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Evaluation of aggressiveness of female mice using a semi-automated apparatus for measurement of aggressive biting behavior toward an inanimate object

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HIGHLIGHTS

- We used the Aggression Response Meter (ARM) to measure female aggressive behaviors.
- Aggressive biting behavior toward an inanimate object (ABI) was measured using ARM.
- ABI did not change significantly during an estrous cycle.
- ABI changed upon isolation stress and serotonin 1A receptor agonist administration.
- ABI is available for evaluation of drug efficacy on aggressiveness in female mice.

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ABSTRACT

Background: Most laboratory research on aggressive behavior has focused on intraspecific intermale aggression tests. The intraspecific confrontation is not available for the evaluation of female aggressiveness, since androgens are critical for maintenance of this behavior, whereas *aggressive biting behavior toward inanimate objects* (ABI) occurs in both males and females.
New method: We propose an experimental method for evaluating female aggressiveness. We improved the previously developed semi-automated apparatus (Aggression Response Meter, ARM) to apply it to measurement of female ABI, and measured changes of ABI in stressed mice and drug actions on ABI.
Results: ABI assessment was performed daily in sexually mature female mice using ARM. The intensity and number of ABI in one session did not significantly change during an estrous cycle, suggesting that ABI is not influenced by the dynamics of sex hormones. Additional female mice were socially isolated for 7 weeks and then re-socialized for 2 weeks, and ABI was monitored weekly. ABI significantly increased during the isolation period, and then significantly decreased during re-socialization; both were time-dependent. In prolonged-isolated aggressive mice, a serotonin 1A receptor agonist, buspirone, significantly decreased ABI.
Comparison with existing method: There are no experimental methods or apparatus available for evaluating female aggressiveness using one individual repeatedly. We could measure ABI semi-quantitatively using the ARM.
Conclusions: ABI is a useful behavioral paradigm in the evaluation of aggressiveness in female mice, regardless of the estrous cycle, and can also be used for evaluating the actions of drugs on aggressiveness.

1. Introduction

Most laboratory research on aggressive behavior has focused on intraspecific aggression tests, such as the resident-intruder test. In this test, an intruder animal is placed in the home cage of a conspecific resident; animals then engage in combative behavior, and the latency to attack, frequency of biting and/or other measures of

Abbreviations: ABI, aggressive biting behavior toward an inanimate object; ARM, aggression response meter.

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aggressive behaviors are recorded (Blanchard and Blanchard, 1977; Clipperton-Allen et al., 2011; Malkesman et al., 2006; Miczek and O'Donnell, 1978; Mineur et al., 2003; Mucignat-Caretta et al., 2004; Vergnes et al., 1986). Intraspecific aggressive behavior is peculiar to males and appears to occur in order to protect their territory and/or female; androgens are involved in the mediation of this behavior in male mice (Barkley and Goldman, 1977; Beeman, 1947; Edwards, 1969). In contrast, female mice typically do not display overt aggressive behavior toward strangers except during the peripartum period (Ieni and Thurmond, 1985; Olivier et al., 1990; Svare and Gandelman, 1976; Veenema et al., 2007). Consequently, female aggressiveness has been studied less frequently; the majority of reported preclinical studies on aggressive behaviors have focused exclusively on males.

In contrast to the above, *aggressive biting behavior toward inanimate objects* (ABI) occurs in both males and females. ABI is a defensive behavior; it is often triggered by mild stimulation, such as a light touch or an object moving in front of the eyes in some psychiatric disorder model mice, such as stress-disorder (Kuchiiwa and Kuchiiwa, 2014; Yen et al., 1959). The neurobiological mechanisms underlying the development of ABI are largely unknown; however, it is conceivable that ABI is one sign of a psychiatric disorder because it is scarcely observed in normal laboratory animals. It has been shown that ABI is induced by social aversive experimental factors such as social isolation (Kuchiiwa and Kuchiiwa, 2014). Social isolation increases the intensity and incidence rate of ABI in a time-dependent fashion in male mice, suggesting that we can evaluate aggressiveness induced by psychiatric disorders by measuring changes in the strength and frequency of ABI (Kuchiiwa and Kuchiiwa, 2014).

