Differential effects of oxytocin receptor antagonist on social rank and other social behavior in mice

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Abstract

Social rank within a group is essential for survival in many animals. Rank in the community helps to avoid unnecessary conflicts and establish stable relationships with others. Recently, oxytocin has received increasing attention for its function in social behavior. However, the causal relationship between oxytocin levels and social rank has remained unclear. Here, we examined the effects of intraperitoneal injection of the oxytocin receptor antagonist L-368-899 on (1) social rank, (2) sex preference, and (3) dyadic interaction in male mice. In the tube test, injection of the oxytocin receptor antagonist had no effect on first-place mice, but caused rank fluctuation of the social rank in second-place mice, suggesting that the function of oxytocin in the maintenance of the social rank is rank-dependent. Second, injection of the oxytocin receptor antagonist dose-dependently impaired the sex preference of the male mice, confirming that oxytocin is essential in sexual behavior. Third, injection of the oxytocin receptor antagonist had no effect on the dyadic interaction between the male mice, suggesting that oxytocin is not necessary in direct social interaction. Taken together, these results demonstrate that the role of the oxytocin in male mice is limited to a specific context of social behavior.

Highlights

- The stable social rank of the male mice in their cages was clearly observed in the tube test.
- Administration of an oxytocin receptor antagonist to first-place mice caused no change in social rank.
- Administration of an oxytocin receptor antagonist to second-place mice caused changes in social rank in some groups.
- Sex preference, but not social and dyadic interactions, were impaired after the injection of the oxytocin receptor antagonist.

Introduction

Social rank is formed through interactions with other individuals and experiences in the community. The existence of social rank helps animals to avoid unnecessary conflicts, reduce energy costs spent in competing for limited resources, and secure comfortable living spaces. Thus, social rank has a profound impact on an individuals' survival, reproductive success, health, and other behaviors.[1]

Despite their importance to societies and individuals, the psychological and neurobiological mechanisms underlying social rank remain unclear. Tube tests have been used to examine the social rank of rodents.[1] In the tube test, two mice were inserted from either end of a thin tube and faced each other at the center of the tube. The rank in rodents can be measured by examining the performance of the mice in a round-robin match within a cage, where the dominant mouse pushes the opponent backward in the tube and pushes him out of the tube. Mice with the highest rank in the tube test are known to groom lower-rank mice excessively and remove the whiskers and/or body hair of lower-rank mice, suggesting that there is a strong correlation between the rank in the tube test and the frequency of dominant behavior over other individuals.[2] In addition, dominant mice tend to exhibit more courtship behavior towards female mice by omitting ultrasonic vocalization than subordinate male mice.[2,3] In a hot spot task, a behavioral task in which four mice were placed on a cold floor and placed to compete for a single warm spot, higher rank mice in the tube test spent more time dominating the hot spot.[4] These studies have demonstrated the utility of the tube test as a reliable behavioral assay for examining social rank in rodents.

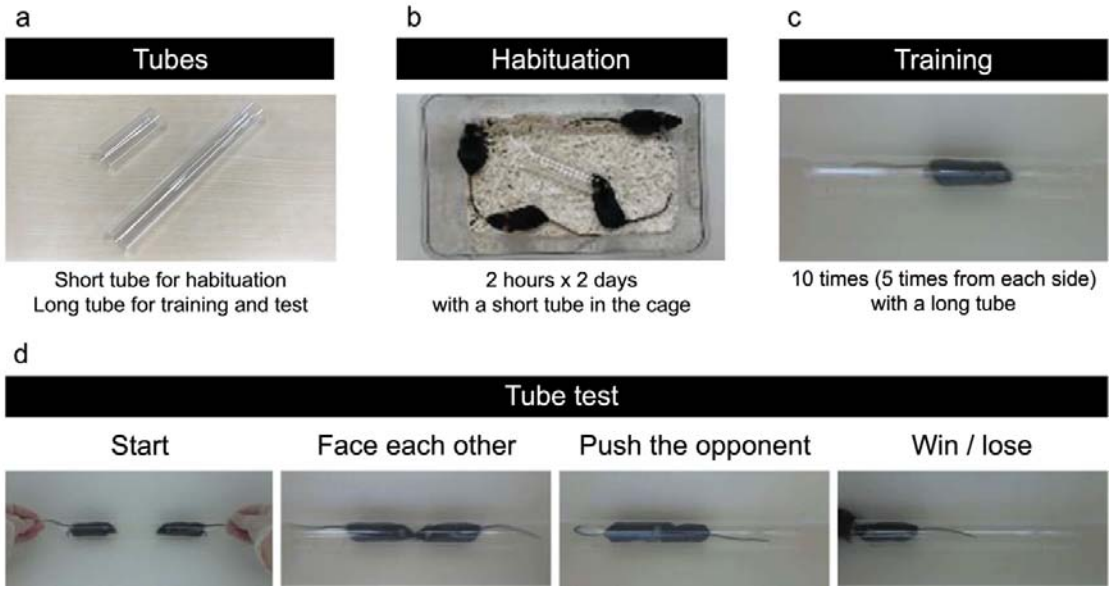
Oxytocin has received increasing attention for its role in social behaviors.[5] Oxytocin has been shown to be involved in a variety of social behavior, including pair-bonding,[6] maternal behavior,[7] aggression,[8] social decision making,[9] observational fear conditioning,[10] and social recognition[11] in many species. Oxytocin is emerging as a candidate pharmacological target for the treatment of mental disorders, especially those with social dysfunction, such as autism spectrum disorder,[12,13] social anxiety disorder,[14] and schizophrenia.[15] A recent study indicated that oxytocin receptor expression in the brain is different in mice with different social ranks.[16] However, the causal relationship between oxytocin levels and social rank remains unclear.

Here, we examined the effects of intraperitoneal administration of a blood–brain barrier penetrating oxytocin receptor antagonist, L-368,899, on various social behaviors. First, we examined the effect of oxytocin receptor antagonist on the tube test to examine the causal relationship between oxytocin and social rank. Second, we examined the effect of the oxytocin receptor antagonist on sex preference to

validate the effect of the oxytocin antagonist on the discrimination of other individuals and on social interaction, and to rule out the possibility that the oxytocin receptor antagonist is not working. Third, we examined the effect of oxytocin antagonists on dyadic interactions between male mice in an open-field box to confirm the effects of oxytocin receptor blockade on direct social interaction between male mice.

