defined as an exploratory movement of the head and shoulders over the edge of the maze [25].

2.4. Zero-maze test

The zero-maze apparatus was circular and made of polyethylene and rough-surfaced polypropylene sheets. The maze was elevated 40 cm above the floor, and the diameter of the maze was 46 cm. The maze was divided into two opposing “open” quadrants without walls and two opposing “closed” quadrants with 20-cm-high walls (Fig. 1b). The illumination of the room was set to 400 lux. Mice were placed in a closed quadrant, and the behavior of each mouse was monitored by a CCD camera during a 5-min testing period. The same parameters as those in the elevated plus-maze were measured but were counted manually by an experimenter who was blinded to the genotype of the mice. Note that we counted the number of risk assessment behaviors in the boundary between open and closed quadrants because the zero-maze does not have a central area. In the case that the locomotor activity increased, the traveling speed in the open or closed quadrants was also calculated (traveling speed = distance traveled in open or closed quadrants / time spent in open or closed quadrants). In addition,

freezing, self-grooming, and head dipping were counted by hand to help interpretations of the above main measures.

2.5. Contextual fear conditioning test

In session 1, each mouse was acclimated in a foot shock box (16.0 × 16.0 × 12.5 cm^3) for 5 min. This was followed by 2-s foot shocks administered at 30-s intervals. Ten foot shocks (shock intensity, 0.6 mA) were inflicted. Thirty seconds after the last foot shock, the mice were returned to their home cage. The freezing rate in session 1 was used as a measure of the acquisition of fear conditioning. In session 2, approximately 24 h after the end of session 1, each mouse was returned to the foot shock box for 30 min without being shocked. The freezing rate in the first 10 min of session 2 was used as a measure of retrieval of fear memory, and the time-course change of the freezing rate in session 2 was used as a measure of within-session extinction. In session 3, approximately 24 h after the end of session 2, each mouse was returned to the foot shock box again for 10 min without being shocked. The freezing rate in session 3 was used as a measure of retrieval or consolidation of fear extinction memory, and the day-to-day change of the freezing rate in the first 10 min between sessions 2 and 3 was used as a measure of between-session extinction. The freezing behavior was defined by a lack of movement except for respiration, and it was used as a measure of fear

memory. In the 30-min testing period, the presence or absence of freezing was estimated by an automatic system (FreezeFrame, Actimetrics) using a pixel difference method.

2.6. Statistical analysis

Two-tailed unpaired *t*-tests were performed to examine the effects of the genotype on behavior in the open field, elevated plus-maze, zero-maze, and the contextual fear conditioning tests. If Levene's test had significant results, Welch's *t*-test was used instead of the Student's *t*-test. If the Shapiro–Wilk normality test had significant results, Mann–Whitney's *U* test was performed.

For the time-course analysis of the locomotor activity in the open field and the contextual fear conditioning tests, each parameter was analyzed by a two-factor mixed-design ANOVA with the time phases as the within-subject factor and the genotype as the between-subject factor. In cases in which there were significant interactions, it was followed by a one-factor ANOVA for each level.

Two mice groups were separately subjected to factor analysis. One group of mice experienced an open field, an elevated plus-maze, and contextual fear conditioning tests, while another group of mice experienced an open field, a zero-maze, and contextual fear conditioning tests (Supplementary Table S1). We did not conduct factor analysis separately for each genotype because the number of samples for each genotype became smaller than the

number of variables. Each behavioral measure was standardized; a simple least-squares method was conducted and followed by an oblique (promax) rotation. However, we eventually used the original results of the factor analysis because the rotation was not helpful in the present data. Factor pattern matrices were identified using the Kaiser criterion; eigenvalues of factors must be 1 or greater. Only loadings greater than 0.5 were used to interpret the results. In the case where a ceiling or floor effect was observed, we excluded these measures from the analysis.

All data are expressed as mean ± standard errors of the mean (SEM). The alpha level was set at 0.05 for all comparisons. All statistical procedures were conducted using SPSS (version 23.0).

3. Results

3.1. Open field test

The total locomotor activity for 30 min in 5-HT_{2C} receptor KO mice was significantly higher than that in wild-type littermates (total distance traveled, $t_{55} = 3.23$, $p = 0.002$, Fig. 2a; numbers of total crossing, $t_{55} = 3.63$, $p = 0.001$, Fig. 2b). Moreover, the time-course analysis revealed that the higher locomotor activity in 5-HT_{2C} receptor KO mice was observed only in the late phase of the test (total distance traveled, genotype × time interaction, $F_{2,110} = 7.27$, $p =$

0.001; followed by one-factor ANOVA for each time : $F_{1,55} = 2.00$, $p = 0.16$, in 0–10 min; $F_{1,55} = 15.59$, $p = 0.001$, in 10–20 min; $F_{1,55} = 12.60$, $p = 0.001$, in 20–30 min , Fig.2c; numbers of total crossing, genotype × time interaction, $F_{2,110} = 14.41$, $p < 0.001$; followed by one-factor ANOVA for each time : $F_{1,55} = 1.17$, $p = 0.28$, in 0–10 min; $F_{1,55} = 19.17$, $p < 0.001$, in 10–20 min; $F_{1,55} = 17.54$, $p < 0.001$, in 20–30 min, Fig.2d).

Insert Figure 2 About Here

The central activity for the first 5 min in 5-HT_{2C} receptor KO mice was significantly higher than that in wild-type littermates ($t_{55} = 2.07$, $p = 0.044$, Table 1) while the marginal activity did not differ between genotypes ($t_{55} = 0.31$, $p = 0.76$, Table 1). Grooming behavior was frequently observed in wild-type littermates compared with 5-HT_{2C} receptor KO mice during the first 5 min of the open field test ($t_{55} = 2.40$, $p = 0.02$, Table 1). The number of rearing behaviors did not differ between genotypes ($ts_{55} < 1.1$, $ps > 0.29$, Table 1).

Insert Table 1 About Here

3.2. Elevated plus-maze test

The genotype did not affect the time spent in the open arms, a measure of anxiety-like behavior (t_{35} = 1.86, p = 0.07, Fig. 3a). Additionally, it did not affect the total traveled distance or the number of total crossing, the measures of locomotor activity (t_{35} = 1.06, p = 0.30, Fig. 3b; numbers of total crossing, t_{35}=1.099, p = 0.28, Fig. 3c) in the elevated plus-maze test. However, a hesitating attitude was observed more in 5-HT_{2C} receptor KO mice than in wild-type littermates (time spent in the central area, U = 98.00, p = 0.039, Fig. 3d; risk assessment, t_{35} = 2.96, p = 0.006, Fig.3e).

Insert Figure 3 About Here

The number of head dipping or grooming behaviors did not differ between genotypes (ts_{35} < 1.7, ps > 0.11, Table 1).

3.3. Zero-maze test

Unlike the results of the elevated plus-maze test, 5-HT_{2C} receptor KO mice showed anxiolytic phenotype (time spent in open quadrants, U = 79.00, t = 0.007, Fig. 4a) and increased

locomotor activity (total distance traveled, $t_{26.75} = 2.76$, $p = 0.01$, Welch's *t*-test, Fig. 4b) in the zero-maze test. No difference in risk assessment behavior was observed between genotypes ($t_{36} = 1.70$, $p = 0.098$). Two-factor ANOVA revealed a significant interaction effect (genotype × quadrants interaction, $F_{1,34} = 6.53$, $p = 0.015$). Following one-factor ANOVA showed a significant effect of quadrants ($F_{1,34} = 14.55$, $p = 0.001$) on the traveling speed in wild-type mice as well as that of the genotype ($F_{1,34} = 18.24$, $p = 0.0001$) on the traveling speed in closed quadrants.

Insert Figure 4 About Here

The number of head dippings in 5-HT_{2C} receptor KO mice was significantly higher than that in wild-type littermates ($t_{36} = 2.89$, $p = 0.006$, Table 1) while the number of grooming behaviors in 5-HT_{2C} receptor KO mice was significantly lower than that in wild-type littermates ($t_{26.92} = 2.61$, $p = 0.015$, Table 1).

3.4. Contextual fear conditioning test

Two-factor ANOVA revealed significant main effects of time ($F_{19,1653} = 418.33$, $p < 0.001$), genotype ($F_{1,87} = 7.46$, $p = 0.008$), and interaction effects ($F_{19,1653} = 3.52$, $p = 0.001$) on the freezing behavior during session 1 (Fig. 5b). The following one-factor ANOVA showed

the significant effects of the genotype on freezing behavior in the 240- to 270-s ($F_{1,87}$ = 5.03, p = 0.027), 330-to 360-s ($F_{1,87}$ = 8.40, p = 0.005), 360- to 390-s ($F_{1,87}$ = 10.15, p = 0.002), 390- to 420-s ($F_{1,87}$ = 8,38, p = 0.005), and 420- to 450-s ($F_{1,87}$ = 4.94, p = 0.029) phases during session 1.