In this study, we determined whether the estrous cycle affects ABI or not, and whether the ABI paradigm is suitable for semi-quantitative evaluation of aggressiveness and drug actions on aggressiveness, using female mice. It has been shown that sex differences exist in the efficacy of psychotropic drugs in rats and humans (Khan et al., 2005; Wilson and Roy, 1986; Young et al., 2009). The results of the present study are discussed in terms of sex differences with reference to the results of our previous study using male mice (Kuchiiwa and Kuchiiwa, 2014). The purpose of this study is to establish an experimental method for evaluating aggressiveness that is also available for female mice.

2. Materials and methods

2.1. Apparatus

Measurement of ABI was performed using the Aggression Response Meter (ARM), which is an improvement on the previously reported apparatus. A detailed explanation of the apparatus is available in our previous paper (Kuchiiwa and Kuchiiwa, 2014). Briefly, it is equipped with a new highly precise load sensor to detect small movements and a newly developed noise reduction program to remove mechanical noise and motion of a mouse accompanying typical activity such as breathing (MODEL TRM-001; Muromachi Kikai Co. Ltd., Tokyo, Japan). A transparent, acrylic, cylindrical animal chamber is set on top of the ARM. The chamber is 95 mm in length and 35, 40, or 45 mm in inner diameter. A pair of slits is present in the floor of the chamber at an interval of 11 mm (15 mm center-to-center distance). Each slit is 4 mm in width and 90 mm in length. A pair of metal sticks is set on the stick-driving unit perpendicular to the floor of the chamber just below the slits. Each stick is 3 mm in diameter with a dome-shaped head, to avoid inflicting pain on the mouse. The stick-driving unit is loaded onto a drive-sliding unit for manual shifting to the right and left, controlled automatically with a computer moving the sticks in an up-and-down motion through the slits of the animal chamber, allowing application of a light touch or visual stimulation to the mouse in the chamber. When the mouse attacks the sticks, the dynamic strength of the biting behavior is detected three-dimensionally by the load sensor attached to the bases of the sticks, as well as the duration of the behavior. The values of the intensity and incidence rate of ABI are displayed on a monitor graphically as well as numerically, and then all data of ABI are recorded in a computer file. The data of intensities are expressed in numerical values, as strength × time (Millinewton × second: mNs).

2.2. Animals

The present experiment was carried out on ddY strain mice (Sea:ddY; Kyudo Co., Ltd., Tosu, Japan). Male and female mice were housed together for mating to obtain offspring, and a total of 77 female offspring weighing 15–55 g (4–20 weeks old) were used in this study. The mice were housed in plastic cages lined with wood shavings. The size of the cages for the isolation-reared mice was 16 × 23 × 12 cm, while that for the group-housed mice was 29 × 34 × 17 cm. The acrylic animal chamber used in the ABI tests was always placed in each home cage for habituation, and mice entered it regularly. All animals were housed under controlled environmental conditions (temperature, 22 °C ± 1 °C; humidity, 55% ± 10%; lighting, 12:12-h light-dark cycle). The mice were allowed food and drink *ad libitum*. Whole cage exchange was performed every 10 days.

All experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23) revised in 1996, and they were conducted strictly in accordance with the guidelines of the Committees of Animal Experimentation, Kagoshima Immaculate Heart University and Kagoshima University. Experiments also complied with the current laws of Japan.

2.3. Measurement of ABI

The ABI test was carried out following our previously described protocol (Kuchiiwa and Kuchiiwa, 2014). Briefly, a pair of stimulation sticks of the ARM were set to move at a rate of 100 mm/s, to extend 10 mm above the floor of the animal chamber and to remain for 1 s at the most extended position, which was set at 10-s intervals. The animal chamber was adjusted to the size of the animal so that the mouse could turn around, avoiding narrow space stress. Measurement of ABI was performed in the mouse-breeding room to prevent novel environmental stress. Some of the experiments were performed by an observer blind to the experimental conditions.