Results

In experiment 1, we examined whether oxytocin antagonists affected the social rank of first- and second-rank mice in the tube test. Following habituation (Supplementary Movie 1) and training in the tube (Supplementary Movie 2), the tube test was started (Figure 1, Supplementary Movie 3). When all ranks of the four mice in the cage were kept stable for three consecutive days, we defined that the rank in the cage was stabilized. All cages tested in this experiment showed a linear social rank structure (rank 1, 2, 3, and 4). Total time spent for the match during the stable three consecutive days was different between the rank pairing (Figure 1f, F (2.111, 99.20) = 39.20, p < 0.0001, repeated measures one-way ANOVA). The pair between first- and second-rank mice took longer time until the winner/looser was determined. The pairs including fourth-place mice took significantly shorter time until the winner/looser was determined. There was no correlation between body weights and ranks (Figure 1g, r^2 = 0.0017, N = 60, p = 0.7556, simple linear regression).



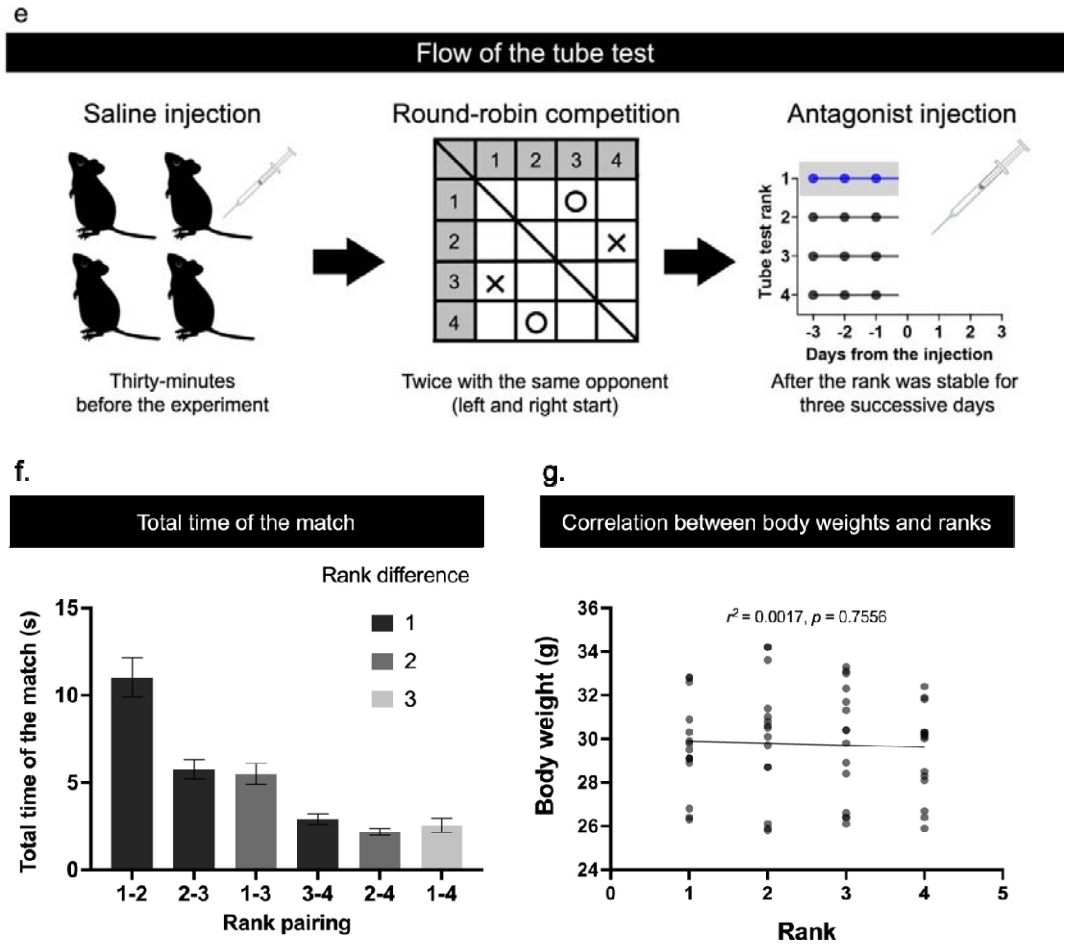


Figure 1. Procedure and results of the tube test

a. A short tube for habituation (35 mm diameter, 2 mm thickness, 30 cm long, acrylic) and a long tube for the tube test (35 mm diameter, 2 mm thickness, 10 cm long, acrylic). **b.** Habituation for the tube in the home cage. Mice were group-housed with four individuals. The short tube was placed in the cage, and the mice were allowed to have free contact with the tube for two hours on two days. **c.** Training for the tube test. During the training phase, the same tube as used in the test was used, and each mouse was trained to pass through the tube 10 times. Mice were started from each entrance on the left and right side five times each, for two days. **d.** After all the ranks in the cage had stabilized for three consecutive days, the first- or second-place individual was given intraperitoneal injections of 10 mg/kg of oxytocin receptor antagonist on the next day. The other individuals were given intraperitoneal injections of saline. **f.** Total time spent in the match of each rank pair is shown. Total time spent for the match during the stable three consecutive days was different between the rank pairing (F (2.111, 99.20) = 39.20, p < 0.0001, repeated measures one-way ANOVA). The pair between first- and second-rank mice took longer time until the winner/looser was determined. The pair includes fourth-place mice took significantly shorter time until the

winner/looser was determined. Error bars indicate standard error of mean. g. Correlation between ranks and weights of the mice is shown. There was no correlation between body weights and ranks (r^2 = 0.0017, N = 60, p = 0.7556, simple linear regression).

The first- or second-place individual was administered intraperitoneal injections of 10 mg/kg of the oxytocin receptor antagonist L-368,899, which crosses the blood-brain barrier and selectively antagonizes central oxytocin receptors, on the next day. When the oxytocin receptor antagonist was administered to first-place mice, there was no change in the rank of all five groups tested (Figure 2a). When oxytocin receptor antagonists were administered to the second-place mice, two groups of second-place mice increased in rank on the day of oxytocin receptor antagonist administration and one group of second-place mice decreased in rank on the day following administration, while the other two groups of second-place mice showed no effect (Figure 2b). We compared the effect of the oxytocin receptor antagonist on the rank change after the injection. There was a significant difference of the effects of the oxytocin receptor antagonist on social rank in between first- and second-place mice ($\chi^2(1) = 4.286$, p = 0.038, Chi-square test). In both first- and second-place mice, injection of the oxytocin receptor antagonist did not change obvious aggressive and affiliative behavior toward the opponent. In addition, there was no change of the body weights after the injection of the oxytocin receptor antagonist in both first- and second-place mice (Supplementary figure 1a-b, first-place mice: t (14) = 1.916, p = 0.076, paired t-test, second-place mice: t (14) = 0.7433, p = 0.470, paired t-test).

