



# Effects of the Female Estrous Cycle on the Sexual Behaviors and Ultrasonic Vocalizations of Male C57BL/6 and Autistic BTBR T+ tf/J Mice

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A primary characteristic of autism, which is a neurodevelopmental disorder, is impaired social interaction and communication. Furthermore, patients with autism frequently show abnormal social recognition. In mouse models of autism, social recognition is usually assessed by examining same-sex social behavior using various tests, such as the three-chamber test. However, no studies have examined the ability of male mice with autism to recognize the estrous cycle of female partners. In this study, we investigated the sexual behaviors, especially mounting and ultrasonic vocal communication (USV), of BTBR T+ tf/J (BTBR) mice, which are used as a well-known mouse model of autism, when they encountered estrus or diestrus female mice. As expected, C57BL/6 mice mounted more female mice in the estrus stage compared with the diestrus stage. We found that BTBR mice also mounted more female mice in the estrus stage than female mice in the diestrus stage. Although the USV emission of male mice was not different between estrus and diestrus female mice in both strains, the mounting result implies that BTBR mice distinguish sexual receptivity of females.

**Key words:** Autism, Social recognition, Estrous cycle, Sexual interaction, Ultrasonic vocalization

## INTRODUCTION

Most female mammals experience the estrous cycles, which are physiological cycles regulated by fluctuations of gonadotropic hormones. The cycle is usually divided into four stages, the proestrus, estrus, metestrus, and diestrus, according to the levels of sexual hormones, such as estrogen and progesterone [1, 2]. The duration of each cycle varies among species and ranges from a

few days to several months. For mice, the average cycle duration is about 4~5 days [3].

The estrous cycle affects many behavioral and psychiatric states, including mood, anxiety, stress responses, sexual receptivity, learning, and memory [4-9]. In particular, sexual receptivity is highest at a stage in which copulation is crucial for successful reproduction [10]. Furthermore, the sniffing and mounting behaviors of male mice, which are primary actions that occur during copulation, are more frequent in response to urine from estrous females compared with urine from metestrus or diestrus mice [11]. The findings of these experiments suggest that male mice can recognize the sexual receptivity of female mice.

However, until now, little is known about how abnormal conditions affect the responses of male mice to females in

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different estrous states. In particular, patients with autism, which is a neurodevelopmental disorder with a primary characteristic of impaired social interaction, frequently exhibit deficits in the perception of social contexts and recognition of feelings or emotional states of others [12-15].

Sexual interaction is obviously a kind of social interaction, and patients with autism manifest abnormal sexual behaviors. Some patients show inappropriate sexual behaviors in public places, and there are reports which show that sexual offenders are more likely to have autistic symptoms [16-18]. Not only human patients, but also autistic mouse models have been characterized by abnormal sexual behaviors. For example, the copulatory behavior was decreased and intromission latency was increased in Balb/c mice [19, 20]. An environmental autistic mouse model, which is prenatally exposed to valproate, showed increased mounting [21]. Based on these abnormal sexual behaviors in autism mouse models, we wondered if male autistic mice could distinguish appropriately the estrous cycle of female mice during sexual interaction.

Thus, we investigated it using BTBR mice, which are a well-known autistic mouse model [22, 23], comparing their behaviors with those of the highly social C57BL/6 (B6) mice. Specifically, we analyzed the mounting behaviors, and ultrasonic vocalization (USV), which is thought to be implicated in social communication and sexual interactions [24-26], during interactions of male and estrus or diestrus females

## MATERIALS AND METHODS

### Mice

Eight to fifteen-week-old BTBR and C57BL/6 mice were used in all experiments. Mice were kept on a 12 h light/dark cycle, and the behavioral experiment was performed during the dark phase of the cycle. Food and water were provided *ad libitum*. The mice were kept in a clean rack at 24~25°C and 50~60% humidity. The Institutional Animal Care and Use Committee of Seoul National University approved the animal protocols and experiments (SNU-130807-3-3). Before each experiment, the mice were placed on a shelf for 30 min for acclimatization. Male mice used in all experiments were sexually inexperienced before the tests.