At first, the mouse was led into the animal chamber of ARM and left for a few minutes until its voluntary exploratory locomotion ceased, and then the test was initiated. The test consisted of two sessions. The first session was carried out to provoke the mouse and induce irritation and anger. In this session, touch stimulation to the hindlimb or abdomen was repeated 30 times for 5 min. If the mouse increased aggressiveness, it often exhibited kicking behavior, namely, it kicked the sticks away intensely with both hindlimbs throughout the session. Within a minute after the first session, the second session was started to measure the intensities and incidence rates of ABI. In this session, the sticks were elevated at head level and touched the mandible and/or whiskers or approached the face without touching it. When the mouse attacked them (biting attack, sometimes including scratching the sticks), the load sensor detected the dynamic strength and duration of the behavior. When the mouse paid no attention to the movement of the sticks, by, for example, exhibiting exploratory behavior or turning away, the challenge was repeated while maintaining the measurement. The

measurement was performed 30 times for 5 min during the second session, and the average intensity and number of responses were recorded.

2.4. Measurements of vaginal electrical resistance and ABI

The first experiment was conducted to investigate the effects of each stage of the ovarian cycle on ABI. After weaning at postnatal week 3, 6 litters of female offspring were housed in same-litter groups, and additional female mice were housed individually; then, at postnatal weeks 18–20, sexually mature female mice were assigned to this experiment. The group-housed mice were randomly selected from the 6 litter groups ($n = 9$). The isolated mice were prescreened for ABI 2 days before the beginning of the experiment using the ARM, and mice that obviously showed the ABI paradigm (over 7 mNs or more in intensity) were assigned to this experiment ($n = 9$). On each day of the experiment, the intensity and incidence rate of ABI were measured using the ARM and then, immediately after the test, measurement of vaginal electrical resistance was carried out with a Vaginal Impedance Checker (MK-10A; Muromachi Kikai Co., Ltd., Tokyo, Japan). The estrous cycle of group-rearing animals is stable; however, that of isolated animals is apt to be disturbed. Thus, the measurement of vaginal electrical resistance was carried out daily for a consecutive 8 days in group-housed mice, and for a consecutive 12 days in isolated mice. Usually, 2 peaks appear during these periods. The estrous cycle was divided into 4 phases: diestrus, proestrus, estrus, and metestrus (Agrawal et al., 2009; Bartos, 1977; Koto et al., 1987; Weixelbaumer et al., 2014). We determined the stage of the estrous cycle of each mouse and analyzed the data of 4 consecutive cycles, including the first peak. The experiments were conducted daily at 13:00–16:00. It is known that the repeated performance of ARM tests has little effect (Kuchiiwa and Kuchiiwa, 2014).

2.5. Socially isolated and re-socialized mice

A total of 14 female ddY strain mice were used in this experiment. One day after birth, each litter was adjusted to 8 infants to ensure similar lactation and sufficient breast milk. After weaning at postnatal week 3, female offspring were housed in same-litter groups for 1 additional week. At postnatal week 4, 14 mice were randomly selected from 4 litter groups, and the mice were subjected to the ABI test using the ARM. Just after the tests, they were divided into 2 groups according to housing conditions; that is, an isolation-housing group (1 per cage; $n = 7$) and a group-housing group (7 per cage; $n = 7$). The ABI test was performed again at postnatal weeks 5, 7, 9 and 11 (namely, at 1, 3, 5 and 7 weeks after the beginning of the isolation). Just after the last measurement at postnatal week 11, the isolated mice were re-socialized (7 per cage) and group-reared for an additional 2 weeks, and then tested for ABI again 1 and 2 weeks from the beginning of the re-socialization (postnatal weeks 12 and 13). The control mice were group-reared continuously and tested for ABI weekly. Experiments were conducted at the same time of day and the individual experimental order was fixed during these experiments.