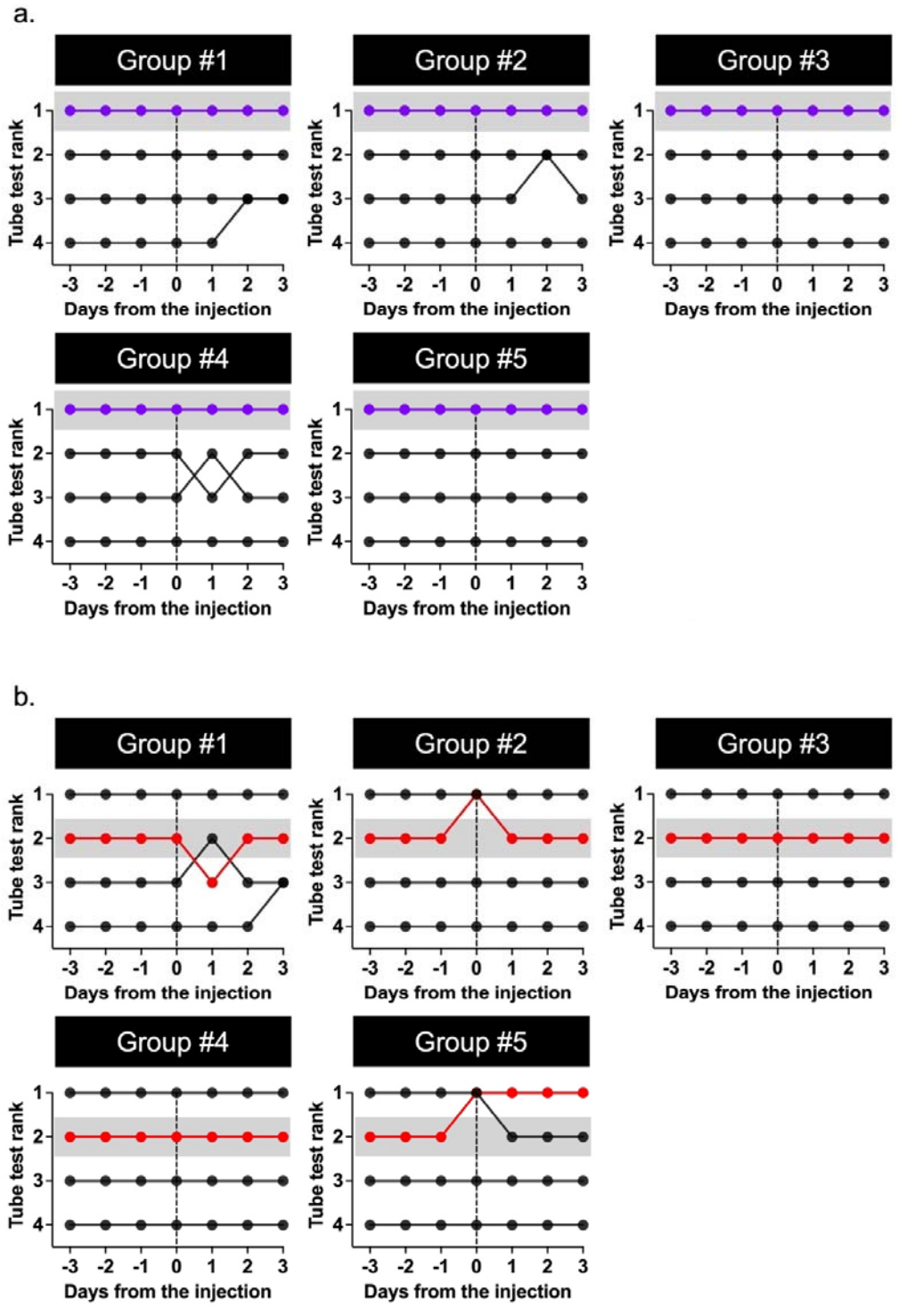


Figure 2. Rank change of first- and second-place individuals after oxytocin receptor antagonist administration

The results of the tube test before and after intraperitoneal injection of the oxytocin receptor antagonist L-368,899 to the first- and second-place mice. a. The results of three days before and after the injection of the oxytocin receptor antagonist on first-place mice are shown. b. The results of three days before and after the injection of the oxytocin receptor antagonist on second-place mice are shown.

To examine whether oxytocin receptor antagonists affect sex preference in male mice, we used a place preference task (Figure 3a). Mice were intraperitoneally injected with saline or 3 or 10 mg/kg of oxytocin receptor antagonists and then placed in a place preference box 30 min after the injection. After 15 min of habituation, the mice were temporally removed from the box, and the stimulus individuals, male and female C57BL/6J mice, were placed in each basket. The subject mice were then placed again in the center of the box and allowed to explore freely in the box for 10 min. We found a dose-dependent impairment of the preference for female mice by the oxytocin receptor antagonist (Figure 3b-d). There was a significant difference in preference for females in the saline group (t (14) = 3.242, p = 0.014, d = 2.192, unpaired t-test) and oxytocin receptor antagonist 3 mg/kg group (t (14) = 2.543, p = 0.023, d = 1.272, unpaired t-test), whereas there was no significant difference in preference for males and females in the oxytocin receptor antagonist 10 mg/kg group (t (14) = 0.673, p = 0.522, d = 0.472, unpaired t-test). We found no significant effect of the oxytocin receptor antagonist on the total time spent with male and female mice between the saline, 3 mg/kg, and 10 mg/kg oxytocin receptor antagonist conditions (F (2,21) = 1.861, p = 0.180, one-way ANOVA).

