The Journal of Veterinary Medical Science

FULL PAPER

Ethology

Two strains of roof rats as effective models for assessing new-object reaction

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J. Vet. Med. Sci.
79(4): 702–708, 2017
doi: 10.1292/jvms.17-0002

Received: 4 January 2017
Accepted: 6 February 2017
Published online in J-STAGE: 16 February 2017

ABSTRACT. Wild animals generally avoid even small and harmless novel objects and/or familiar objects moved to a novel position, which is termed "new-object reaction". Although new-object reaction appears to be a biologically important characteristic for animals, little progress has been made in understanding the neural mechanisms underlying new-object reaction. One reason might be the lack of effective experimental animals. Two strains of roof rats (Sj and Og strains) were established from wild roof rats caught in Shinjuku, Tokyo and one of the Ogasawara Islands, respectively, by a Japanese pest control company. Based on the rat caregivers' informal observations, we conducted behavioral and anatomical tests to assess the validity of Sj and Og strains for the analyses of new-object reaction. In Experiment 1, the Sj strain showed reduced food consumption compared with the Og strain when food was provided in a novel way, suggesting that the Sj strain had a stronger avoidance of novel objects compared with the Og strain. Experiment 2 demonstrated that the basolateral complex of the amygdala and bed nucleus of the stria terminalis in experimental Sj rats had a larger percentage area compared with that of experimental Og rats, indicating these nuclei might be involved in the difference observed in avoidance of novel objects between the strains. Taken together, the present study suggests that Sj and Og strains are effective experimental animals for assessing new-object reaction.

KEY WORDS: basolateral complex of the amygdala, bed nucleus of the stria terminalis, main olfactory bulb, neophobia, olfactory receptor

Changes in the environment often reduce the consumption of food by wild animals at baiting stations because of their hesitant approach, even to small and harmless new objects or familiar objects in a new position. For example, when a small piece of wood was suddenly placed between the entrance hole of a shed and a familiar baiting station in the shed, wild brown rats stopped at the entrance hole, frequently raising their noses high into the air, and then withdrew [3]. It was also observed that wild rats gradually disappeared after much inspection, mostly from a distance, when foods normally placed on the floor of the shed were placed in a shallow metal tray [3]. An avoidance of unfamiliar objects in familiar surroundings or familiar objects in a new position is termed "new-object reaction" [1].

New-object reaction appears to be a biologically important characteristic, because it is a common feature of many species of wild animals. However, because only a few species of animals that are used as laboratory animals show no or weak new-object reaction, less attention has been paid to new-object reaction. Indeed, laboratory rats approach, rather than avoid, novel objects placed in familiar surroundings, which are used as an index in many cognitive tests. Nonetheless, several studies have been conducted in laboratories using wild or wild-derived rats. In one procedure, novel object(s) are simply placed in one compartment of the familiar test apparatus. The change in time spent in the compartment is measured as a direct index of avoidance. However, the presence of novel objects did not reduce the time spent in the compartment, suggesting that this procedure is less effective for observing avoidance [19, 22]. A more effective procedure might be changing objects related to feeding or drinking. The reduction of food or water consumption is measured as an indirect index of avoidance, because the more the animals avoid novel objects, the less the animals approach the food or water sources. As a result, food or water consumption is reduced. For example, wild and wild-derived rats showed reduced food consumption when the food container or the location of the food container was changed [1, 4, 9, 15] or when a novel object was placed on the path to the feeding place [1, 5, 6].

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Although the existence of new-object reaction has been reported, little progress in understanding the neural mechanisms involved has been made over the last 60 years. To the best of our knowledge, only one study has assessed the potential role of the amygdala in wild-derived brown rats. Rats showed an elongated latency to eat food when a metal plate was placed beneath the usual food cup. This reaction was blocked by a lesion in the amygdala, although the lesion area was not shown [8]. One reason for the lack of studies might be the lack of available and effective experimental animals. Although wild animals generally show strong avoidance, it is difficult to prepare a control group of wild animals that show no or a weak avoidance of novel objects. In addition, differences in genetic backgrounds make it difficult to interpret the data when comparing controls with laboratory animals of the same species. Difficulties in the handling of wild animals further obstruct detailed behavioral neuroscientific analyses.