2.6. Administration of buspirone

Female offspring were weaned at postnatal week 3 and then housed in isolation for 8–10 weeks. The isolated animals were prescreened for the ABI paradigm 2 days before the experiment, and mice that had overt ABI were used in this experiment ($n = 45$).

On the day of the experiment, at first, the intensity and number of ABI in one session were measured using the ARM. Just after the ARM test, each mouse was placed on wire netting, picked up by its tail, and administered buspirone hydrochloride (a serotonin 1A receptor agonist; at 1.25, 2.5, 5.0 or 10.0 mg/kg; $n = 9$ each) or vehicle (0.9% saline; $n = 9$) intraperitoneally using a Terumo 29 × 1/2″ ga syringe (Terumo Co., Tokyo, Japan) to minimize pain and fear. Buspirone or vehicle was injected at a volume of 5 ml/kg. Thirty minutes after the injection, ABI was re-measured using the ARM. Buspirone was purchased from Sigma Chemical (St. Louis, MO, USA) and fresh solutions were prepared daily. Saline was purchased from Otsuka Pharmaceutical Co., Ltd. (Tokyo, Japan).

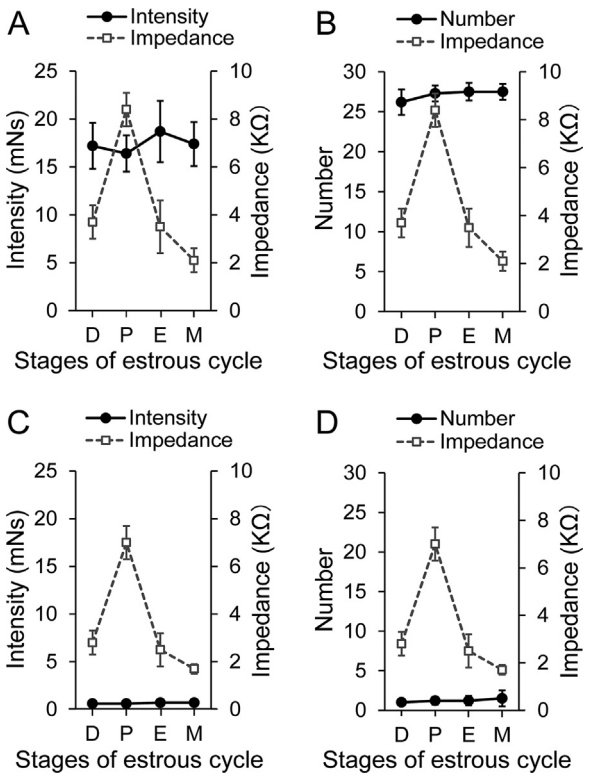

Fig. 1. Changes of ABI during an estrous cycle. (A) and (B), isolated mice ($n = 9$); (C) and (D), group-housed mice ($n = 9$). (A) and (C), intensity of ABI; (B) and (D), number of ABI in one session (30 trials for 5 min). The estrous cycle was divided into 4 phases: diestrus (D), proestrus (P), estrus (E) and metestrus (M). Vaginal electrical resistance was significantly higher during proestrus than at any other stage of the reproductive cycle, in both isolated (A–B) and group-housed mice (C–D). The intensity and number of ABI did not significantly change in socially isolated and group-housed mice during the 4 estrous stages. Intensity data are expressed in numerical values, as strength × time (Millinewton × second: mNs). Values shown are mean ± S.E.M.

2.7. Statistical analysis

Statistical analyses of the results were performed using log-transformation values (R programming language ver. 3.0.2 and SAS-JMP ver. 5.0.1; SAS Institute Inc., Cary, NC, USA). Analysis of variance was used to test for statistically significant differences among measured weeks and was interpreted using Tukey's HSD for multiple comparisons. The Welch t-test (unpaired and paired) was used for comparisons between the isolation-reared mice and the group-housed mice. The levels of statistical significance were set at $^{**}\ p < 0.01$.