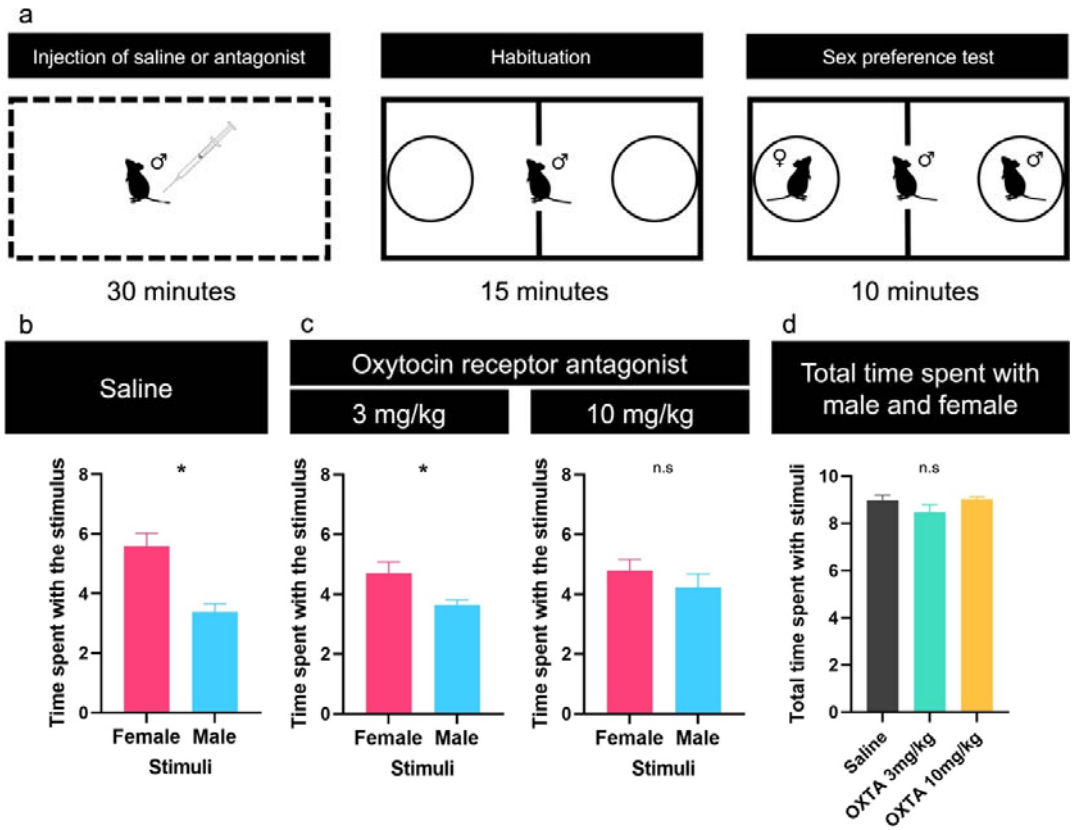
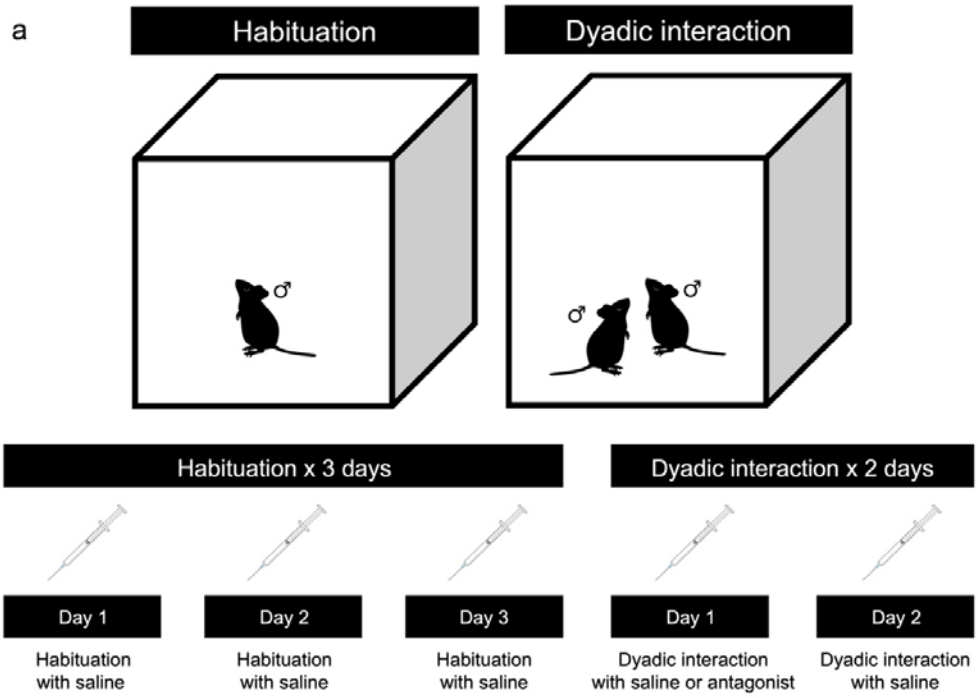


Figure 3. Effect of the oxytocin receptor antagonist on the sex preference in place preference task.

a. The apparatus used for the test was a custom-made white vinyl chloride box (25 cm length × 50 cm width × 30 cm height), with a basket containing the stimulus individuals placed at the center of the 25 cm long box and at both ends of the 50 cm wide box. A wall was attached to the center of the 50 cm width to separate the space on the left and right sides. Mice were intraperitoneally injected with 3 or 10 mg/kg of oxytocin receptor antagonist or saline and then placed in the box 30 min after the injection. After 15 min of habituation, the mice were temporally removed from the box, and the stimulus individuals were placed in each basket. The subject mice were then placed in the center of the box and allowed to move freely for 10 min. White noise (75 dB) was presented throughout the experiment to mask the external noise. **b.** Time spent with the male and female stimuli in male mice 30 minutes after the injection of saline. Male mice preferred to spend more time with female mice. There was a significant difference in time spent in female and male mice (t (14) = 3.242, p = 0.014, d = 2.192, unpaired t-test). **c.** Time spent with the male and female stimuli in male mice 30 minutes after the injection of oxytocin receptor antagonist. Preference of the male mice to the female mice was impaired in the condition of the oxytocin receptor antagonist at

the dose of 10 mg/kg. There was a significant difference in time spent in female and male mice in the dose of 3 mg/kg (t (14) = 2.543, p = 0.023, d = 1.272, unpaired t-test), but no significant difference was found in the dose of 10 mg/kg (t (14) = 0.673, p = 0.522, d = 0.472, unpaired t-test). **d.** Total time spent with male and female mice between saline, 3 mg/kg, and 10 mg/kg oxytocin receptor antagonist conditions. There was no significant difference in total time spent with the stimuli between the conditions (F (2,21) = 1.861, p = 0.180, one-way ANOVA). Error bars indicate standard error of mean.