Two-factor ANOVA revealed significant main effects of time ($F_{2, 174}$ = 309.20, p < 0.001), genotype ($F_{1, 87}$ = 5.42, p = 0.02), and interaction effects ($F_{1, 87}$ = 3.08, p = 0.049) on the freezing behavior during session 2 (Fig. 5c). The following one-factor ANOVA showed the significant effects of the genotype on the freezing behavior in the 10- to 20-min ($F_{1, 87}$ = 6.52, p = 0.012) and 20- to 30-min ($F_{1, 87}$ = 7.23, p = 0.009) phases during session 2.

In session 3, Mann–Whitney's U test showed significant effects of the genotype on freezing behavior (U = 745.00, p = 0.045). Furthermore, between-session extinction was evaluated by comparing the freezing behavior in the initial 10 min during session 2 and that during session 3 (Fig. 5e). Two-factor ANOVA revealed a significant main effect of session ($F_{1, 87}$ = 490.17, p < 0.001) on the freezing behavior, while neither a main effect of the genotype ($F_{1, 87}$ = 2.26, p = 0.14) nor a significant interaction effect ($F_{1, 87}$ = 0.86, p = 0.356) was observed.

Insert Figure 5 About Here

3.5. Factor analysis: open field test, elevated plus-maze test, and contextual fear-conditioning test

Because freezing behavior was rarely observed in the open field and elevated plus-maze tests, we excluded the measures from these tests in further analysis.

As shown in Table 2, five factors accounted for 66.2% of the variance. The measures of locomotor activity loaded highly and positively on factor 1, whereas freezing behavior in the contextual fear-conditioning test loaded highly and negatively on factor 1. Rearing loaded highly and positively on factor 2, whereas the distance traveled in the marginal area of the open field loaded negatively on factor 2. Freezing behavior during the later phases, a measure of extinction, loaded highly and positively on factor 3. No measures loaded highly on factors 4 or 5.

Insert Table 2 About Here

3.6. Factor analysis: open field test, zero-maze test, and contextual fear-conditioning test

Because freezing behavior was rarely observed in the open field and zero-maze tests, we excluded the measures from these tests in further analysis.

As shown in Table 3, six factors accounted for 74.9% of the variance. Measures of locomotor activity loaded highly and positively on factor 1, whereas grooming behavior loaded negatively on factor 1. Time spent in open quadrants of the zero-maze loaded highly and positively on factor 2, whereas rearing with support to wall, an escape-related behavior, loaded highly and negatively on factor 2. Locomotor activity in the former phase of the open field test, grooming in the zero-maze test, and freezing behavior during the former phase loaded positively on factor 3. Freezing behavior during the latter phase, which are measures of extinction, loaded highly and positively on factor 4. No measures loaded highly on factors 5 or 6.

Insert Table 3 About Here

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4. Discussion

We found that locomotor activity declined slowly in 5-HT_{2C} receptor KO mice compared with wild-type littermates, indicating that 5-HT_{2C} receptor KO mice showed slower

habituation to the novel environment (Fig. 2). The slower habituation could explain previous inconsistent findings. Although previous studies have used the averaged locomotor activity or total locomotor activity [10, 11], such analyses would decrease the statistical power and lead to variable results; this would depend on small differences in experimental conditions because the differences in locomotor activity appear only in the late phase of the test. It should be noted that Hill et al. [11] repeated the tests and detected the differences in locomotor activity between genotypes while Heisler et al. [10] conducted the test only once and failed to detect the differences. We speculate that Hill et al. detected the differences because the repeated tests accentuated the differences in habituation. Thus, we may now conclude that a pharmacological blockade of either the 5-HT_{2C} receptor or gene KO of 5-HT_{2C} receptor moderately increases locomotor activity. However, further studies are required in order to determine whether the pharmacological blockade could induce slower habituation to a novel environment; a previous study demonstrated that a pharmacological blockade of the 5-HT_{2C} receptor increased locomotor activity without time-dependent effects, though they did not use a novel environment [9].

Moreover, we found that 5-HT_{2C} receptor KO mice displayed a hesitating attitude, staying in the central area, as well as risk assessment behavior in the elevated plus-maze test (Figs. 3d and e). This phenotype could explain the reason why Hill et al. [11] failed to find an increase

of time spent in the open arms, which is a measure of anxiety, in the elevated plus-maze test while Heisler et al. [10] found the increase of time spent in open arms in the zero-maze test. Please note that the zero-maze test lacks a central area (Fig. 1). The increased time spent in the central area will decrease the time spent in the open arms and reduce the sensitivity for detecting the differences in anxiety levels [26]. Supporting this view, the time spent in the open quadrants was longer in 5-HT_{2C} receptor KO mice than wild-type littermates, while risk-assessment behavior did not differ between genotypes when the zero-maze test was used (Figs. 4a and c).

Although locomotor activity in the zero-maze test was higher in 5-HT_{2C} receptor KO mice than in wild-type littermates (Fig. 4b), it is unlikely that the higher locomotor activity affected the time spent in open quadrants, a measure of anxiety-like behavior, because of two reasons. First, the time spent in open quadrants is not directly affected by the change of locomotor activity because the parameter was calculated as a percentage (i.e., time in open / [time in open + time in closed quadrants]). Second, the difference in the traveling speed between open and closed quadrants differed between genotypes (Fig. 4d), indicating that the difference in the traveling distance means not only the difference of overall activity but also the difference of behavioral patterns. That is, wild-type mice might have accelerated their movements in open quadrants because they wanted to flee down to closed quadrants as soon as possible; in

contrast, 5-HT_{2C} receptor KO mice moved at a constant speed regardless of whether they were running in open or closed quadrants because they were not as motivated to flee down to closed quadrants. Thus, we conclude that the suppression of 5-HT_{2C} receptor functions exerts anxiolytic effects at least under some conditions.

Additional measures also support the above conclusions. Higher activity in the central arena of the open field test and frequent head dipping in the zero-maze test support the idea that the suppression of 5-HT_{2C} receptor functions exerts anxiolytic effects. Because higher activity will reduce the rate of self-grooming, less frequent grooming in 5-HT_{2C} receptor KO mice could be a byproduct of higher locomotor activity (Table 1).

In the contextual fear-conditioning test, 5-HT_{2C} receptor KO mice showed a slow acquisition of fear conditioning and rapid within-session extinction of fear, but normal retrieval and between-session extinction. However, these characteristics of 5-HT_{2C} receptor KO mice might be confounded with their higher locomotor activity because the decline curve of the freezing rate (Fig. 5c) was like a mirror image of the decline curve of locomotor activity (Fig. 2c). Indeed, our factor analysis suggests that locomotor activity and freezing behavior could be regulated by common psychological factors (factor 1 in Table 2) though factor 3 (Table 2) implies that freezing behavior in the latter phase of the contextual fear-conditioning test is relatively independent of locomotor activity. Because factor analysis is a

retrospective analysis and the sample size in the present study is small for factor analysis, we will refrain from drawing any conclusions. Rather, our analysis suggests that freezing behavior in the latter phase of the contextual fear-conditioning test could be isolated from locomotor activity by devising some behavioral tasks. This possibility should be pursued in future studies. Thus, we need to suspend conclusions about the roles of the 5-HT_{2C} receptor in contextual fear.

Taken together, we provided reasonable explanations about previous inconsistent findings and filled some gaps between pharmacological and genetic findings. One possible interpretation of the present results is that the anxiolytic effects caused by suppressing 5-HT_{2C} receptor functions are weak or moderate because the anxiolytic effects were not observed in the elevated plus-maze test (Fig. 3). However, we suggest that suppressing 5-HT_{2C} receptor functions could exert substantial anxiolytic effects without evoking risk-taking behavior because the hesitating attitude remained intact in 5-HT_{2C} receptor KO mice (Figs. 3e and 4c). Furthermore, it is unlikely that suppressing 5-HT_{2C} receptor functions attenuates memory-dependent fear in a sustained manner because it did not facilitate between-session extinction (Fig. 5e). Recent animal studies have suggested that some drugs, such as memantine [27], KNT-127 [28], and riluzole [18], could facilitate between-session extinction. These drugs might be better candidates for treating fear memory-related problems, though more

experiments are required to use them for treating post-traumatic stress disorder. Therefore, the 5-HT_{2C} receptor would still be a promising target for developing anxiolytics if the target disease is appropriately selected.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgements

This work was supported by Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan awarded to Y.O. [18K07545] and M.Y. [16H05371]. We would like to thank Hitomi Sasamori for help with breeding the transgenic mice, and Enago (www.enago.jp) for the English language review.