### Determining the estrous stages of female mice

The estrous stages of female mice were determined 1 h before each behavior experiment. Vaginal smears were flushed with 10  $\mu$ l of saline 3~5 times and 2  $\mu$ l of the fluid was spread on a slide glass. Images were then obtained using a light microscope.

### Reciprocal sexual interaction

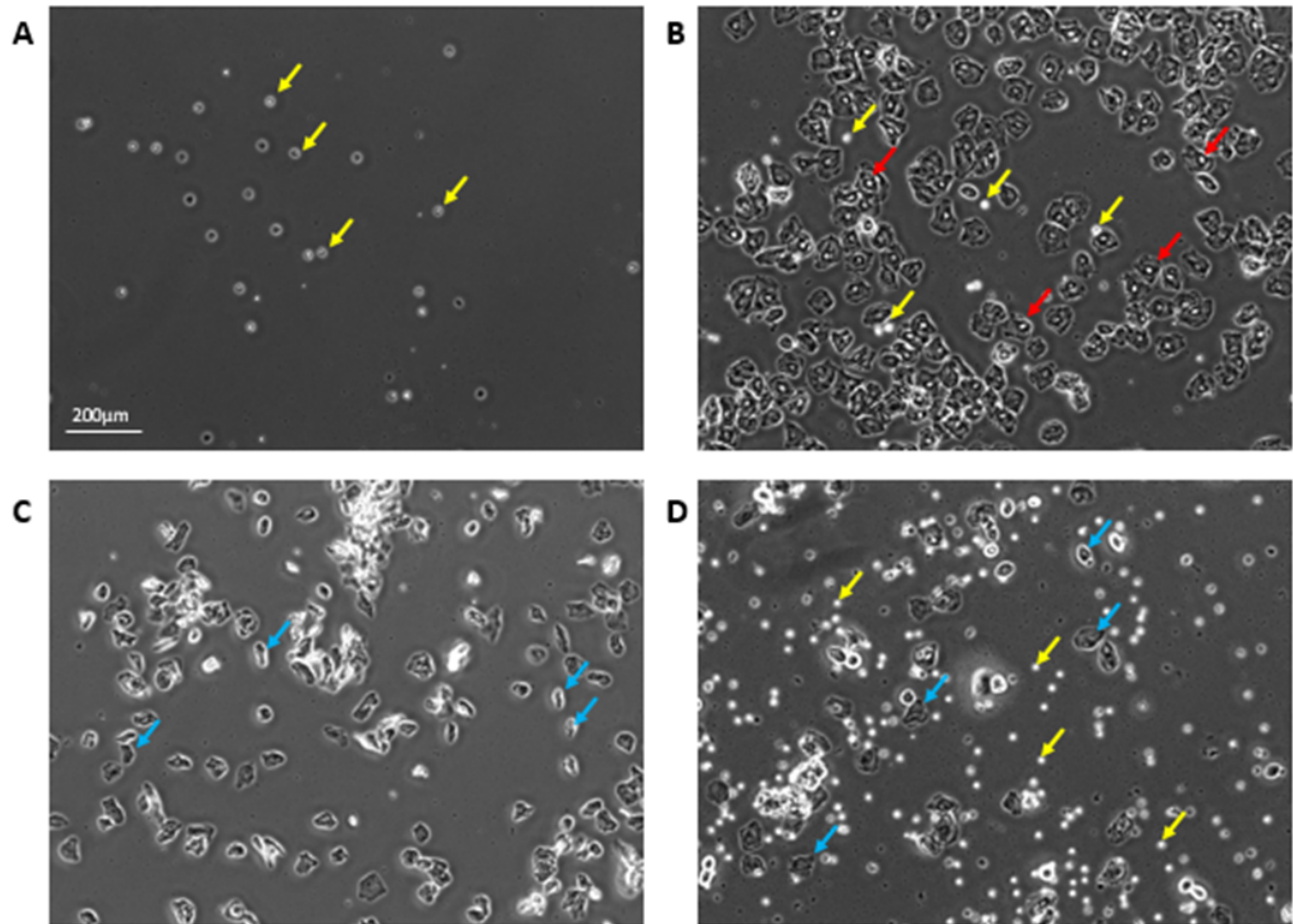
Male mice were individually housed in a standard cage for at least 7 days. On the test day, a male mouse and its home cage were brought into the test room, and the mouse was allowed to habituate for 30 min under dim light. After habituation, an estrus or diestrus female mouse was introduced into the cage, and the behavior of the male mouse was video recorded for 10 min. The numbers of mounting and sniffing incidents were manually counted by an examiner who was blind to the estrous stage of the females.