Two closed colonies of roof rats (*Rattus rattus*) were established at a pest control company in Japan. One colony (Sj strain) was derived from warfarin-resistant roof rats caught in Shinjuku, a large downtown area in Tokyo. Another colony (Og strain) was established from warfarin-sensitive roof rats caught at Chichijima, one of the Ogasawara Islands. Physiological analyses revealed that the Sj strain metabolized warfarin faster than the Og strain [21]. In addition to the differences in warfarin sensitivity, caregivers noticed that the Sj and Og strains might have different responses to changing objects related to feeding. Because the rats are maintained as a group in each colony room, food is supplied in a container placed in the center of the colony room. The Sj strain appeared not to eat when the food container was moved from the usual place or when novel objects were placed next to the food container. In contrast, these manipulations did not appear to affect the feeding behavior of the Og strain. Based on these informal observations, these two strains might be effective experimental animals to investigate new-object reaction. Therefore, it is important to confirm that the Sj strain shows a stronger avoidance of novel objects than the Og strain.

These two strains might also be effective experimental animals, if the difference in avoidance originates from differences in brain activities. Avoidance of novel objects seems to be a fear and/or anxiety response to uncertainness. Previous research has proposed that the basolateral complex of the amygdala (BLA) is a crucial region for both fear and anxiety responses, while the bed nucleus of the stria terminalis (BNST) contributes only to anxiety responses [7, 23]. In addition, the high activity of these regions is likely to be associated with their large size, although this relationship has not been proven. It is known that activation of the BLA induces fear responses, such as fear-potentiated startle reflex [7], while anxiety responses, such as increased time spent in an open arm in the elevated plus maze, were evoked by activation of the BNST [10] in laboratory rats. In addition, a group of stressed laboratory rats that developed an anxiety response in the elevated plus maze, but no fear-potentiated startle reflex, had a larger BNST, but not BLA, compared to the control group of rats [18]. Based on these findings, we hypothesized that the size of BLA and/or BNST is different between Sj and Og strains when the difference in avoidance originates from a difference in brain activities. Specifically, it is possible that the Sj strain has a larger BLA and/or BNST compared to the Og strain.

In the present study, we conducted behavioral and anatomical analyses to assess the validity of the Sj and Og strains for the analyses of new-object reaction. In Experiment 1, we conducted a 3-day behavioral test repeatedly to confirm the informally observed avoidance in each colony room. On Day 1, food was supplied in the usual container. On Day 2, an additional container covered with a cardboard box with an entrance hole was placed next to the usual container. On Day 3, the usual container was removed so that only the container covered with cardboard was available. We measured food consumption as an indirect index of avoidance. In Experiment 2, we conducted anatomical analyses to assess further the validity of Sj and Og strains for the analyses of new-object reaction. Sj and Og rats from each colony room were euthanized, and the sizes of the BLA and BNST were measured.

MATERIALS AND METHODS

All experiments were approved by the Animal Care and Use Committee of the Faculty of Agriculture at The University of Tokyo, according to guidelines adapted from the *Consensus Recommendations on Effective Institutional Animal Care and Use Committees* by the Scientists Center for Animal Welfare.