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Titles and Legends to Figures

Fig. 1. Elevated plus-maze test and zero-maze test

(a) The setup of the elevated plus-maze test. The elevated plus-maze test consists of two open arms (25 × 5 cm) and two closed arms (25 × 5 cm) that extend from the central platform (5 × 5 cm). (b) The setup of the elevated zero-maze test. The zero-maze consists of two opposite closed quadrants with 20-cm-high walls and two open quadrants. Unlike the elevated plus-maze, the zero-maze does not have a central area.

Fig. 2. Effect of the genotype on parameters in the open field test.

(a) The total distance traveled for 30 min. (b) The number of total crossings (crossings of the lines made by the division of the field [30 × 30 cm^2] into 5 cm × 5 cm squares) for 30 min. (c) The total distance traveled was divided into three time phases (0–10, 10–20, and 20–30 min). The dotted line indicates 5-HT_{2C} receptor KO mice. (d) The number of the total crossings was divided into three time phases (0–10, 10–20, and 20–30 min). The dotted line indicates 5-HT_{2C} receptor KO mice. Data are given as mean ± SEM. *$p < 0.05$, 5-HT_{2C} receptor KO versus wild-type mice.

Fig. 3. Effect of the genotype on parameters in the elevated plus-maze test.

(a) Effect of the genotype on the time spent in open arms, a measure of anxiety-like behavior. (b) Effect of the genotype on the total distance traveled, a measure of locomotor activity. (c) Effect of the genotype on the total crossings, a measure of locomotor activity. (d) Effect of the genotype on the time spent in the central area, a measure of hesitating attitude. (e) Effect of the genotype on the number of risk assessment behaviors, a measure of hesitating attitude. Data are given as mean ± SEM. *$p < 0.05$.

Fig. 4. Effect of the genotype on parameters in the zero-maze test.

(a) Effect of the genotype on the time spent in open quadrants, a measure of anxiety-like behavior. (b) Effect of the genotype on the total distance traveled, a measure of locomotor activity. (c) Effect of the genotype on the number of risk assessment behavior, a measure of hesitating attitude. (d) Effect of the genotype on the traveling speed in open and closed quadrants. Data are given as mean ± SEM. *$p < 0.05$.

Fig. 5. Effect of the genotype on freezing behavior in the contextual fear-conditioning test.

(a) Procedure of the contextual fear conditioning test (see *2.5. Contextual fear-conditioning test*). (b) Mice received 10 foot shocks during session 1. The freezing rate was calculated for

30 s each to assess the acquisition of fear conditioning. (c) In session 2, each mouse was returned to the foot shock box without being shocked to assess the retrieval of fear memory and the acquisition of extinction. Within-session extinction was evaluated by dividing the freezing rate into three time phases (0–10, 10–20, and 20–30 min). The dotted line indicates 5-HT_{2C} receptor KO mice. (d) In session 3, each mouse was returned to the foot shock box again without being shocked to assess the consolidation or retrieval of extinction memory. (e) Between-session extinction was evaluated by comparing between the freezing behavior in the initial 10 min during session 2 and that during session 3. Data are given as mean ± SEM. *$p < 0.05$, 5-HT_{2C} receptor KO versus wild-type mice.

Table 1. Additional measures in behavioral tests.

Test	Measure	Wild-type	5-HT_{2C} KO
Open field test (0–5 min)	Central activity (cm)*	107.6 ± 10.4	134.4 ± 7.9
	Marginal activity (cm)	916.2 ± 39.7	934.7 ± 45.2
	Grooming*	4.4 ± 0.4	3.2 ± 0.3
	Rearing with support	14.5 ± 1.3	15.4 ± 1.8
	Rearing without support	6.7 ± 1.0	8.5 ± 1.4
Elevated plus-maze test	Head dipping	6.9 ± 1.8	9.8 ± 0.8
	Grooming	2.5 ± 0.6	1.7 ± 0.2
Zero-maze test	Head dipping*	11.7 ± 1.7	17.8 ± 1.2
	Grooming*	2.1 ± 0.4	1.1 ± 0.2

Open field test: wild-type, n = 28; 5-HT_{2C} receptor KO, n = 29

Elevated plus-maze test: wild-type, n = 15; 5-HT_{2C} receptor KO, n = 22

Zero-maze test: wild-type, n = 20; 5-HT_{2C} receptor KO, n = 18

Data are given as mean ± SEM. *$p < 0.05$, 5-HT_{2C} receptor KO versus wild-type mice.

Table 2. Factor loadings of open field, elevated plus-maze, and contextual fear-conditioning tests.

Behavior	Factor				
	1 Activity	2 Escape	3 Less extinction	4	5
OF 0–5 min grooming	−.325	−.183	−.290	.127	.478
OF 0–5 min rearing without support	.102	**.604**	−.225	−.179	−.122
OF 0–5 min rearing with support to wall	.147	**.842**	−.485	.054	.079
OF 0–5 min central distance	**.768**	.265	−.171	.453	.011
OF 0–5 min marginal distance	**.616**	**−.516**	.235	.010	.176
OF 0–10 min distance	**.858**	.127	−.116	.379	.020
OF 10–20 min distance	**.795**	−.168	.070	−.002	.017
OF 20–30 min distance	**.823**	−.203	−.056	.115	.014
EPM head dip	**.721**	.093	.364	.115	.136
EPM grooming	−.320	−.207	−.152	.103	−.252
EPM open time	**.661**	.262	.421	−.155	.077
EPM distance	**.596**	.060	.232	−.209	−.407
EPM central time	.339	.314	.195	−.474	.463
EPM risk assessment	.407	.052	−.028	−.240	−.217
CFC 0–10 min freezing (session 2)	**−.804**	.193	.267	.094	.089
CFC 10–20 min freezing (session 2)	−.484	.292	**.630**	.299	.070
CFC 20–30 min freezing (session 2)	−.335	.337	**.557**	.194	−.140
% variance	34.4	11.7	9.9	5.4	4.9

The sample size is as follows: total, n = 32; wild-type, n = 13; 5-HT_{2C} receptor KO, n = 19. Loadings higher than 0.5 (or less than −0.5) were printed in bold. OF, open field; EPM, elevated plus-maze; CFC, contextual fear-conditioning.

Table 3. Factor loadings of open field, zero maze, and contextual fear-conditioning tests.

Behavior	Factor					
	1 Activity	2 Boldness	3 Unknown	4 Less extinction	5	6
OF 0–5 min grooming	**−.527**	−.142	.356	.153	.427	.218
OF 0–5 min rearing without support	.254	−.227	.447	−.486	.008	.354
OF 0–5 min rearing with support to wall	.291	**−.697**	.024	.134	.120	.071
OF 0–5 min central distance	**.560**	−.372	.234	.492	−.094	−.135
OF 0–5 min marginal distance	.449	.045	.337	−.363	.171	−.059
OF 0–10 min distance	**.658**	−.133	**.567**	.381	.020	.012
OF 10–20 min distance	**.775**	−.104	.424	−.150	.308	−.289
OF 20–30 min distance	**.863**	.092	.046	−.229	.120	−.261
ZM head dip	**.791**	.403	.023	−.071	.022	.410
ZM grooming	−.456	−.097	**.632**	−.131	−.486	−.240
ZM open time	.417	**.883**	−.155	−.077	.154	−.074
ZM distance	**.801**	−.175	−.136	.353	−.176	.204
ZM risk assessment	.436	−.403	−.195	−.051	−.143	.085
ZM open speed	−.266	−.384	−.262	.074	.316	.150
ZM closed speed	**.806**	.431	−.268	.217	−.163	.006
CFC 0–10 min freezing (session 2)	−.199	.444	**.501**	.132	−.181	.372
CFC 10–20 min freezing (session 2)	−.281	.451	.279	.047	.020	.006
CFC 20–30 min freezing (session 2)	−.426	.439	.245	**.546**	.250	−.129
% variance	31.0	15.6	11.2	7.8	4.9	4.5

The sample size is as follows: total, n = 19; wild-type, n = 13; 5-HT_{2C} receptor KO, n = 6. Loadings higher than 0.5 (or less than −0.5) are printed in bold. OF, open field; ZM, zero-maze; CFC, contextual fear-conditioning.