3. Results

3.1. Changes of vaginal electrical resistance and ABI during an estrous cycle

In the first experiment, the vaginal electrical resistance was significantly higher during proestrus than at any other stage of the

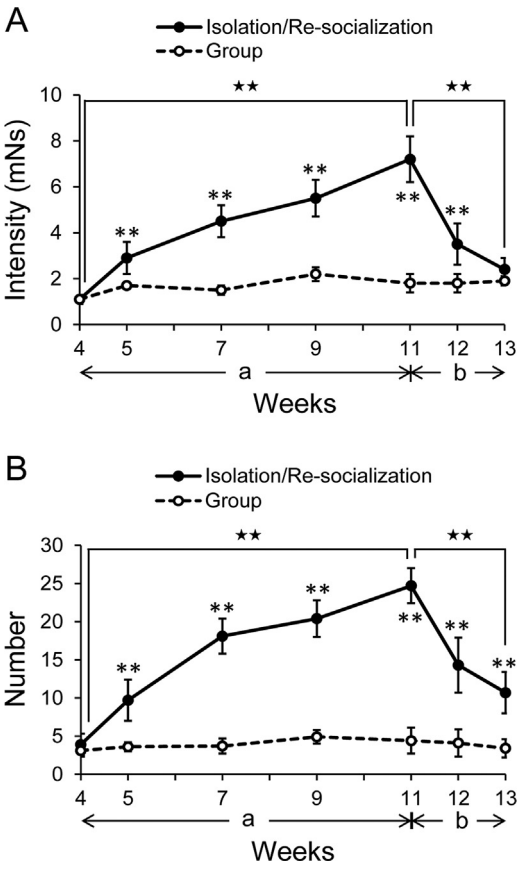

Fig. 2. Changes of ABI during socially isolated and re-socialized periods. In the isolation/re-socialized mice ($n = 7$), both intensity (A) and number of responses in one session (B) were significantly increased during the 7 weeks of isolation rearing (A-a, B-a; postnatal weeks 4–11, ** $p < 0.01$), and then significantly decreased during the 2 weeks of re-socialization (A-b, B-b; postnatal weeks 11–13; ** $p < 0.01$). In control group-housed mice ($n = 7$), the intensity or number of ABI was not significantly changed during the experimental period. ** $p < 0.01$, comparison of isolation/re-socialized group with control group. Intensity data are expressed in numerical values, as strength × time (Millinewton × second: mNs). Values shown are mean ± S.E.M.