### USV

A male mouse that had been caged alone at least for 7 days was brought in its home cage into the test room under dim light. The home cage was placed inside a sound-attenuating Styrofoam box for USV recording. A microphone (CM16/CMPA, Avisoft Bioacoustics e.K., Glienicke, Germany) was fitted onto a transparent lid with a hole in the center. The microphone was connected to a USV recording device (UltraSoundGate 116H, Avisoft Bioacoustics e.K.). After a 30-min habituation period, an estrus or diestrus female mouse was introduced into the cage, and the USV calls during sexual interactions were recorded for 5 min. The sampling rate was set at 250 kHz and the bit depth was formatted to 16 bits for recording by RECORDER Hardlock software program (Avisoft Bioacoustics e.K.). The recorded data were processed with SASLab Pro software program (Avisoft Bioacoustics e.K.). Signals with frequencies less than 35 kHz and noise with vertical patterns were removed. Each call in the recorded data was automatically determined (Hold time: 10 ms, Overlap: 75%, Hamming window). Dominant frequency, bandwidth, and duration were determined automatically with SASLab.

## RESULTS

We examined if BTBR mice, which are used as a well-known animal model of autism, respond differently to the different estrous stage of females. Thus, we determined the estrous stage of adult female mice (B6 or BTBR) by examining vaginal smears [1, 27]. During diestrus, which involves the regression of the corpus luteum, leukocytes were dominant, and little epithelial cells were observed (Fig. 1A). During proestrus, when the follicles in the ovaries begin to grow, nucleated epithelial cells were dominant, and leukocytes were still present (Fig. 1B). During estrus, when the female is sexually receptive, mostly cornified cells were observed (Fig. 1C). During metestrus, cornified epithelial cells and leukocytes were present (Fig. 1D). We chose to examine the estrus



**Fig. 1.** Classification of estrous cycles of female mice. (A) Diestrus. The leukocytes (yellow arrow) are dominant, and epithelial cells are rare. (B) Proestrus. Nucleated epithelial cells (red arrow) are dominant, and leukocytes are still present. (C) Estrus. Mostly cornified cells are observed. (D) Metestrus. Cornified epithelial cells (cyan arrow) with leukocytes are observed.