Figure 1

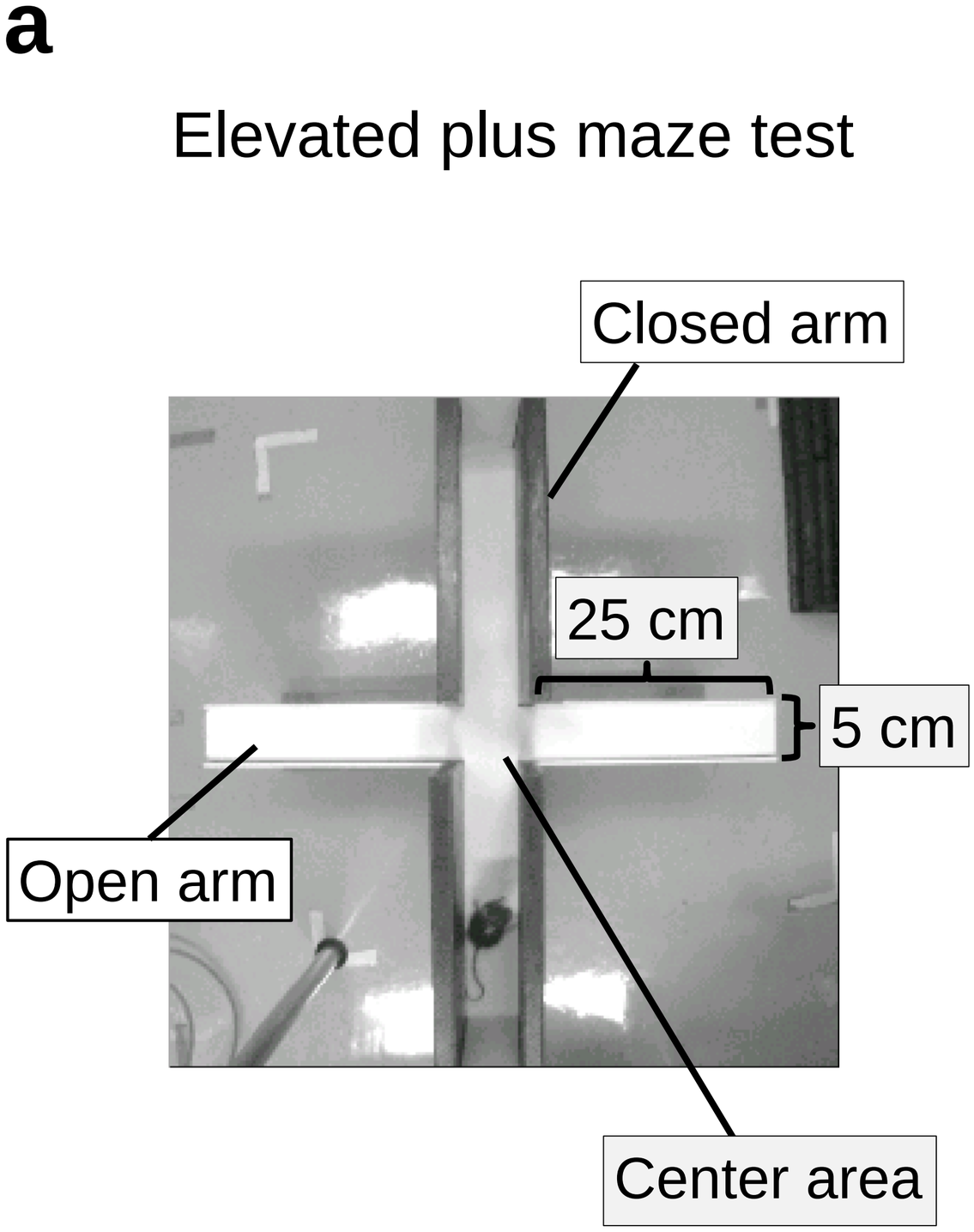
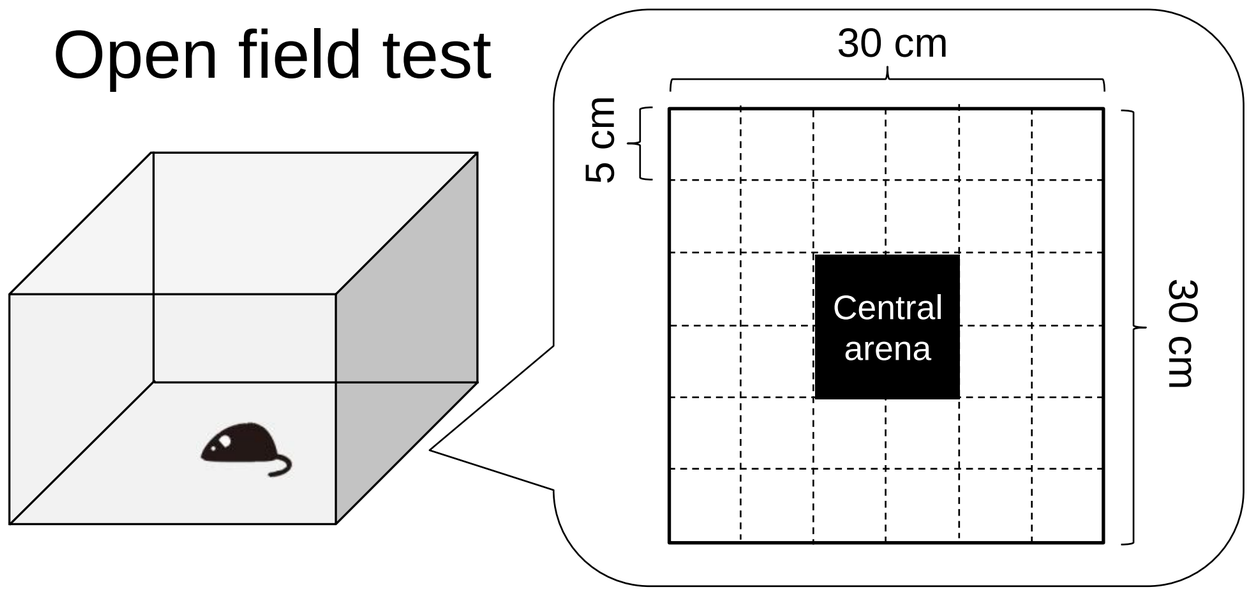