Experiment 1

Sj and Og rats were obtained from Ikari Shodoku Corporation (Tokyo, Japan). The Sj strain was established from 4 male and 6 female warfarin-resistant roof rats caught in Shinjuku in 2007. The Og strain was established from 3 male and 3 female warfarin-sensitive roof rats caught at one of the Ogasawara Islands in 1989. Because these strains did not reproduce when they were kept in laboratory cages, rats were maintained as a group in separate semi-natural colony rooms (Sj: 2.7 × 3.0 × 2.5 m; and Og: 2.7 × 3.0 × 3.0 m) with an ambient temperature of 20 ± 5°C and a 12-hr light/12-hr dark cycle. Lights were switched on at 5:30. The colony rooms consisted of 3 concrete walls and 1 glass front wall with an entrance door. Nesting places were provided by piling up 8–9 concrete blocks and wooden boards, one by one, near the neighboring two concrete walls. Food and water were available *ad libitum.* Water was supplied by an automatic watering system at the remaining concrete wall. Food powder (CE-2, Clea Japan, Tokyo, Japan) was supplied in a container (17.3 × 30.4 × 11.0 cm) placed in the center of each room. Food was complemented between 9:00–11:00 every day to maintain a sufficient amount within the container at all times.

A 3-day test was conducted 6 times in each colony room with intervals of more than 1 week. This interval was determined by caregivers' experiences that the reduction of food consumption by a previous manipulation recovered to the normal level of consumption at about 2 days after the end of the manipulation and that the effects of learning were negligible with this interval. The caregiver also informally confirmed that food consumption was stable at the beginning of the experiment. On Day 1, the usual container was placed in the center of the room. On Day 2, we additionally placed an identical container (17.3 × 30.4 × 11.0 cm), but covered with a cardboard box (44.0 × 32.0 × 26.5 cm) with an entrance hole (10 cm square on the long side of the cardboard

box). On Day 3, we removed the usual container so that only the container covered with a cardboard box was placed in the room. Although food was provided in a novel way, the food *per se* was identical for all 3 days.

Food consumption of each strain was measured between 9:00–11:00 every day by weighing the container when the food was complemented. The amount of consumption from Day 1 morning to Day 2 morning was regarded as the food consumption on Day 1. Food consumption on Days 2 and 3 was similarly measured, although food consumption from the two containers was combined on Day 2. We defined food consumption on Day 1 as the basal consumption and calculated the percentage of food consumption on Days 2 and 3 with respect to the basal consumption. Food consumption during the 3 days was analyzed by two-way repeated ANOVA. The food consumption from each container on Day 2 was additionally analyzed by Student's *t*-test.

Experiment 2

To sample the brain, 16 Sj rats (male 8, female 8) and 16 Og rats (male 8, female 8) were randomly selected from the colony room described in Experiment 1. They were kept individually in a wire-mesh box (23 × 14 × 10 cm) for 1 or 2 days before sampling. After being slightly anesthetized with ether, rats were deeply anesthetized with sodium pentobarbital and weighed. Then, rats were intracardially perfused with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Sampled brains were vertically cut at the caudal end of the cerebellum and weighed. Body weight and brain weight were analyzed by Student's *t*-test. Additional analyses are provided in the Supplemental Materials.

The sampled brains were immersed overnight in 4% paraformaldehyde in 0.1 M phosphate buffer before being placed in 30% sucrose/phosphate buffer for cryoprotection. We collected six BLA-containing coronal sections (50 μm) every five sections from the bregma −3.48 mm to the anterior. We also collected six consecutive BNST-containing coronal sections from the bregma −0.12 mm to the posterior. After processing with 0.3% H_2O_2 in phosphate buffer saline for 30 min, the sections were incubated with citric acid buffer (Mitsubishi Chemical Medicine, Tokyo, Japan) for 2 hr, followed by incubation with a primary antibody to neuronal nuclei protein (MAB377; Millipore, Billerica, MA, U.S.A.) overnight and then with biotinylated anti-mouse secondary antibody (BA-2000; Vector Laboratories, Burlingame, CA, U.S.A.) for 2 hr. Finally, sections were processed with the VECTASTAIN Elite ABC kit (Vector Laboratories) and developed using a diaminobenzidine solution with nickel intensification.

