Existence of wild brown rats (*Rattus norvegicus*) that are indifferent to novel objects

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ABSTRACT. Exposure to novel objects typically evokes avoidance behavior in wild animals, which is called neophobia. We previously found that wild brown rats (*Rattus norvegicus*) that were trapped in a park in downtown Tokyo, Japan, exhibited neophobia. We also found that this behavior was accompanied by the activation of the basolateral complex of the amygdala (BLA). Previous studies have suggested that genetic factors are the primary determinants of neophobia. Since rats in cities form populations with distinct genetic characteristics, it is reasonable to assume that wild rats caught at different locations in urban centers will exhibit different levels of neophobia. Here we assessed the intensity of neophobia in wild rats trapped at a wholesale market in Tokyo. Although we performed exactly the same experiment in which neophobia was observed in wild rats trapped at the park, the presence of novel objects did not affect the behaviors of wild rats trapped at the market. Conversely, laboratory rats showed approach and exploratory behaviors as seen in the previous study, suggesting that the experiment was performed appropriately. Compared to the laboratory rats, the lack of behavioral changes in the wild rats was accompanied by fewer Fos immunoreactive cells in the BLA. In addition, the numbers of Fos immunoreactive cells in the bed nucleus of the stria terminalis and ventromedial hypothalamus were similar between the two types of rats. The results demonstrated the existence of wild rats that were indifferent to novel objects.

KEY WORDS: brown rat, neophilia, neophobia, new-object reaction

Avoidance behavior is commonly evoked in wild animals that encounter novel objects for the first time, even if the objects are small and seemingly harmless [15, 26, 36, 38]. This behavioral response was originally referred to as new-object reaction [2] and was first described approximately 60 years ago in wild brown rats [7]. However, little progress in our understanding of new-object reaction has been made, partly because laboratory animals typically show approach behaviors, rather than avoidance behaviors, toward novel objects.

To obtain a better understanding of new-object reaction, we have been conducting studies on wild-trapped brown rats (*Rattus norvegicus*) [31]. Laboratory rats, a domesticated form of wild brown rats, are well suited for use as control animals for wild brown rats. Laboratory and wild brown rats are conspecific and are capable of producing hybrids [6], even though the oldest strain of laboratory rat (Wistar strain) has been maintained in laboratories for more than 100 years [32]. We previously established an experimental design for observing new-object reaction in home cages [30]. When we placed novel objects (two pumpkin head plastic dolls or two plastic bear dolls) at one end of the home cage, wild and laboratory rats decreased and increased the time that they spent in the objects’ half of the cage, respectively. The avoidance behavior in wild rats was accompanied by the activation of brain nuclei related to defensive behaviors, such as the basolateral complex of the amygdala (BLA), ventral bed nucleus of the stria terminalis (vBNST), and ventromedial hypothalamus (VMH), suggesting that new-object reaction is a defensive behavior. However, the dorsal bed nucleus of the stria terminalis (dBNST) was not activated under these conditions in wild rats. Given that defensive behaviors can be divided into dBNST-dependent anxiety responses and dBNST-independent fear responses [12, 25], new-object reactions are considered to be a fear response, i.e., neophobia.

Although the assumption that any wild rats should show neophobia is prevalent, the findings of ecological studies have questioned that assumption. It is known that genetic factors are primarily responsible for neophobia. For example, laboratory...
studies on the descendants of wild brown rats [2, 10, 20, 34] and wild roof rats [9, 11, 26] showed that they retained neophobia, even after being kept in a laboratory for generations. In addition, handling by experimenters from postnatal day 10 through 110 had no effect on decreasing neophobia [17]. However, these findings did not assess whether neophobia is a ubiquitous phenotype among wild rats. In urban environments, wild rats spend their entire lives within a small area, which produces numerous genetic clusters of rat populations within a city. For example, in the city of Baltimore, USA, it was estimated that the kinship coefficient was estimated zero when two rats were separated more than 1,700 m [18]. In support of this finding, the leave-one-out test using genetic information at eight loci could correctly assign 95.3% of 277 trapped rats to one of 11 trapping areas [18]. Similarly, wild rats in Vancouver, New Orleans, New York City, and Salvador exhibited statistically significant autocorrelation only within 200, 1,000, 1,000, and 2,500 m, respectively [8]. Therefore, it is reasonable to assume that wild rats caught at different locations within the same city exhibit different levels of neophobia because these rats belong to genetically distinct clusters.