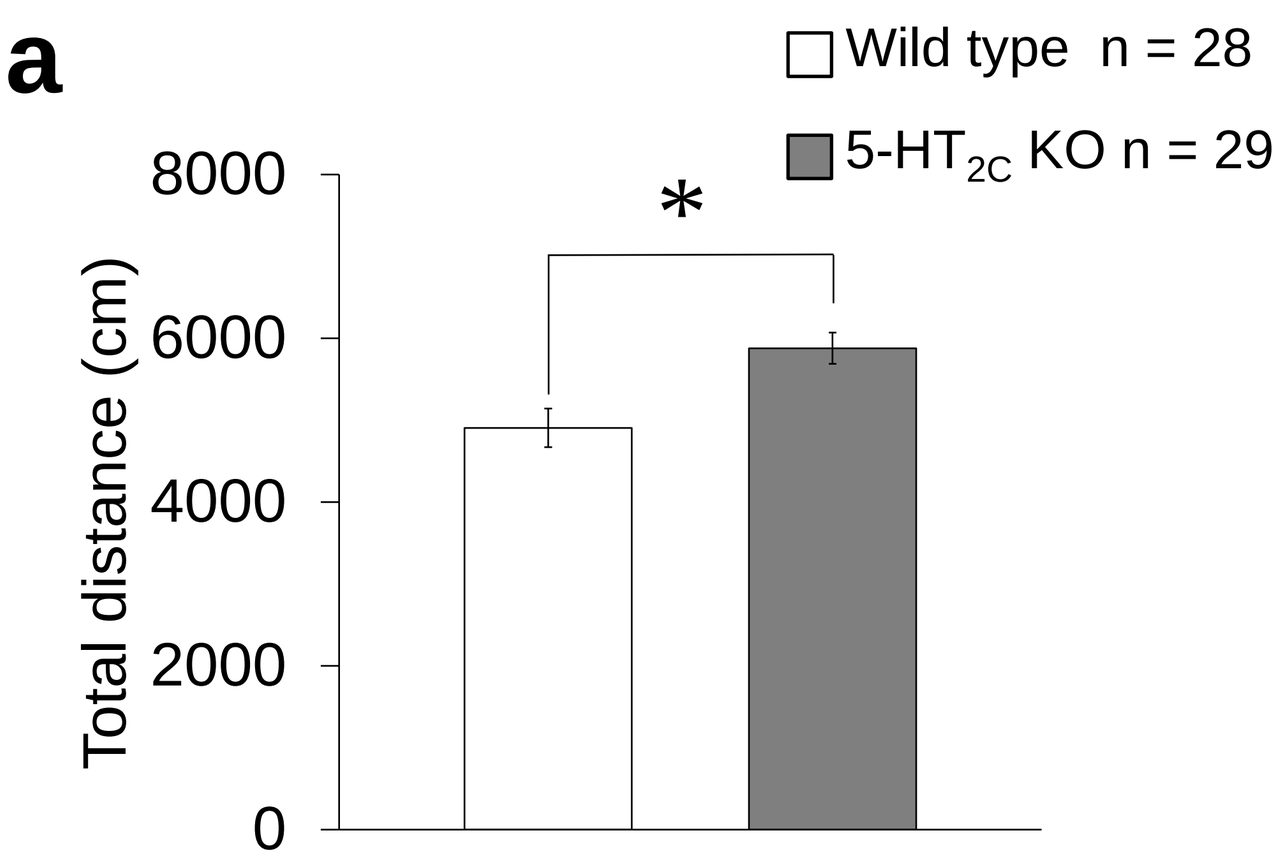
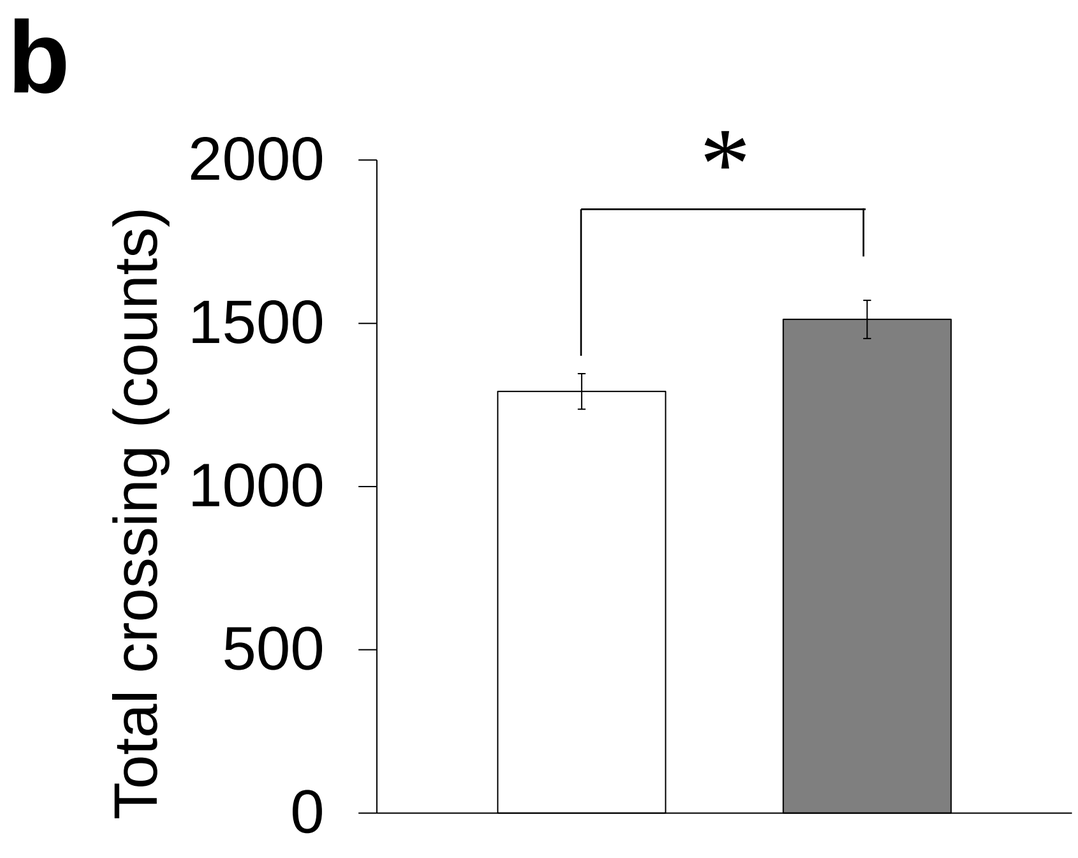
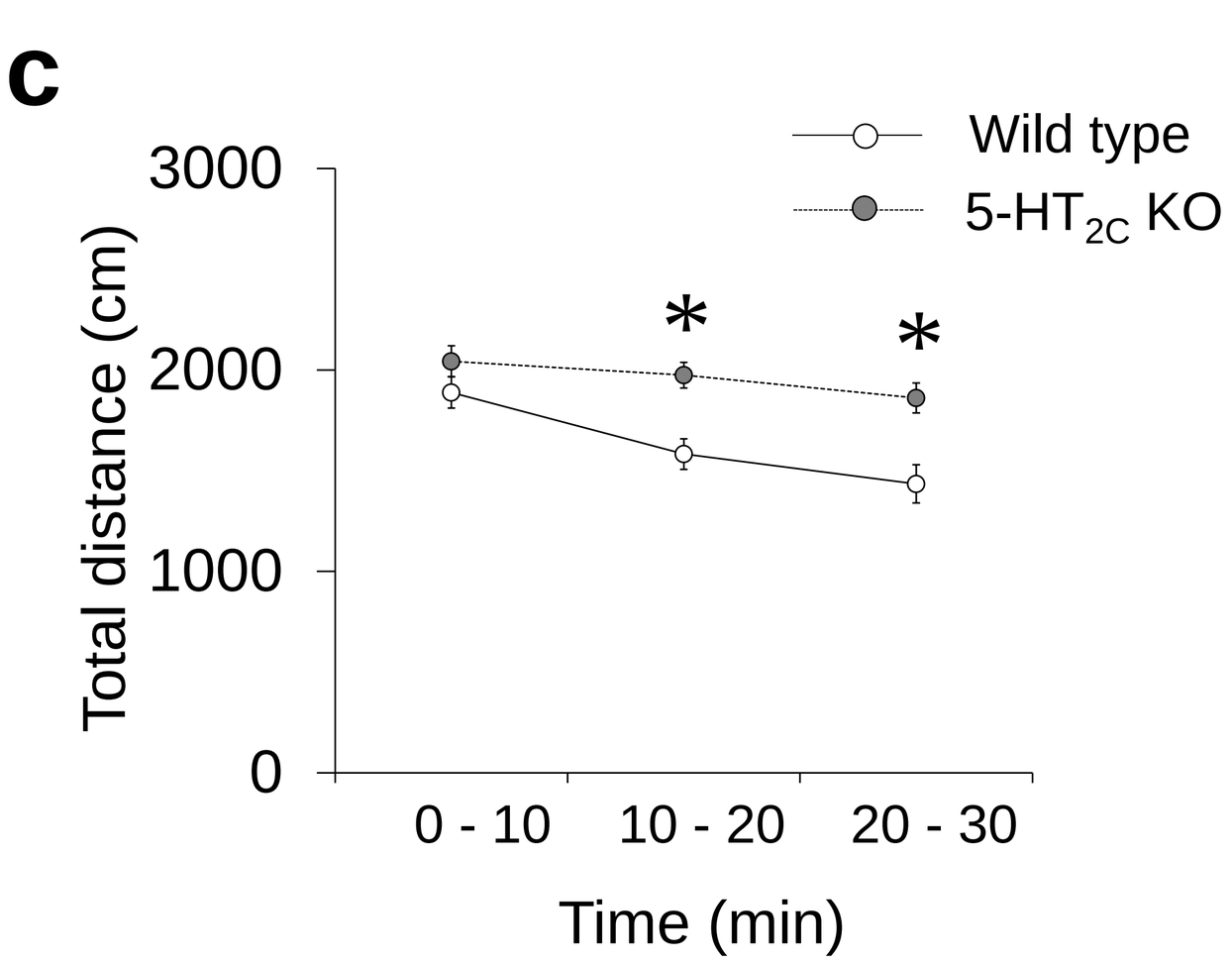