Images of sections were captured using a microscope equipped with a digital camera (DP30BW, Olympus, Tokyo, Japan). Experimenters who were blinded to the strains measured the size of areas of interest using ImageJ 1.45s software. To compare the size of the BLA and BNST between strains, we measured the area of the whole section and the area of the nucleus bilaterally. Then, the sizes of BLA and BNST were adjusted with respect to the area of the whole section by calculating the percentage of the nuclei relative to the whole section [2, 14]. All data were analyzed by the Student's *t*-test. Additional analyses are provided in the Supplemental Materials.

RESULTS

Data are expressed as the mean ± standard error of the mean. *P* values <0.05 were considered significant for all statistical analyses.

Experiment 1

Although we did not know the exact number of Sj and Og rats in the rooms, caregivers had an impression that the Sj strain grew slower than the Og strain and that there were less numbers of Sj rats compared with Og rats in the colony room. In accordance with this impression, the food consumption of the Sj strain on Day 1 (344 ± 94 g) was smaller than for the Og strain (1,131 ± 178 g) (t_{10}=−3.90, P<0.01). Therefore, we defined the food consumption on Day 1 as the basal consumption for each strain and calculated the percentage of food consumption on Days 2 and 3 relative to the basal consumption. Statistical analyses revealed that the food consumption was affected by strain ($F_{(1,10)}$=16.3, P<0.01) and day ($F_{(2,20)}$=26.6, P<0.01). The interaction between these two factors was also significant ($F_{(2,20)}$=11.6, P<0.01) (Fig. 1). These results suggest that the Sj strain had reduced food consumption compared with the Og strain when food was provided in a novel way.

One possible interpretation is that both Sj and Og strains have the characteristic of avoidance of novel objects but that it is stronger in the Sj strain. Another possibility might be that the Sj strain has the characteristic of avoidance of novel objects, whereas the Og strain does not. To clarify this point, the food consumption from each container on Day 2 was additionally analyzed. On Day 2, unlike on Day 3, the Sj and Og strains could choose to eat food from the usual container or from the novel container covered with a cardboard box. It was demonstrated that offering a choice between the usual container and novel container detected a weak difference in the avoidance of novel objects compared to presenting only a novel container [15]. If the former interpretation is true, the Og strain should prefer to eat food from the usual container.

Statistical analyses revealed that food consumption from the container covered with a cardboard box was lower than from the usual container both in the Sj (t_{10}=4.67, P<0.01) and Og strains (t_{10}=13.3, P<0.01) (Table 1). These results demonstrated that the Og strain also ate less food from the novel container, suggesting that both the Sj and Og strains have the characteristic to avoid novel objects.

Taken together, we confirmed the caregivers' informal observation that the avoidance of novel objects was stronger in the Sj strain than in the Og strain. These results suggest that these two strains are effective experimental animals for the analyses of new-object reaction.

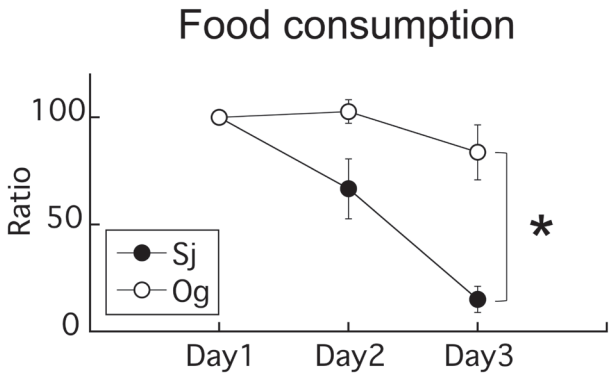

Fig 1. Food consumption of Sj and Og strains in Experiment 1. On Day 1, food was provided in the usual container. On Day 2, an additional container covered with a cardboard box with an entrance hole was placed next to the usual container. On Day 3, only the container covered with a cardboard box was used. Food consumption was expressed as a percentage relative to the consumption on Day 1. **P*<0.05 by two-way repeated ANOVA.