To examine the effects of an oxytocin receptor antagonist on dyadic interactions between two male mice, we observed dyadic interactions in an open-field box (Figure 4a, Supplementary Movie 4). In this experiment, we injected the oxytocin receptor antagonist or saline into both male cagemate mice 30 min before the experiment, and examined the time spent in dyadic interaction and the average distance between the two mice (Figure 4b-c). Intraperitoneal injection of oxytocin receptor antagonist had no effect on the contact duration (F (1,8) = 0.1425, p = 0.7156, two-way repeated measures ANOVA) or distance between the individuals (F (1,8) = 0.0888 p = 0.7732, two-way repeated measures ANOVA). We found a significant decrease in the contact duration (F (1,8) = 11.21, p = 0.0101, two-way repeated-measures ANOVA) and the distance between the mice (F (1,8) = 6.216, p = 0.0373, two-way repeated-measures ANOVA) from day 1 to day 2. The experimental day × drug interaction had no significant effect on the contact duration (F (1,8) = 0.03277, p = 0.8609, two-way repeated-measures ANOVA) or distance between mice (F (1,8) = 2.332, p = 0.1653, two-way repeated-measures ANOVA).


a
Habituation
Dyadic interaction
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Day 1
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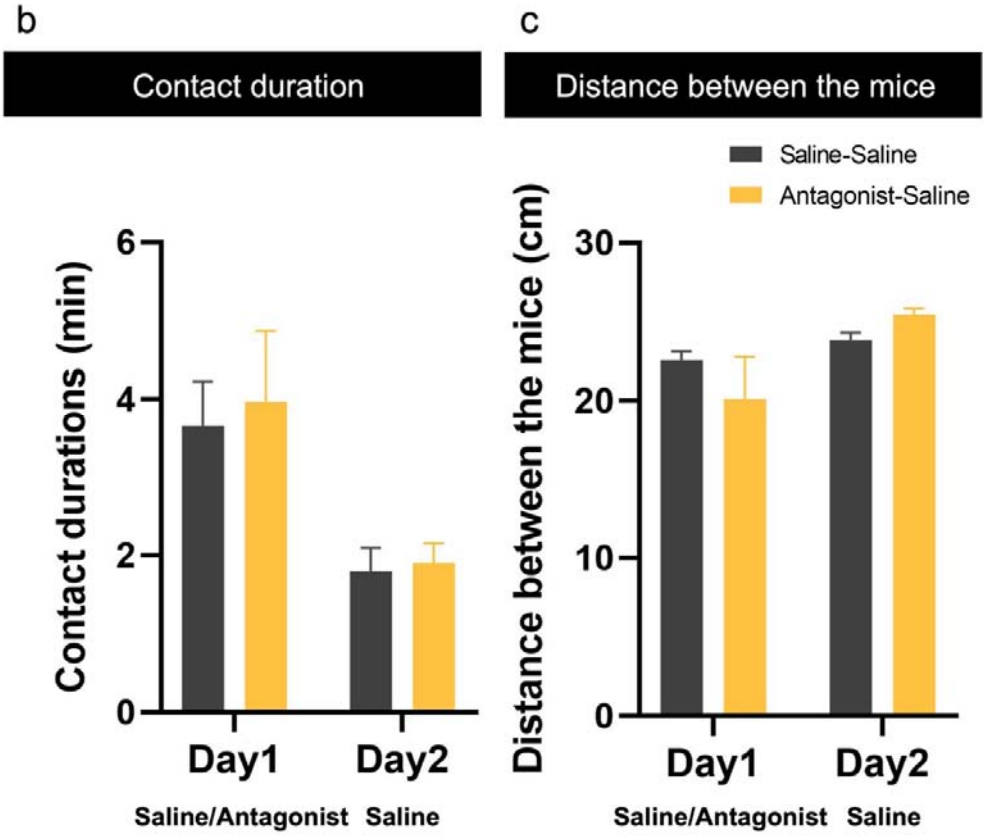
b
Contact duration
Contact durations (min)
0
2
4
6
Day1
Day2
Saline/Antagonist
Saline
c
Distance between the mice
Saline-Saline
Antagonist-Saline
Distance between the mice (cm)
0
10
20
30
Day1
Day2
Saline/Antagonist
Saline

Figure 4. Effects of the oxytocin receptor antagonist on contact time and distance between individuals in dyadic interaction in the open-field box

a. Habituation to the injection and apparatus was performed for 30 min per day for three days prior to the start of the experiment. The apparatus was a custom-made white vinyl chloride box measuring 50 cm in length × 50 cm in width × 50 cm in height. The cameras (Logicool HD Webcam C270n, Logicool) were placed 106 cm above the bottom of the box. The video was recorded on a Windows PC. During habituation, the mice were placed in an open-field box 30 min after receiving an intraperitoneal injection of saline and allowed to explore freely. The experiment was conducted over two successive days. On day 1, an oxytocin receptor antagonist or saline was injected intraperitoneally 30 min prior to the experiment. On day 2, saline was injected intraperitoneally 30 minutes before the experiment. The duration of the experiment was 30 minutes. White noise (75 dB) was presented throughout the experiment to mask the external noise. **c.** We examined the effects of intraperitoneal injection of an oxytocin receptor antagonist on dyadic interactions in an open field box. In this experiment, we injected the oxytocin receptor antagonist or saline into male cagemate mice 30 min before the experiment, and then examined the contact duration and average distance between the two mice. The intraperitoneal injection of oxytocin receptor antagonist had no effect on the contact duration (F (1,8) = 0.1425, p = 0.7156, two-way repeated-measures ANOVA) or distance between the individuals (F (1,8) = 0.0888 p = 0.7732, two-way repeated-measures ANOVA). The experimental day had a significant effect on the contact duration (F (1,8) = 11.21, p = 0.0101, two-way repeated-measures ANOVA) and distance between the mice (F (1,8) = 6.216, p = 0.0373, two-way repeated-measures ANOVA). The experimental day × drug interaction had no significant effect on the contact duration (F (1,8) = 0.03277, p = 0.8609, two-way repeated-measures ANOVA) or distance between mice (F (1,8) = 2.332, p = 0.1653, two-way repeated-measures ANOVA). Error bars indicate standard error of mean.