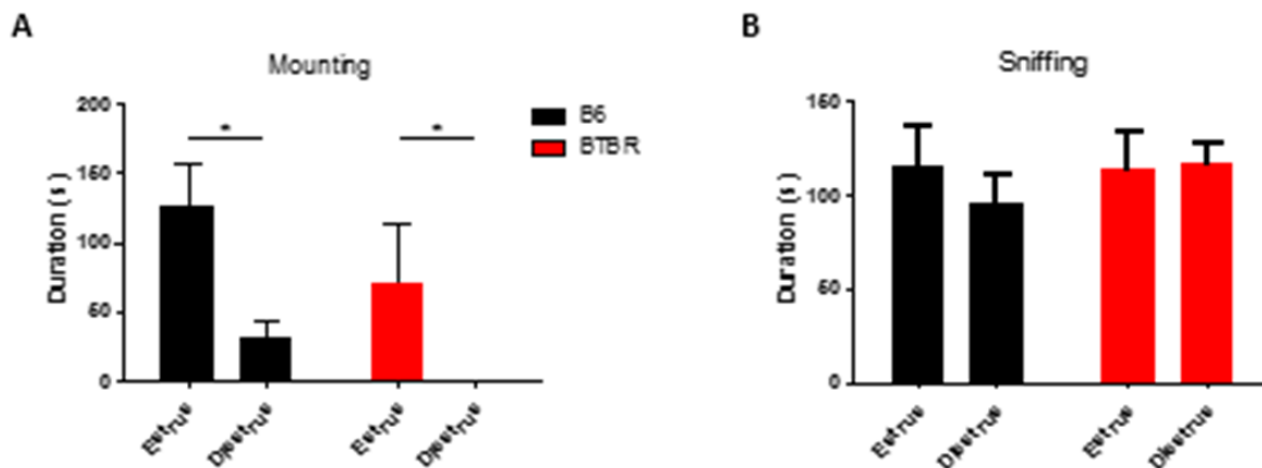
and diestrus stages in this study because the preferences for male urine and lordosis behavior are considerably decreased during diestrus than during estrus [28-30].

The total mounting duration for 10 min was analyzed, and as expected, the mounting time of male B6 mice was significantly higher with estrus females than with diestrus females (with estrus:  $n=13$ , with diestrus:  $n=9$ , student's  $t$ -test,  $*p<0.05$ , Fig. 2A).

None of the eight male BTBR mice that we tested mounted diestrus female partner at all whereas some mice (5 out of 9 mice) mounted estrus females (Table 1). In BTBR mice, the proportion of mounting was significantly higher with estrus females than with diestrus females (Fisher's exact test,  $*p<0.05$ , Fig. 2A). These results suggested that BTBR mice could recognize the sexual state of its mating partner. It seems that the mounting duration of BTBR was less than that of B6, although the tendency did not reach statistical significance (two-way ANOVA, strain  $\times$  estrous cycle, strain:  $p=0.153$ , Fig. 2A). Sniffing behavior, which is a more general social

behavior compared with mounting, was not affected by the female estrous cycle in either B6 or BTBR mice (Fig. 2B).

Next, we investigated if the patterns of USV emissions by male mice were affected by the female estrous cycle. We analyzed the total numbers and latencies for the first call of the USV that was emitted by male mice during 5 min of sexual interaction. However, both the numbers and first call latencies of the USV of male B6 mice did not differ between those matched with estrus females and diestrus females (Fig. 3A~C). The parameters of BTBR mice were also not affected by the female estrous cycle, but the total number and first call latencies of the USVs tended to be higher in BTBR mice compared with B6 mice (two-way ANOVA, strain  $\times$  estrous cycle, strain:  $*p<0.05$ , Fig. 3B, C). We then analyzed specific properties, such as dominant frequency, bandwidth, and duration, of each USV call as the parameters of male USV calls. However, the parameters of each USV calls in both strains did not differ between estrus and diestrus females (Fig. 3D~F).



**Fig. 2.** Comparison of sexual behaviors exhibited by B6 and BTBR mice with females in the estrus and diestrus stages of the estrous cycle. (A) Total mounting duration of B6 and BTBR male mice. The mounting duration of B6 mice is higher with estrus females than with diestrus females (with estrus:  $n=13$ , with diestrus:  $n=9$ , Student's  $t$ -test,  $*p<0.05$ ). Mounting behavior is not seen in BTBR male mice with diestrus females. The number of BTBR mice showing mounting is significantly higher with estrus females than with diestrus females (with estrus: 5/9, with diestrus: 0/8, Fisher's exact test,  $*p<0.05$ ). (B) Total sniffing durations of both B6 and BTBR mice. The durations are not affected by the estrous cycle (B6, with estrus:  $n=13$ , with diestrus:  $n=9$ ; BTBR, with estrus:  $n=9$ , with diestrus:  $n=8$ , Student  $t$ -test,  $p>0.05$ ). All values are presented as mean $\pm$ SEM.