Table 1. Food consumption on Day 2 in Experiment 1

	Sj strain	Og strain
Usual container	65.8 ± 13.8	92.4 ± 5.5
Container covered with a cardboard box	0.9 ± 0.9*	10.3 ± 2.8*

Data are expressed as a percentage relative to food consumption on Day 1. **P*<0.05 by Student's *t*-test compared to food consumption from the usual container.

Experiment 2

To assess further the validity of Sj and Og strains for the analyses of new-object reaction, we conducted anatomical analyses of the BLA and BNST. Some Sj and Og rats were randomly selected from each colony room. Although the body weight was not significantly different between the experimental Sj and Og rats (Fig. 2A), the brain weight was lower in Sj rats compared with Og rats (t_{30}=−2.04, P<0.05) (Fig. 2B).

We prepared BLA-containing sections of experimental Sj and Og rats (Fig. 3A). The BLA area was not significantly different between the experimental Sj and Og rats (Fig. 3B), while the area of the BLA-containing section was smaller in Sj rats than in Og rats (t_{30}=−2.19, P<0.05) (Fig. 3B). To assess the possible contribution of BLA to the difference in avoidance of novel objects between the strains, we expressed the area of the BLA as a percentage relative to the area of BLA-containing section. Statistical analyses revealed that the percentage area of BLA was higher in Sj rats than in Og rats (t_{30}=2.70, P<0.05) (Fig. 3B).

We also prepared BNST-containing sections of experimental Sj and Og rats (Fig. 4A). Statistical analyses revealed that the BNST area was larger (t_{30}=4.86, P<0.01), whereas the BNST-containing section area was smaller (t_{30}=−2.79, P<0.01) in the experimental Sj rats compared with Og rats (Fig. 4B). When we expressed the area of the BNST as a percentage relative to the area of the BNST-containing section, the percentage of BNST was higher in Sj rats than in Og rats (t_{30}=6.13, P<0.01) (Fig. 4B).

These results suggest that the Sj strain has a larger BLA and BNST compared with the Og strain. Therefore, the BLA and/or BNST might be a candidate nucleus for the difference in avoidance of novel objects between the strains. Taken together, these results further support the validity of the use of the Sj and Og strains for the analyses of new-object reaction.

DISCUSSION

In Experiment 1, when food was provided in a novel way, the Sj strain showed a greater reduction in food consumption compared with the Og strain. Additional statistical analyses revealed that both the Sj and Og strains preferred to eat food from the usual container when they were offered a choice of food from the usual container or from the container covered with a cardboard box. These results suggest that the Sj strain has a stronger avoidance of novel objects than the Og strain. To further assess the validity of these two strains for the analyses of new-object reaction, we measured the size of the BLA and BNST in Sj and Og rats in Experiment 2. We found that experimental Sj rats had a larger percentage of BLA and BNST relative to the area of the whole section compared with experimental Og rats. These results suggest the BLA and BNST are potential nuclei involved in the difference in the avoidance of novel objects between the strains. Based on these findings, the present study suggests that the Sj and Og strains are effective experimental animals for the analysis of new-object reaction.

Among many differences between the Sj and Og strains, we focused on the difference in the BLA and BNST. However, other