Discussions

To clarify the role of oxytocin receptors in social rank and other social behaviors, this study examined the effects of intraperitoneal injection of the oxytocin receptor antagonist L-368,899, which crosses the blood-brain barrier and selectively antagonizes central oxytocin receptors in male mice. In experiment 1, we obtained a stable social rank within a cage of four C57BL/6J mice using the tube test after habituation and training. Following the determination of social rank in the group for three consecutive days, we systemically administered the oxytocin receptor antagonist to first- and second-place mice. Intraperitoneal injection of the oxytocin receptor antagonist had no effect on first-place mice but had an effect on second-place mice in the social rank, suggesting that the role of oxytocin in the maintenance of the social rank is rank-dependent. In experiment 2, sex preference was impaired without affecting social preference by the intraperitoneal injection of oxytocin receptor antagonist, supporting the existing finding that oxytocin plays an important role in sexual behavior or motivation[17-20] but not in social interactions.[11,19,21,22] In experiment 3, contact time and distance between the male mice in dyadic interaction in the open-field situation were not affected by the intraperitoneal injection of the oxytocin receptor antagonist, confirming that oxytocin is not important in social interaction. Taken together, our results demonstrate that the role of oxytocin in the social behavior of male mice is limited to specific contexts.

In experiment 1, stable social rank was obtained using a tube test. Total time spent for the match during the stable three consecutive days was different between the rank pairing. This result is consistent with previous findings using a tube test.[1,2,4,23] In addition, there was no correlation between the body weight and the ranks. The C57BL/6J mice used in this experiment were inbred, and all mice were almost genetically identical. Because the mice were reared in the same cage from birth, their environmental factors throughout development were almost identical. Nevertheless, a stable and linear social rank relationship was demonstrated for all the cages. This result suggests that social rank is generated even if there are no genetic or inherited differences in the given situation. In this sense, social rank is a product of learning, a long-term change in behavior caused by experiences in their environment.

In the tube test, there was no change in the rank of the first-place mice after injection of the oxytocin receptor antagonist, demonstrating that oxytocin receptor antagonist administration had no effect on social dominance. Meanwhile, the oxytocin receptor antagonist affected the rank of second-place mice. In three of the five groups of oxytocin receptor antagonist-injected second-place mice, the rank fluctuated on the

day or day after the oxytocin receptor antagonist injection. The body weight of the second-place mice after the oxytocin receptor antagonist was not different from the body weight before the injection, ruling out the possibility that the gain or loss of the body weight affected the fluctuated rank. One possible explanation for this result is the difference in the neurobiological states between dominant and subdominant mice. In a study that utilized autoradiography to determine the degree of oxytocin and vasopressin receptor binding in many brain regions in mice of different social ranks, the differential expression of oxytocin receptors in the brain was observed in dominant, subdominant, and subordinate mice.[16] Different baseline expression levels of oxytocin receptors might explain the differential effects of oxytocin antagonists on dominant and subdominant mice in the present study.

Another possible explanation for the rank-dependent effects of oxytocin receptor antagonists is the difference in the necessity of individual discrimination between first- and second-place mice. For first-place mice, there is little need for individual discrimination, since every mouse to compete with was a subordinate for the first-place mice. For second-place mice, however, they need to change their behavior according to whether the opponent is dominant or subordinate, so individual recognition is important. If individual discrimination or response selection based on information is impaired, the rank of second-place mice fluctuates. The difference in the necessity of individual discrimination might explain the differential effects of the oxytocin receptor antagonist on first- and second-place mice.

In the tube test, the oxytocin receptor antagonist was administered after the social rank of the mice stabilized. The finding that the oxytocin receptor antagonist has no effect on first-place mice indicates that oxytocin receptors do not play an important role in the "maintenance" of social dominance. The role of the oxytocin receptors on the “acquisition” of social dominance is still unknown. Oxytocin receptor antagonists inhibit the acquisition of partner preference in female prairie voles.[24] According to the social salience hypothesis of oxytocin,[25] the critical role of oxytocin may be related to the acquisition but not the maintenance of social signals. Further studies are needed to clarify the effect of oxytocin receptor manipulation on the acquisition of social rank.

In experiment 2, we examined the role of oxytocin receptors in sex preference in male mice. A significant preference for mice of the opposite sex was observed in the saline group, confirming that male mice prefer to spend more time with females. The preference for females in the male mice decreased in a dose-dependent manner in the oxytocin receptor antagonist group. In particular, there was no significant preference for female mice in the dose of the oxytocin receptor antagonist at 10 mg/kg.

In addition, there was no significant difference in the total time spent with male and female mice, implying that social preference, irrespective of sex preference, was not impaired by injection of the oxytocin receptor antagonist. Although we found significant sex preference at a dose of 3 mg/kg, these results support the findings of Haskal de la Zerda et al.,[19] who demonstrated the importance of the oxytocin receptor in sex preference but not social preference using L-368,899, the same oxytocin receptor antagonist used in this study. These results in intact social preference after the blockade of the oxytocin receptors are also consistent with the results obtained from oxytocin knockout mice[11,21,22] but are in contrast to the oxytocin receptor knockout mice.[26,27]

In experiment 3, the intraperitoneal injection of the oxytocin receptor antagonist to two familiar cagemate mice had no effect on the contact duration and distance between the male mice in the dyadic interaction in the open-field box. This result indicates that oxytocin is not important for dyadic interactions between male mice. This result confirmed that the impaired sex preference of male mice in experiment 2 was not due to an increased preference for the same sex in male mice. Both the contact duration and distance between the male mice decreased on the day after injection of the oxytocin receptor antagonist. This result suggests that habituation to the existence of the other in the open-field context occurred in both oxytocin receptor antagonist and saline injection groups. Habituation is also a type of learning and the subject mice are well habituated to the box and experimental room before the dyadic interaction. The result indicates that the oxytocin receptor is not critical in learning the context of the dyadic interaction in the open-field box.

Taken together with the results found in experiments 1, 2, and 3, intraperitoneal injection of the oxytocin receptor antagonist might affect the response selection based on individual recognition. Compared to primates and birds, mice and other rodents have not developed vision during the course of evolution. In the perceptual world of mice, olfaction likely plays an important role in discriminating between individuals. Indeed, oxytocin receptors are known to be distributed in many olfaction-related brain regions,[28] and inhibition of oxytocin in the anterior olfactory nucleus impairs the olfactory perception of individuals without affecting non-social odor discrimination.[29] A previous important finding suggests that sensory signals, such as olfactory and somatosensory cues, may not play a critical role in determining social rank in the tube test.[29] Altered individual discrimination induced by the impairment of olfactory perception contributes to impaired sex preference by the oxytocin receptor antagonist, but not to rank-dependent social rank alternation.

Recently, many laboratories have reported clear behavioral differences between dominant and subordinate mice in the same cage. Chou et al.[30] further showed that the most dominant mice spent more time in the tube even if the material in the tube prevented further forward movement to the exit of the tube with no opponent, implying that one of the critical factors that determines the social rank in a cage may not be the condition of the dominant mice, but the conditions of the subordinate mice. Kunkel and Wang[23] reported an increased social preference in dominant mice. A recent finding suggests that social dominance and mnemonic abilities are correlated in young human children and mice.[31] These results demonstrate that the behavior of the dominant mouse is different from that of other cagemates, implying that various social and nonsocial behaviors are modulated by social rank.