a
Elevated plus maze test
Closed arm
25 cm
5 cm
Open arm
Center area
b
Zero-maze test
46 cm
5 cm

Figure 2


Open field test
30 cm
5 cm
30 cm
Central
arena


a
Wild type n = 28
5-HT2C KO n = 29
Total distance (cm)
8000
6000
4000
2000
0
*


b
Total crossing (counts)
2000
1500
1000
500
0
*


c
Wild type
5-HT2C KO
Total distance (cm)
3000
2000
1000
0
*
*
0 - 10
10 - 20
20 - 30
Time (min)

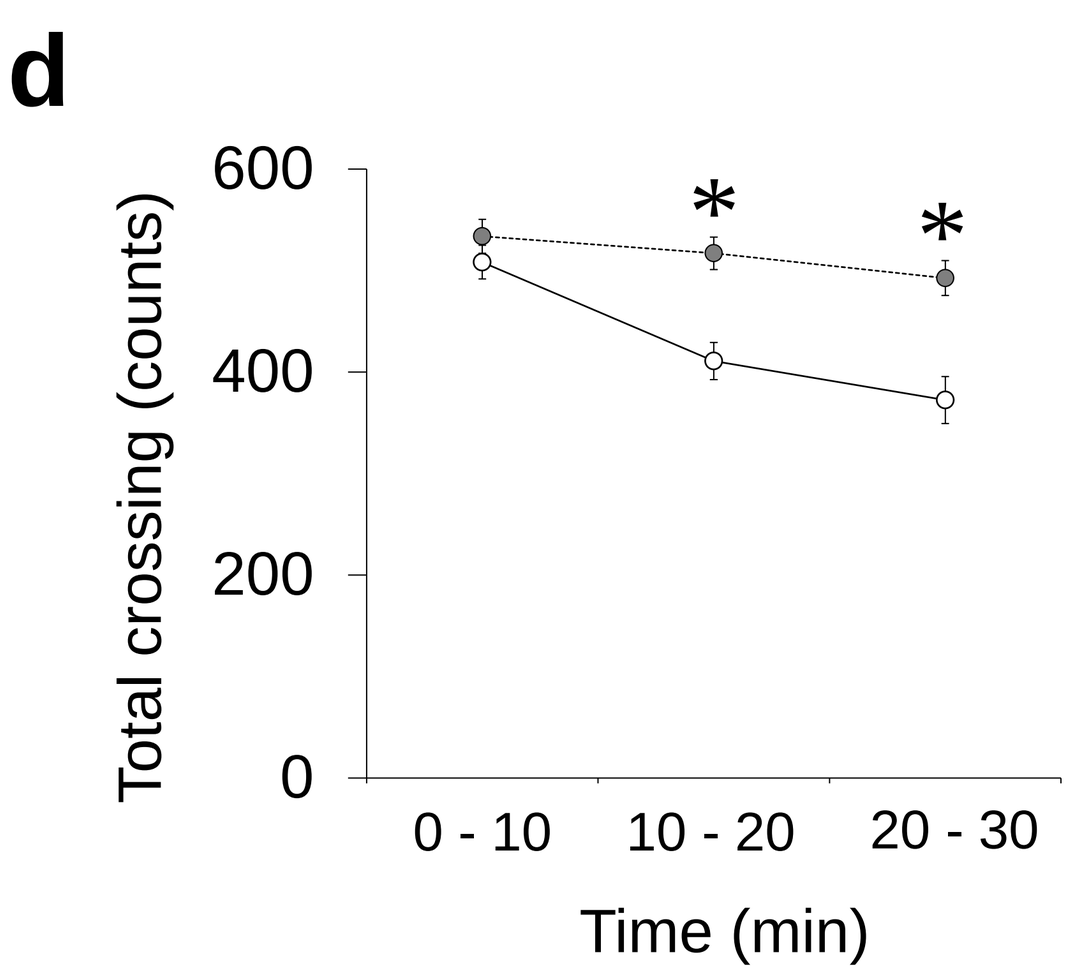

d
Total crossing (counts)
600
400
200
0
*
*
0 - 10
10 - 20
20 - 30
Time (min)

Figure 3

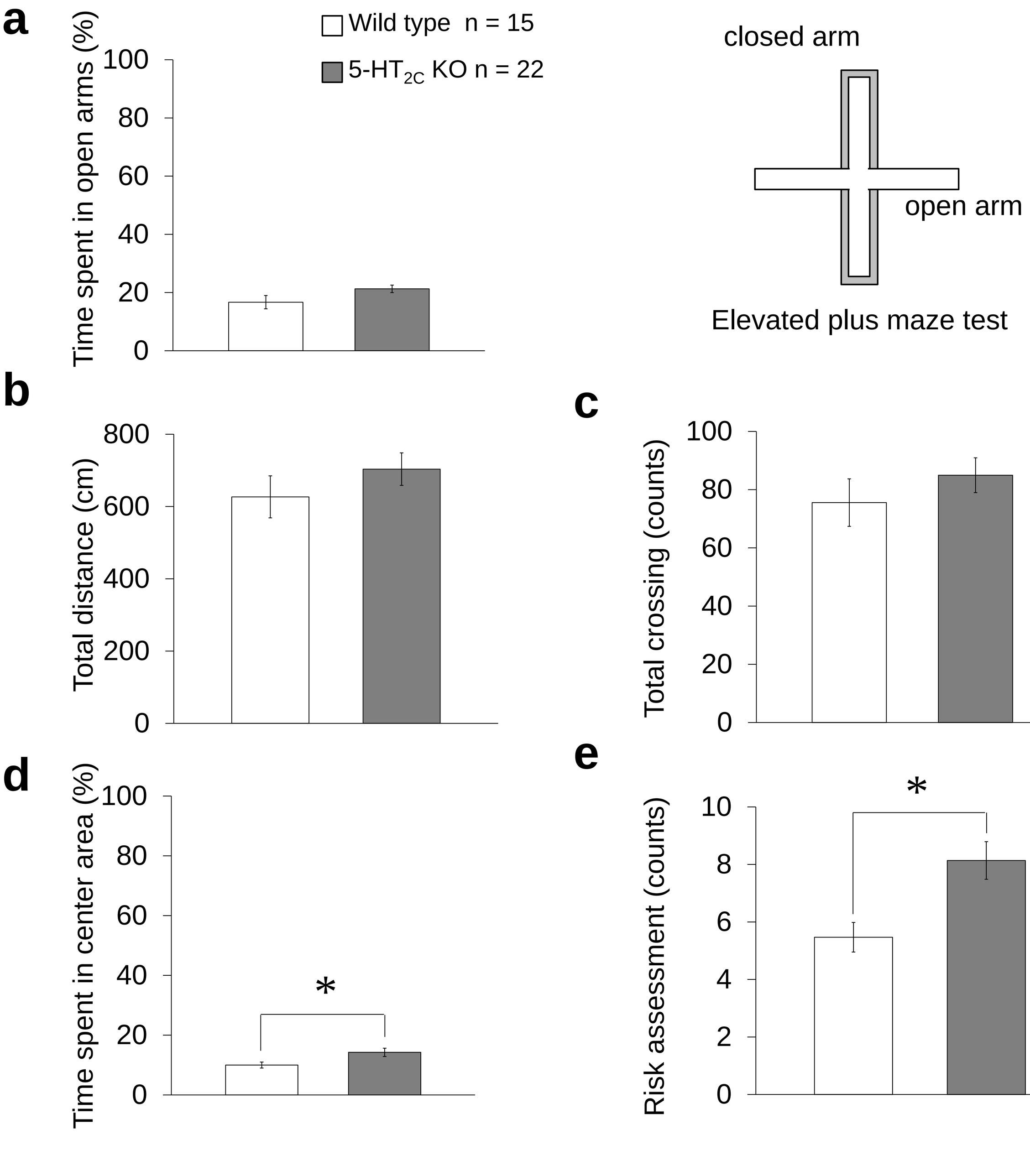
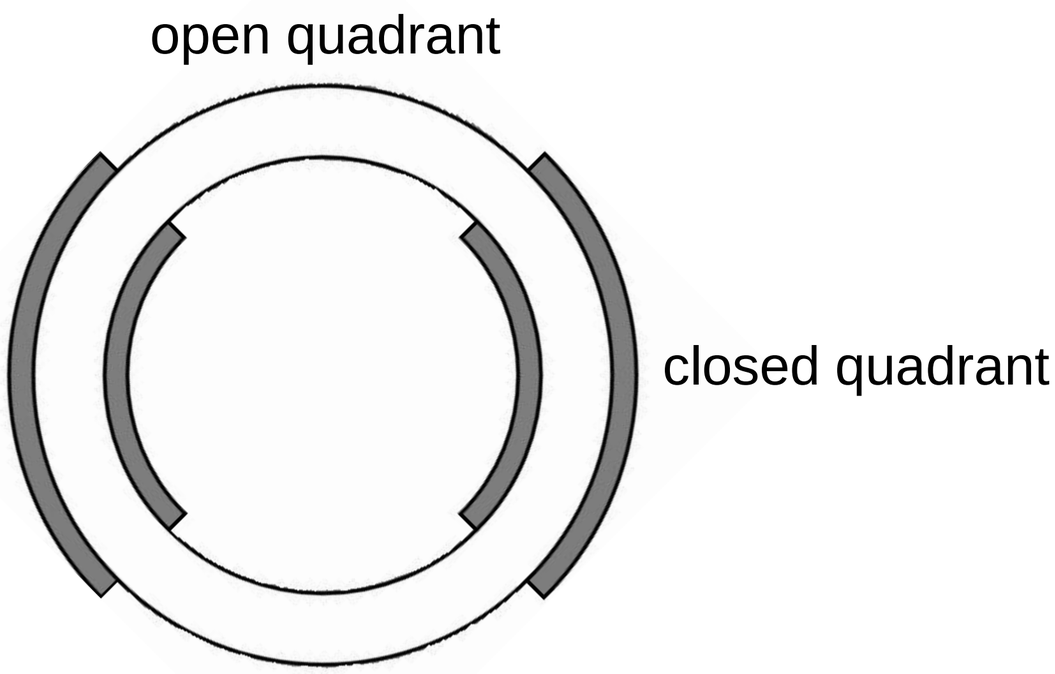

a
Wild type n = 15
5-HT2C KO n = 22
Time spent in open arms (%)
100
80
60
40
20
0
closed arm
open arm
Elevated plus maze test
b
Total distance (cm)
800
600
400
200
0
c
Total crossing (counts)
100
80
60
40
20
0
d
Time spent in center area (%)
100
80
60
40
20
0
*
e
Risk assessment (counts)
10
8
6
4
2
0
*