**Table 1.** The number of male mice in B6 (A) and BTBR (B) that mounted or did not mount on each estrous cycle

	B6		BTBR	
	With estrus	With diestrus	With estrus	With diestrus
Mounting	11	8	5	0
No mounting	2	1	4	8
p value (Fisher's exact test)	$p>0.05$		$p=0.0294$	

## DISCUSSION

The receptivity of female mice is the highest in the estrus stage, and sexual behaviors, including mounting of male mice, are most frequent with estrus females [10, 11]. We investigated the sexual interactions of male B6 and BTBR mice with female mice at different estrous stages. Particularly, the mounting durations with estrus female were increased compared to those with diestrus females in both strains. Although the mounting duration of BTBR tended to be lower compared with B6, both strains of mice distinguished the different sexual states of the females according to the estrous cycle. This suggested that BTBR mice could somewhat recognize different sexual states of female mice affected by estrous cycle. However, in order to examine if estrous cycle itself could be recognized by BTBR mice excluding the effects of the sexual receptive behavior of female mice, urine from females of different estrous stages should be used.

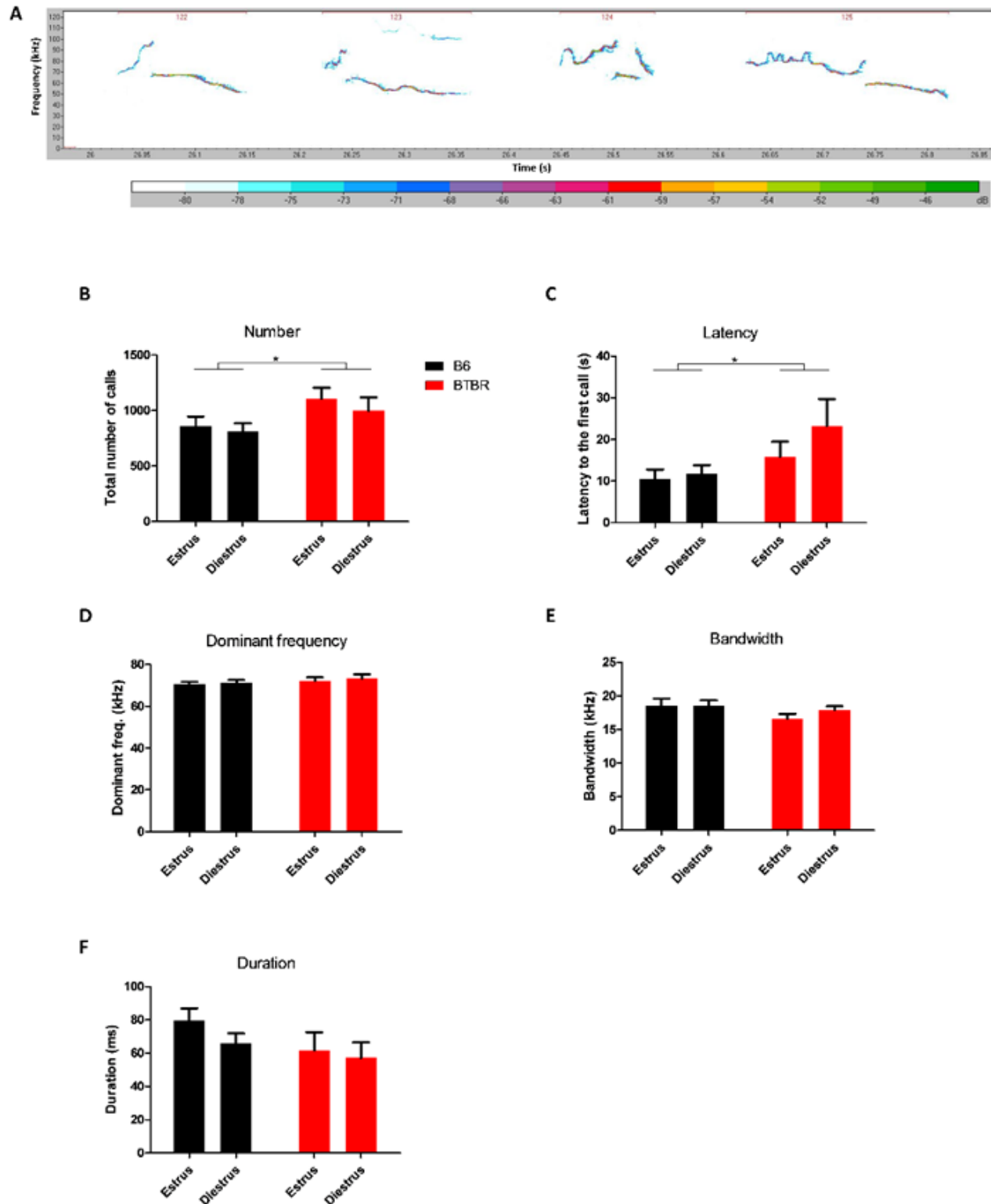
Sniffing was not affected by the estrous cycle in either strain. This behavior is a more general social behavior compared to mounting, and it is presented in many situations, including same-sex social