The relationship between oxytocin and social memory has been repeatedly demonstrated in oxytocin knockout mice[11] and in microinjections of the oxytocin receptor antagonist in the medial amygdala and lateral septum.[32] However, the result of the dyadic interaction indicates that individual recognition of familiar mice might not be impaired by the oxytocin receptor antagonist because there was no impairment in the contact time and distance between the male mice in the dyadic interaction on the day and day after the injection of the oxytocin receptor antagonist. In this experiment, we used familiar mice in the cages of two mice. In this situation, there are only dominant and subordinate mice in the cage, which is different from the cage of four mice that have a more complicated social structure. For mice in a large social network, the necessity of individual discrimination and response selection based on this information is critical and complicated. The difference in the size of the social structure might cause differential effects of the oxytocin receptor antagonist on rank-dependent fluctuations in social rank. Our results extend the previous finding by demonstrating that the oxytocin receptor antagonist may play an essential role in the complicated social context that requires response selection based on individual recognition. Further studies are necessary to elucidate the psychological and neurobiological mechanisms underlying social ranking.

The concentrations of 3 or 10 mg/kg of the oxytocin receptor antagonist used in this study were higher than those used in previous studies on rodents[19,33,34] and primates.[17] A concentration of 10 mg/kg was chosen as it had clear effects in experiment 2 and in a study that examined the effects of the oxytocin receptor antagonist on observational fear conditioning in mice.[10] A non-human primate study with rhesus monkeys validated that peripherally administered L-368,899 entered the cerebrospinal fluid (CSF) and accumulated in the hypothalamus, septum, orbitofrontal

cortex, amygdala, and hippocampus, but not in other areas.[17] In that study, the authors also demonstrated that peripherally administered L-368,899 at 1 and 3 mg/kg reduced or eliminated interest in infant and sexual behavior. Studies in mice have also shown that an oxytocin receptor antagonist dose of 3 mg/kg affects sex preference and other tasks.[19] Despite the strong oxytocin receptor blockade, oxytocin receptor antagonist administration had no effect on social dominance in the first-place mice, confirming that oxytocin is not important for dominant behavior.

In general, research on oxytocin has focused a great deal of attention on its role in social behavior. However, the majority of the research has been based on findings from studies in which oxytocin itself, that is, oxytocin agonists, is administered to alter social behavior. Few studies have examined the effects of centrally acting selective antagonists of oxytocin receptors on behavior, compared to the attention given to oxytocin agonists. The experimental results of the present study, in which the effects of oxytocin receptor antagonists were limited to specific situations, suggest that oxytocin receptors do not play an important role in various social behaviors. This evidence is supported by the "social salience hypothesis" of oxytocin,[24] which suggests that oxytocin may play an important role in the salience of social stimuli by way of its relationship with the neurotransmitter dopamine.

Conclusions

In this study, we examined the effect of systemic administration of the oxytocin receptor antagonist L-368,899, which crosses the blood-brain barrier and selectively antagonizes central oxytocin receptors, on (1) the social rank of the mice using a tube test, (2) sex preference with place preference task, and (3) dyadic interaction between the mice in the open-field task. We obtained a stable rank for mice reared in the same cage. Intraperitoneal injection of the oxytocin receptor antagonist to first-place mice had no effect on social rank, suggesting that oxytocin is not critical for the maintenance of social rank. On the other hand, the injection of the oxytocin receptor antagonist to second-place mice caused fluctuations in social rank, suggesting that the role of oxytocin in social rank is rank-dependent. Second, intraperitoneal injection of the oxytocin receptor antagonist impaired the sex preference of male mice in the place preference task, confirming that oxytocin plays an essential role in sexual behavior. Third, intraperitoneal injection of the oxytocin receptor antagonist had no effect on the dyadic interaction between male mice in the open-field box, suggesting that oxytocin is not essential for social interactions between male mice in a semi-naturalistic social context. Taken together, these results indicate that the role of oxytocin in male mice is limited to a specific context of social behavior. We have only begun to learn how the brain represents social rank.[35-37] Future studies need to specify the psychological factors that determine social rank in depth, and the neurobiological causal and correlational relationship between social rank and its underlying neurobiological mechanisms need to be elucidated.

STAR Methods

Subjects

All data were collected from 2-6 to month-old adult male wild-type C57BL/6J mice. The mice were maintained on a 12:12 light cycle. All the experiments were conducted during the dark phase of the light cycle. The mice had unrestricted access to food and water in their cages. Their weights were monitored daily. All animal procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Research Committee of Keio University. This study is reported in accordance with ARRIVE guidelines (https://arriveguidelines.org).

Procedure

Experiment 1: Tube test

In experiment 1, we examined the effects of intraperitoneal administration of the oxytocin antagonist L-368,899 on social rank in mice. We used 3-6 month old 24 adult male C57BL/6J mice. All the mice were reared and group-housed with four individuals in their home cages. All mice were born to the same parents in each cage. The procedure of the tube test followed the protocol described by Fan et al.,[38] with some modifications. The tube test experiment was divided into three phases: habituation, training, and testing. In the habituation phase, a short tube (35 mm in diameter, 2 mm thickness, 10 cm long, acrylic, Figure 1a) was placed in the cage, and the mice were allowed free contact with the tube for two hours for two days (Figure 1b, Supplementary movie 1). During the training phase, a long tube (35 mm diameter, 2 mm thickness, 30 cm long, acrylic, Figure 1a) was used, and each mouse was trained to pass through the tube 10 times in total (Figure 1c, Supplementary movie 2). Mice were started from each entrance on the left and right sides five times each for two days. In the testing phase, the mice competed in a round-robin competition for the tube test (Figure 1d). We conducted the tube test in the 63.5 cm length x 40.0 cm width x 60.0 cm height box (MED Associates) with the door kept opened. A long tube (35 mm diameter, 2 mm thickness, 30 cm long, acrylic, Figure 1a) was used in the test phase. White noise (75 dB) was presented throughout the experiment to mask the external noise. Cameras (Logicool HD Webcam C270n, Logicool) were placed on the side of the tube and were located 30 cm apart from the tube. While the mice performed the tube test, a movie was recorded on a Windows PC. Because there were left and right entrance conditions, the mice had to compete against the same opponent twice from each starting location. The order of matches was set randomly using a random number generator. To eliminate potential order fluctuations induced by the effects of needle penetration and injection,