Figure 4



Zero maze test

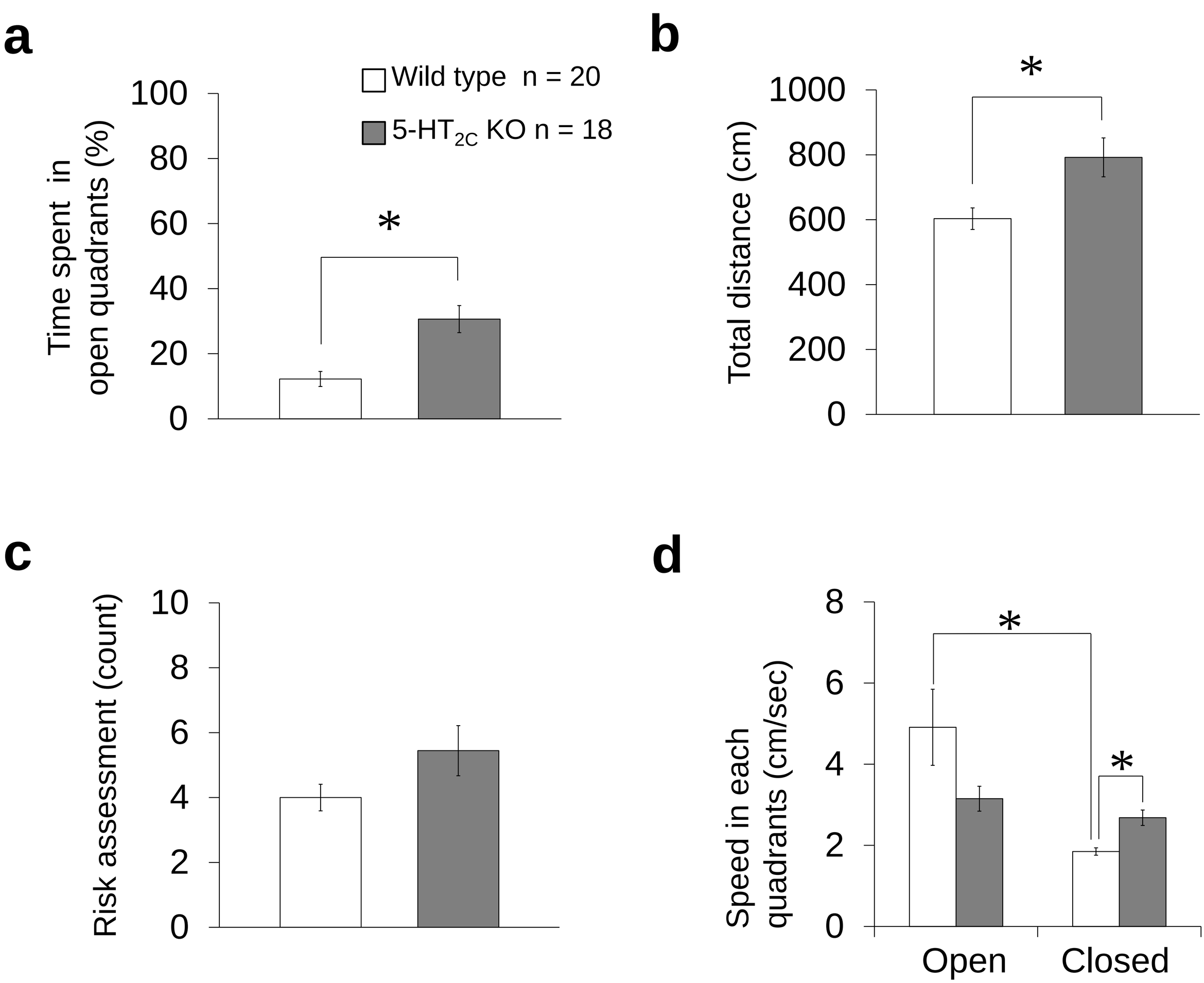
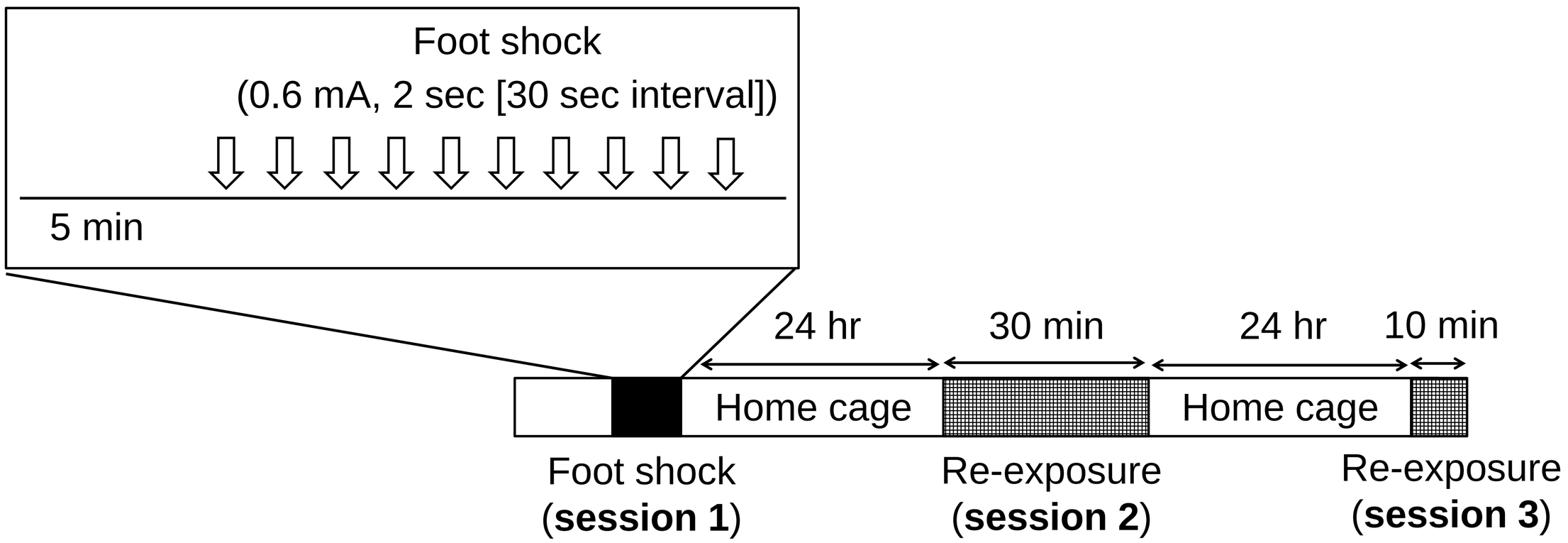
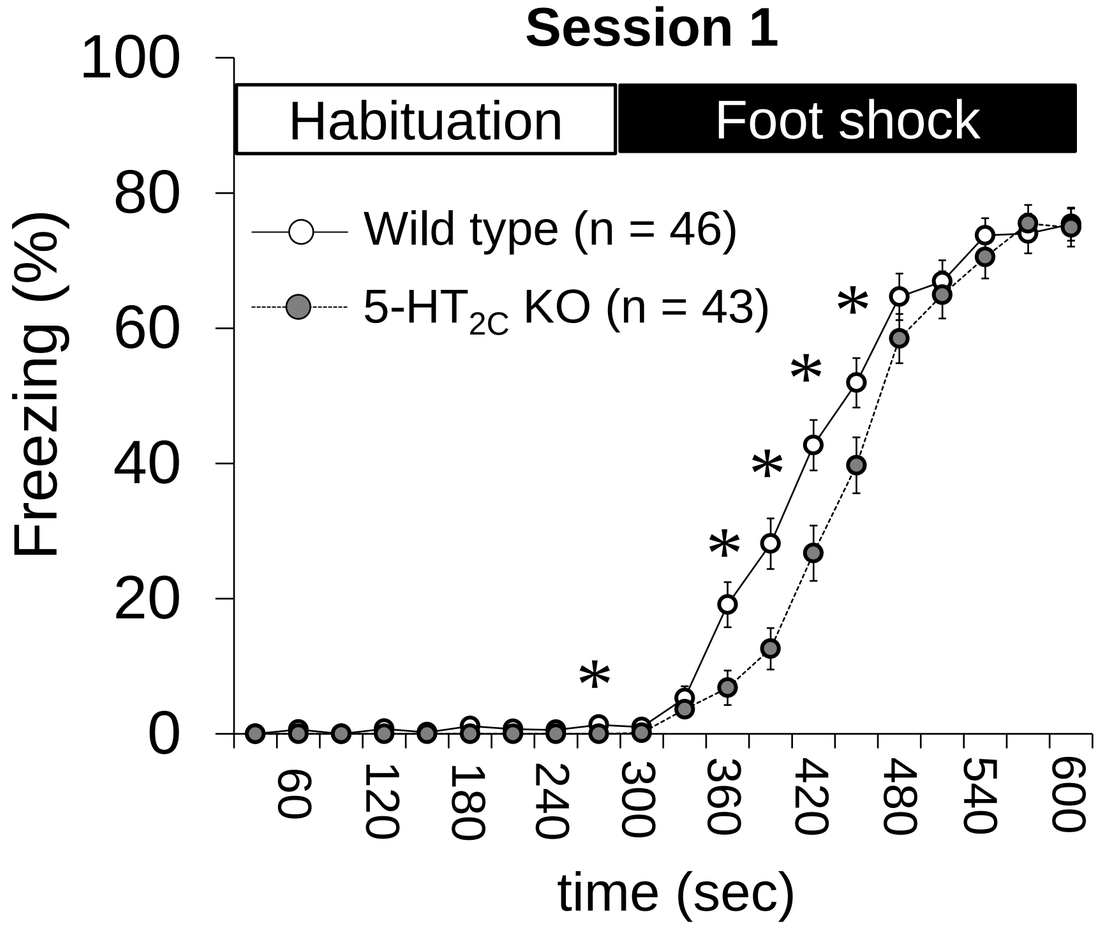