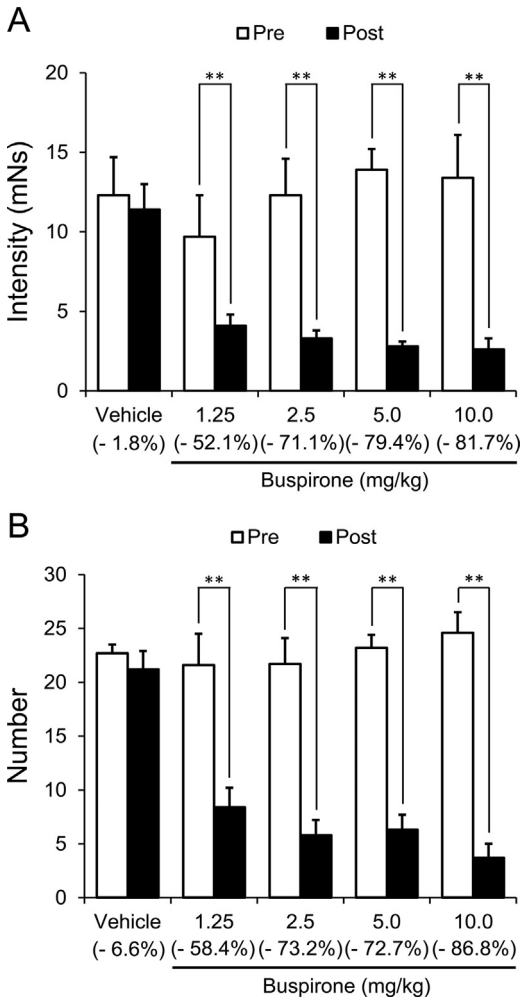

Fig. 3. Changes of ABI after administration of buspirone. (A), intensity of ABI. (B), number of ABI in one session. Intensity and number of ABI were measured before and after intraperitoneal injection of buspirone hydrochloride (1.25, 2.5, 5.0 or 10.0 mg/kg; $n = 9$ each) or vehicle (0.9% saline; $n = 9$). "Pre" and "Post" indicate before and after the administration of buspirone or vehicle, respectively. The intensity and number of ABI decreased in similar manners. The numbers in parentheses show the rate of decrease after each administration. Data are expressed in numerical values, as strength × time (Millinewton × second: mNs). Values shown are mean ± S.E.M. ** $p < 0.01$.

reproductive cycle (Fig. 1). This result is consistent with previously reported observations in mouse and rat (Agrawal et al., 2009; Bartos, 1977; Chern et al., 2010; Koto et al., 1987). The intensity and number of ABI did not change significantly in either socially isolated or group-housed mice during the 4 estrous stages, suggesting that ABI in female mice is not really influenced by any stage of the estrous cycle or the dynamics of sex hormones.

3.2. Changes of ABI during isolation and re-socialization periods

At postnatal week 4, prior to isolation rearing, no female mice exhibited distinct ABI in response to moving of the sticks in the ARM test; however, a few very weak biting-like motions were observed in one session with 30 stimulus deliveries in some mice. Because we could not draw a distinct line between whether the motions were aggressive behaviors or exploratory ones, we judged movements with a value of 3 mNs (Millinewton × second) or more to be ABI responses. As a rule, the response of the isolation-group mice was the same as the behavior of the group-housed control mice.

Both intensity and number of responses in one session were significantly increased during the 7 weeks of isolation rearing ($p < 0.01$; Fig. 2). After 7 weeks of isolation rearing (postnatal week 11), for most isolated mice, many ABI events occurred in one session. When the isolated mice were re-socialized, ABI began to decrease. Both intensity and number of responses in one session were significantly decreased during the 2 weeks of re-socialization ($p < 0.01$). After 2 weeks of re-socialization, no significant differences in the intensity of ABI were found between the isolated/re-socialized and group-housed mice in the ARM test, although the significant difference in the number of ABI did not disappear. On the other hand, in the group-housed control mice, no obvious ABI event was seen throughout the experimental period (Fig. 2).

3.3. Effects of buspirone on ABI

Before the administration of buspirone or vehicle, all isolation-reared mice used in this experiment exhibited obvious ABI behavior in the ARM trials. After the administration of buspirone, both intensity and number of ABI in one session decreased significantly in each dose of buspirone (Fig. 3). In the control mice that received an injection of vehicle, the intensity of ABI and the number of ABI in one session did not change significantly.

4. Discussion

Long-term social isolation in laboratory animals causes stress, which induces a variety of behavioral abnormalities including increased anxiety-related behavior, hyper-locomotion, cognitive deficits and a deficit in pre-pulse inhibition of the acoustic startle reflex, as well as increased aggressiveness (Hatch et al., 1963; Ibi et al., 2008; King et al., 2009; Miczek and O'Donnell, 1978; Roncada et al., 2009; Valzelli, 1969, 1973; Voikar et al., 2005; Wei et al., 2007; Wongwitdecha and Marsden, 1995). Isolated male animals have been used for the analysis of aggression mechanisms and evaluation of the actions of drugs on aggressiveness, and have been used as models of psychiatric disorders including schizophrenia and/or depression (Brenes and Fornaguera, 2009; Day-Wilson et al., 2006; Fone and Porkess, 2008; King et al., 2009; Koike et al., 2009; Leng et al., 2004; Miura et al., 2002; Weiss and Feldon, 2001). In contrast, female aggressive behavior has been studied less frequently because female mice do not display intraspecific aggression except during the peripartum period (Olivier et al., 1990; Svare and Gandelman, 1976). In the present study, we indicated that socially isolated female mice reliably attacked inanimate objects following a painless, noninvasive stimulus; furthermore, it was possible to collect measurements, such as frequency and force of attack, using the same individual repeatedly. To the best of our knowledge, there are no methods for the continuous evaluation of changes of aggression in individual female mice during a long-term period.