saline was administered intraperitoneally to all four mice 30 min before the start of every experiment. The tube test was initiated when the experimenter placed the mice in the tube and simultaneously released their hands from their tails (Supplementary Movie 3). In the test, the winner was determined when either the mouse pushed the other mouse out of the tube or stepped back and out of the tube. The mouse whose hind leg went outside the tube was the loser, and the mouse that stayed in the tube longer than the opponent was the winner. The winner/loser was determined in all competitions without setting a time limit. When all ranks of the four mice in the cage were kept stable for three consecutive days, we determined that the rank in the cage was stabilized. Obtaining a stable rank in the cage took at least three days. After the rank had stabilized for three consecutive days in the test, the first- or second-place individual was administered intraperitoneal injections of 10 mg/kg oxytocin receptor antagonist on the next day. The other mice were administered an intraperitoneal injection of saline. On the day after oxytocin receptor antagonist administration, all four mice received saline, and the experiment continued until the third day after oxytocin receptor antagonist administration (Figure 1e).

Experiment 2: Sex preference in the place preference task

To examine whether oxytocin receptor antagonists affect sex preference in male mice, we used a place preference task (Figure 3). We used 2-4 month old 24 adult male C57BL/6J mice and 2-4 month old 6 adult male and female C57BL/6J mice as stimuli. All mice were reared and single- or group-housed with 2-4 individuals in their home cages. A custom-made white vinyl chloride box (25 cm length × 50 cm width × 30 cm height) was used for the test. Male and female mice were placed in a white cylindrical pen stand (8 cm diameter × 10 cm height). The pen stands containing the stimulus individuals were placed at the center of both ends of a 50 cm wide box. A 500 mL empty glass bottle was placed on top of the pen stand to prevent the subject mouse from climbing. Two partition walls (5 cm length × 5 mm thickness × 30 cm height) were attached to both sides of the center of the 50 cm width box to separate the spaces on the left and right sides. The cameras (Logicool HD Pro Webcam C920r, Logicool) were placed 80 cm above the bottom of the box. While the mice performed the sex preference test, a movie was recorded on a Windows PC. Mice were intraperitoneally injected with 3 or 10 mg/kg of oxytocin receptor antagonist or saline and then placed in the box 30 min after the injection. After 15 min of habituation, the mice were temporally removed from the box, and the stimulus individuals, male and female C57BL/6J mice, were placed in the basket. The subject mice were then placed again in the center of the box and allowed to explore freely in the box for 10 min. White noise (75 dB) was

presented throughout the experiment to mask the external noise.

Experiment 3: Dyadic interaction in the open-field box

To examine the effects of an oxytocin receptor antagonist on dyadic interactions between two male mice, we observed dyadic interactions in an open-field box (Figure 4, Supplementary Movie 4). We used 2-3 month old 20 adult male C57BL/6J mice. All the mice were group-housed with two individuals in their home cages. Habituation to the saline injection and apparatus was performed for 30 min per day for three days prior to the start of the experiment. The apparatus was a custom-made white vinyl chloride box measuring 50 cm in length × 50 cm in width × 50 cm in height. The cameras (Logicool HD Webcam C270n, Logicool) were placed 106 cm above the bottom of the box. While the mice performed the dyadic interaction in the open-field box, a movie was recorded on a Windows PC. During habituation, the animals were placed in an open-field box immediately after receiving an intraperitoneal injection of saline and were allowed to behave freely. The experiment was conducted over two successive days. On day 1, an oxytocin receptor antagonist or saline was injected intraperitoneally 30 min prior to the experiment. On day 2, saline was injected intraperitoneally 30 minutes before the experiment. The duration of the experiment was 30 minutes. White noise (75 dB) was presented throughout the experiment to mask the external noise.

Drug

The oxytocin receptor antagonist L-368,899 (CAS 160312-62-9, Tocris Bioscience) was dissolved in saline, divided into small aliquots, and stored in a deep freezer at -80°C until further use. Prior to the experiment, we defrosted the oxytocin receptor antagonist solution and administered it intraperitoneally at a dose of 3 mg/kg in experiment 2 or 10 mg/kg in experiments 1, 2, and 3. For habituation and control conditions, saline was administered intraperitoneally, as in the drug condition. The concentration of the oxytocin receptor antagonist used in this study (3 or 10 mg/kg) was relatively higher than that used in previous studies (1 or 3 mg/kg), which reported the behavioral effects of such administration.[17,19,32,33] A concentration of 10 mg/kg was chosen because it yielded the maximal effect in experiment 2 and a previous study in mice.[10]

Analysis

In all experiments, RStudio and GraphPad Prism were used for data analysis. In experiments 2 and 3, we used an open-source visual programming framework, Bonsai[39] to track the activities of mice using computer vision analysis. The videos were converted to grayscale, smoothened, and monochrome inverted. The mice were identified by setting a contrast threshold. The locomotor activity of the mice in the box

was quantified by measuring the changes in the central coordinates of the mice. In experiment 2, we defined the mice as spending time with the stimulus if they were located within 7.5 cm from the edge of the box containing stimulus mice. In experiment 3, we determined the distance between two male mice. If the distance between the two mice was within 5 cm, we defined the mice as being in contact with each other.

Data availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Code availability

The original codes written for the analyses are available from the corresponding author upon reasonable request.

Competing interests

The authors declare no competing interests.

Acknowledgements

This research was supported by JSPS KAKENHI 20J21568 (KY), 18KK0070 (KT), 19H05316 (KT), 19K03385 (KT), 19H01769 (KT), 22H01105 (KT), Keio Academic Development Fund (KT), and the Keio Gijuku Fukuzawa Memorial Fund for the Advancement of Education and Research (KT). We thank Kohei Yamamoto, Shohei Kaneko, and Yasuyuki Niki for their assistance and valuable discussions. We also thank Dr. Takaaki Ozawa for his valuable comment on the manuscript.

Author contributions

D.N. and K.T. designed all the experiments. D.N. collected all the tube test data. D.N. and K.T. collected data on sex preference and dyadic interaction tasks with the help of H.H. and S.Y. H.H., R.T., and Y.U. helped to set up the experimental apparatus. D.N. and K.T. analyzed the data. K.T. wrote the manuscript. D.N. and K.T. created all the figures. D.N., K.Y., Y.U., and K.T. discussed the data and commented on the manuscript accordingly. K. T. revised the manuscript.

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