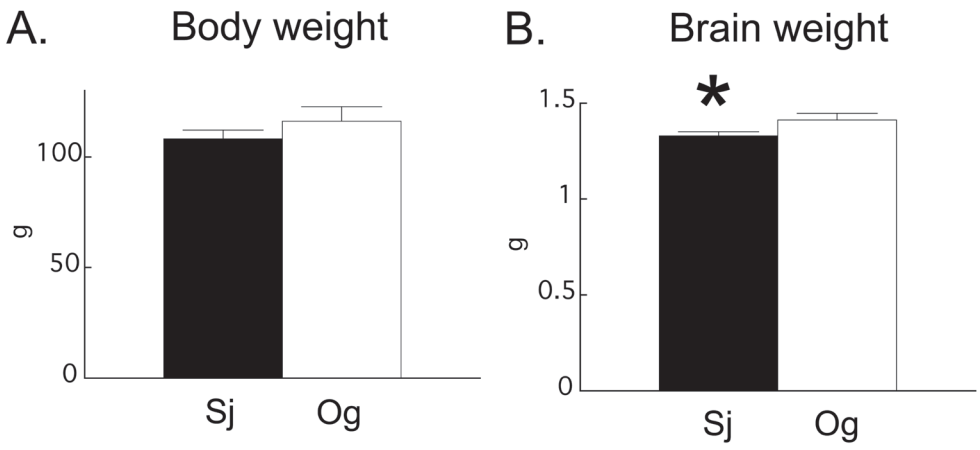

Fig. 2. General information of experimental Sj and Og rats in Experiment 2. (A) Body and (B) brain weights of experimental Sj and Og rats.

differences might also contribute to the difference in the avoidance of novel objects between the strains. For example, because the olfactory bulb was larger in experimental Sj rats than in Og rats (see Supplemental Results), the Sj strain might have a more effective sense of olfaction than the Og strain (see Supplemental Discussion). This may increase the degree of recognized novelty in the Sj strain compared with the Og strain, even if the same stimuli are presented. In addition, an alternative possibility is that the difference in physiological requirement for feeding produced the difference in the avoidance of novel objects between the strains. Although body weight was similar between experimental Sj and Og rats in Experiment 2, these rats do not necessarily represent the Sj and Og strains in Experiment 1. Difference in warfarin-sensitivity may also affect the difference in physiological requirements for feeding. Further research using the Sj and Og strains would clarify these points.

Based on the present and previous findings, we hypothesized that a difference in genetic factors, rather than in environmental factors, produced the difference in new-object reaction between the strains. The progenitors of Sj and Og strains were caught in Shinjuku and the Ogasawara Islands, respectively. The experience of the pest control operators that roof rats in Shinjuku, but not in the Ogasawara Islands, avoided traps suggested that the progenitors of the Sj strain were more likely to show stronger avoidance of novel objects than the progenitors of the Og strain. We confirmed that this difference was maintained even after being kept in similar environments for more than 10 years. Similarly, the descendants of wild roof rats [4, 6] and wild brown rats [1, 9] also maintained the avoidance of novel objects, indicating that the characteristic of new-object reaction might be determined by genetic factors.

This notion is further supported by the fact that new-object reaction was observed in a Wistar strain descended from 4 pairs of albino rats brought to the Wistar Institute in 1906 [13], i.e., rats kept in laboratories for more than 100 years. For example, Wistar rats preferred pressing a lever to obtain food from a familiar container to eating food without any labor from a novel container [16]. In addition, when Wistar, Long-Evans and wild rats were offered a choice between a familiar and a novel container containing identical food, all strains of rats did not initially eat food from the novel container [15]. However, it was also reported that wild rats (59 days) required more days than Wistar (27 days) and Long-Evans (31 days) rats to eat an equal amount of food from both containers [15], suggesting that wild rats have a stronger new-object reaction than laboratory rats. This might be because the selection of laboratory rats is not based on the intensity of new-object reaction after being kept in laboratories. In contrast, wild rats continue to receive strong pressure to increase the intensity of new-object reaction to avoid traps in the city. As a result, the number of genetic factor that contributes to new-object reaction is increased in wild rats. To support this, the intensity of avoidance of novel objects was weak even in wild rats when they were isolated from human activities [5].