Figure 5

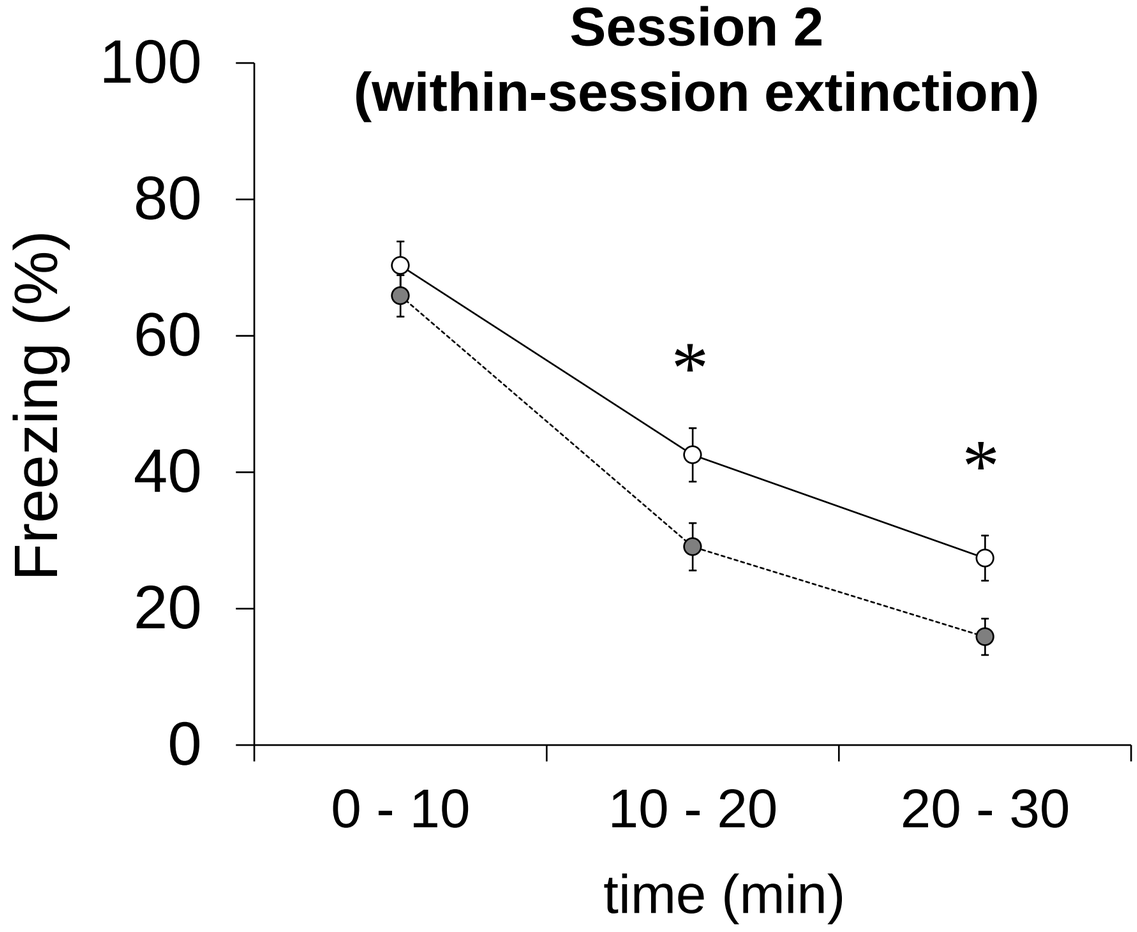
a



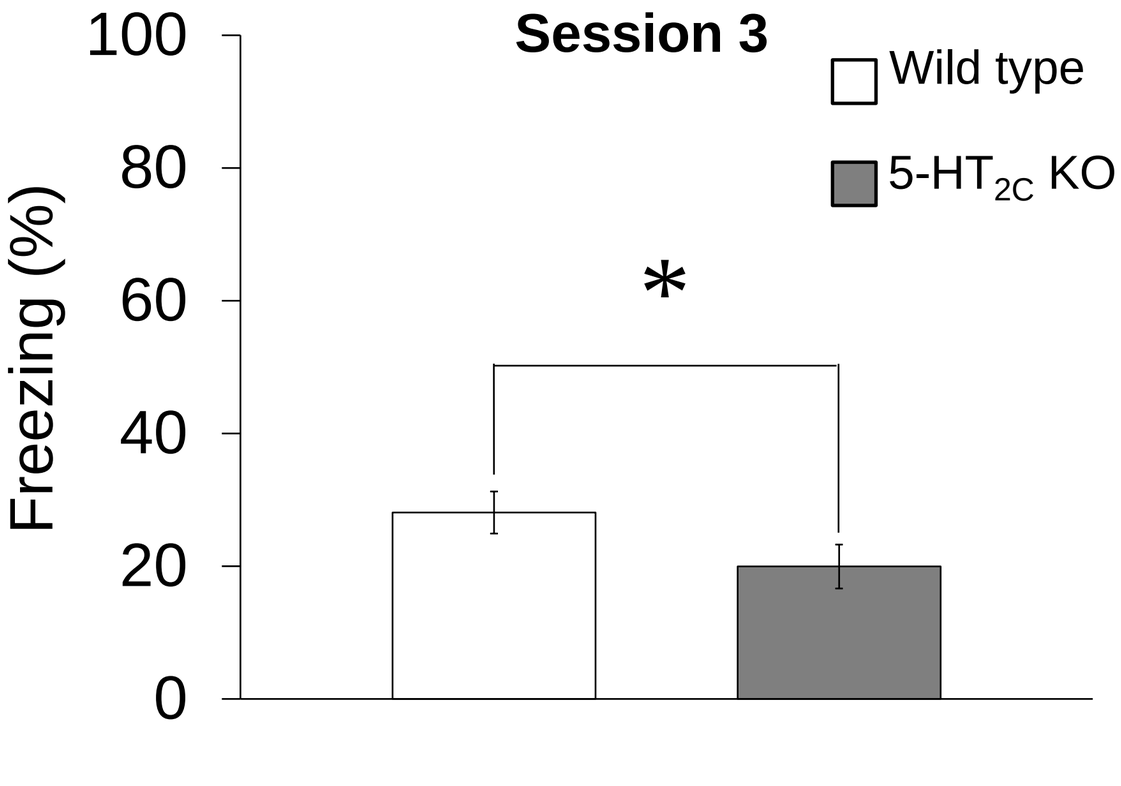
b



c



d



e