interactions and interactions under nonsocial contexts.

USVs are composed of various call types although their meanings are not yet clear. USVs are associated with sexual behaviors. Mice with autism, including BTBR mice, exhibit abnormal patterns of USV emissions [23, 31-33]. However, the USV call number and latency were not affected by the estrous cycle in either strain in our study. Thus, although USVs are involved with sexual behavior, they do not appear to be affected by the estrous cycle. However, we cannot exclude the possibility that a specific type of USV call may be influenced by the estrous cycle.

In contrast to the findings of a previous report [23], our results showed that the total number of USV calls in BTBR mice was not less than that of B6 mice. However, in the previous study, male mice were group-housed, and male mice encountered the females in cages with fresh bedding, which represented a novel environment. However, in the present study, male mice encountered the females in their own cages where they had been housed for at least 7 days. These methodological differences might explain the different results in the studies.

It would be interesting to determine how autistic BTBR mice can



**Fig. 3.** Ultrasonic vocalizations (USVs) of male B6 and BTBR mice with females in the estrus and diestrus stages. (A) Representative examples of USV calls. (B, C) Total number or latency to the first call of the USV of male B6 and BTBR mice with estrus or diestrus female. The number and latency of the calls with estrus or diestrus female do not differ (B6, with estrus: n=8, with diestrus: n=8; BTBR, with estrus: n=5, with diestrus: n=5, Student *t*-test,  $p>0.05$ ). The number and latency tend to be higher in BTBR mice compared with B6 mice (two-way ANOVA, strain x estrous cycle, strain: \* $p<0.05$ ). (D~F) The properties of each USV call (dominant frequency, bandwidth, and duration) do not differ (B6, with estrus: n=8, with diestrus: n=8; BTBR, with estrus: n=5, with diestrus: n=5, Student *t*-test,  $p>0.05$ ). All values are presented as mean±SEM.



recognize the sexual states of females. One possible explanation is the contribution of oxytocin, a neuropeptide that has been implicated in pair bonding and sexual interactions [34]. Because oxytocin level was even higher in BTBR mice than B6 mice [35], elevated oxytocin might make BTBR mice distinguish the sexual states of females, in spite of their autistic phenotypes. Further studies should be done to understand the molecular mechanisms involved in the recognition of sexual states of female mice. Finally, as there are many autistic mouse models, our findings of sexual behaviors in BTBR cannot be generalized to all autistic model mice. Therefore, it would be interesting to compare the estrous cycle-dependent sexual behaviors among different autistic mouse lines.

### ACKNOWLEDGEMENTS

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