In the present study, we suggested that the BLA and BNST participate in the difference in avoidance of novel objects between the strains. Therefore, these nuclei might play an important role in new-object reaction. Although the similarity of underlying neural mechanisms has not been assessed, the importance of the BLA is suggested in the hesitation of laboratory rats when ingesting novel food or water, i.e., food or taste neophobia. For example, rats spent a longer time eating a usual food than a novel food. Such preference was abolished by a lesion of the BLA [20], which also blocked the reduction of fluid intake when a novel taste was added to the water [11, 17]. In addition, this reduction was accompanied by increased Fos expression in the BLA [12]. In contrast, few studies have suggested a role for the BNST even in this experimental model. One study reported that a novel tasting water that induced a reduction in drinking [11] also tended to increase Fos expression in the BNST (P=0.051) [12]. In addition, BLA-lesioned rats still showed reduced drinking when a novel smell was added to the water [11], suggesting the existence of different neural mechanisms other than the BLA in this experimental model. Therefore, the present findings propose the possible contribution of the BNST in food or taste neophobia.

In summary, we found that the Sj strain showed a greater reduction of food consumption than the Og strain when food was provided in a novel way. These results suggest that the Sj strain has a stronger avoidance of novel objects than the Og strain. When we analyzed the brains of rats, experimental Sj rats had a larger BLA and BNST than experimental Og rats. These results indicate

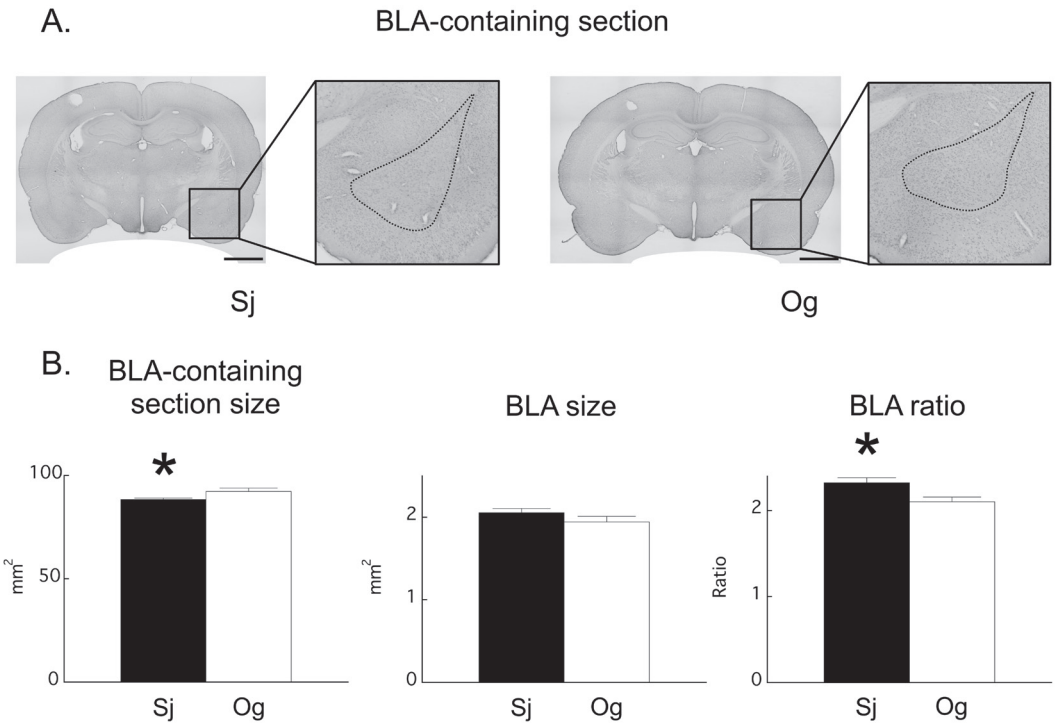

Fig. 3. Anatomical analyses of the basolateral complex of the amygdala (BLA) in Experiment 2. (A) Representative photomicrograph of the BLA-containing section of experimental Sj and Og rats. Horizontal bar indicates 2 mm. (B) The area of the BLA and BLA-containing section and the percentage of the BLA relative to the BLA-containing section of experimental Sj and Og rats. **P*<0.05 by Student's *t*-test.