Compared with the results of our previous ABI study using male mice (Kuchiiwa and Kuchiiwa, 2014), the ABI of female mice induced by social isolation was about half of that of male mice after 7 weeks of isolation rearing. It has been reported that female aggression levels are low in most laboratory rodent species (Greenberg et al., 2014; Yen et al., 1959). Our present results are in agreement with these reports. Furthermore, in the present study, the intensity and incidence rate of ABI decreased more conspicuously in female mice after the administration of buspirone than the results of our previous experiments using male mice in the same protocol (Kuchiiwa and Kuchiiwa, 2014). It is conceivable that, in female mice, signs of serotonin syndrome should already be present following the lowest dose administration, and more than evident following higher doses (Haberzettl et al., 2014). In laboratory animals, the initial metabolism of the antidepressant imipramine was slower in females than in males (Wilson and Roy, 1986); in humans, antidepressants differ in efficacy, with women showing greater sensitivity to selective serotonin reuptake inhibitors (Khan et al., 2005; Young et al., 2009). These reports and our present study indicate that there are sex differences in drug efficacy. These suggest that we should evaluate the actions of drugs on aggressive behaviors in both males and females, at least in preclinical studies targeting innovative drug development. We think that ABI is a useful behavioral paradigm for this purpose.

Aggressiveness induced by social isolation increases combative behavior in male mice during the resident-intruder test (Valzelli, 1969, 1973). Androgen depletion following castration radically lowered the intraspecific intermale aggression level in mice; the administration of testosterone propionate restored the aggressive behaviors to the intact level (Barkley and Goldman, 1977; Beeman, 1947; Edwards, 1969), and intermale aggression can be reversed to near precastration levels in some strains by its metabolites, estradiol or dihydrotestosterone (Simon and Whalen, 1986). This male offensive aggression in resident-intruder confrontation was almost completely suppressed in estrogen receptor-α gene-knockout mice, even when administered exogenous testosterone (Ogawa et al., 1998). These findings indicate that sex hormones are critical for the maintenance of intraspecific aggression in males.

On the other hand, in female mice, the scores of the resident-intruder paradigm are not clearly affected by social isolation (Svare and Gandelman, 1976; Yen et al., 1959). Namely, the resident-intruder paradigm cannot be used for evaluating female aggressiveness. In the present study, social isolation increased both the intensity and the frequency of ABI in a time-dependent fashion in female mice as well as in male ones (Kuchiiwa and Kuchiiwa, 2014). Moreover, it was indicated that the ABI was not affected by the ovarian cycle, suggesting that the ABI test is always available regardless of the estrous cycle.

Intensified ABI induced by mild stimulation without pain is not observed in normal mice. It is considered that the ABI emerges in psychiatric model mice with emotional lability, morbid irritability and/or aggression. If this is correct, the ABI test is more suitable for evaluating aggressiveness developing in mental disorders.

5. Conclusion

We can evaluate the aggressiveness of female mice semi-quantitatively by measuring ABI using the ARM. ABI is not influenced by the ovarian cycle, indicating that the ABI test is always available regardless of the estrous cycle. It is considered that the ABI paradigm is suitable for evaluating the aggressiveness developing in mental disorders in female mice.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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