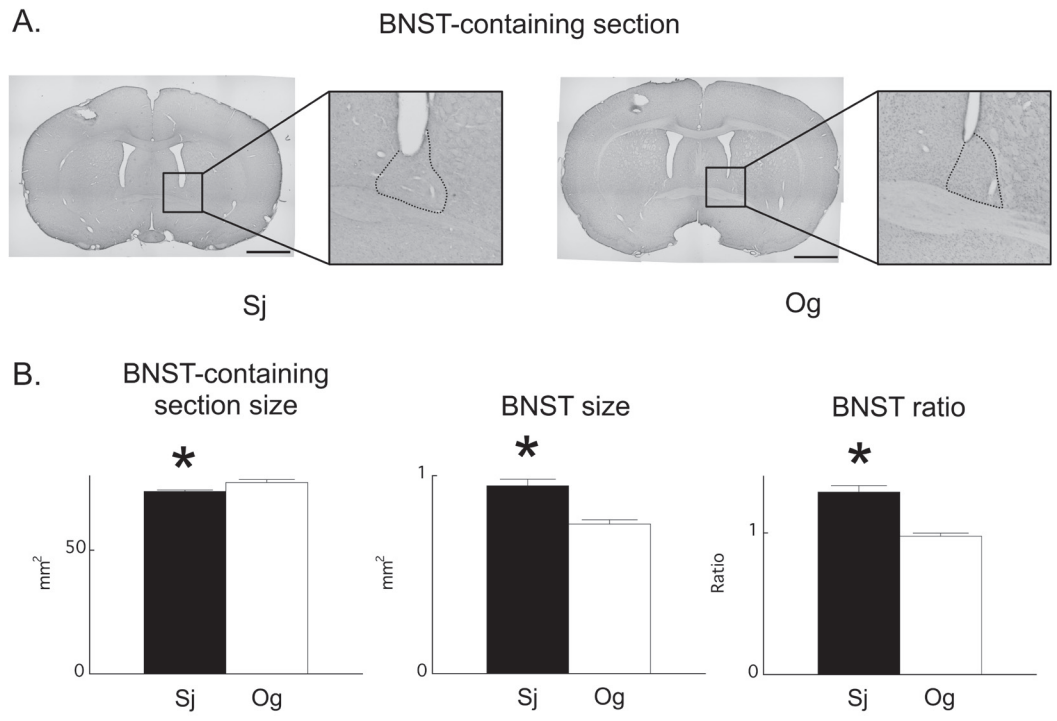

Fig. 4. Anatomical analyses of the bed nucleus of the stria terminalis (BNST) in Experiment 2. (A) Representative photomicrograph of the BNST-containing section of experimental Sj and Og rats. Horizontal bar indicates 2 mm. (B) The area of the BNST and BNST-containing sections and the percentage of the BNST relative to the BNST-containing section of experimental Sj and Og rats. **P*<0.05 by Student's *t*-test.

the BLA and/or BNST are potential nuclei involved in the difference of avoidance of novel objects between the strains. Based on these differences, the present study suggests that the Sj and Og strains are effective experimental animals for the analysis of new-object reaction. To clarify the functional role of BLA and BNST in new-object reaction, it is crucial to establish an experimental model observing avoidance of novel objects in individual animals. A model unrelated to ingestive behavior might be better to exclude the confounding origins that cause differences in physiological requirements. Behavioral neuroscientific analyses in such an experimental model will enable us to increase our understanding of the neurobiology of new-object reaction. Because new-object reaction is a major obstacle to the control of rodents, analyses of new-object reaction might also contribute to the development of effective methods to control rodents in society.

ACKNOWLEDGMENTS. We thank Dr. M. Yokosuka for his advice on the technical procedures. This work was supported by the Japanese Society for Promotion of Science KAKENHI (Grant Numbers 15H05635 and 15H05782).

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