The intensity of neophobia in wild rats is thought to be affected by the human activities in that habitat. For example, intensive culling programs using traps and rodenticides, i.e., new objects, could force wild rats to develop neophobia. Consistent with this notion, non-pest rats, such as long-haired rats (R. villosissimus) and bush rats (R. fuscipes), did not show neophobia, even when trapped individuals were tested in the laboratory [10]. We previously showed that brown rats caught in a park in a downtown area of Tokyo, Japan, showed high levels of neophobia [30]. In municipalities, even a small increase in rat populations prompts local residents to report these increases to the local public health center. Because rats are known to transmit many diseases [1, 14, 16, 29] and an increase in rat numbers in parks is hazardous to public health, the public health center undertakes ad hoc culling exercises until all of the rats appear to have been eradicated. Therefore, wild rats in our previous study likely developed neophobia in order to survive such culling programs. In contrast, neophobia seems to be a less adaptive behavior in habitats where people are tolerant of rats. When the risk of being attacked by humans is low, bolder rats would obtain more food and increase the likelihood of finding mates. In addition, more potential nesting sites are available to bolder rats. Therefore, wild rats in such places would degrade neophobia. In a central metropolitan wholesale market in Tokyo, only two culling programs that culled approximately one fourth of the resident rats were conducted annually. In addition, the staff at the market were also tolerant of rats. Based on these scenarios, it was hypothesized that rats trapped in the market would show either weak or no neophobia.

To test this hypothesis, we trapped wild brown rats at the wholesale market in Tokyo and housed them individually in the laboratory. In addition, laboratory rats were purchased and housed individually under the same conditions. We then placed novel objects at one end of the home cage of each rat and assessed the level of neophobia by measuring the time spent in the half of the cage containing the objects. In addition, we examined Fos expression in the BLA, dBNST, vBNST, and VMH.

**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.

Wild brown rats (5 males) were captured at the metropolitan central wholesale market in Tokyo Japan (Tsukiji Market) using live traps and transferred to the laboratory at Ikari Shodoku Corporation immediately. Upon arrival, the rats were weighed and kept individually in wire mesh cages (23.5 × 40 × 16.5 cm) in a room with an ambient temperature of 20 ± 5°C and a 12-hr light/dark cycle (lights were switched on at 6:00). We allowed the rats to acclimatize to laboratory conditions for approximately 5 weeks. We purchased laboratory rats (5 male Wistar rats) from Charles River Laboratories Japan (Yokohama, Japan) that weighed slightly less than wild rats so that they would be weight-matched at the day of testing. The Wistar rats were treated in the same manner as the wild rats with the exception that they were given approximately 1 week to acclimatize to laboratory conditions. Food (CE-2, Clea Japan, Tokyo, Japan) and water were administered *ad libitum* to all rats. During acclimation, we occasionally observed the subjects and assessed whether they showed any preference for one side of the cage.

Behavioral tests were conducted in the home cage of each subject between 10:00 to 15:00, as reported in our previous study [30]. We first transferred the cage to a table in the colony room and inserted a partition into the center of the cage to confine the subject to the non-preferred side. Two experimental conditions were tested: the with-object condition and the no-object condition. In the with-object condition, two randomly selected novel objects were placed at the end of the preferred side of the cage. The objects comprised two pumpkin-head plastic dolls (height 13 cm) or two plastic bear dolls (height 13.5 cm). After adding the objects (or adding no objects in the no-object condition), the cage was returned to the original location in the colony room. We then removed the partition and used a video camera (HDRHC9, SONY, Tokyo, Japan) to record the subject’s behavior during the subsequent 20-min experimental period. Given the limited number of available wild rats, all subjects underwent the behavioral test once in the no-object condition and once in the with-object condition. The two trials were separated by a 2–3-hr interval. As the test in the with-object condition established contextual fear conditioning in the wild rats [30], all subjects were first exposed to the no-object condition and then to the with-object condition.

Immunohistochemistry for Fos protein was performed as described previously [27, 28]. After the behavioral test, the home cage was left undisturbed for an additional 40 min so that the subjects were exposed to the novel objects for a total of 60 min. Each subject was then deeply anesthetized with sodium pentobarbital, weighed, and intracardially perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were sampled and immersed in the same fixative overnight before being placed in 30% sucrose/phosphate buffer for cryoprotection. We then prepared four successive 40-µm coronal sections containing the dBNST and vBNST (Bregma −0.12 mm), VMH (Bregma −1.80 mm), and lateral (LA) and basal
amygdala (BA) (Bregma –3.12 mm). The sections were incubated with a primary rabbit antibody to c-Fos protein (ABE457; EMD Millipore, Temecula, CA, USA) for 65 hr at 4°C, and with a secondary biotinylated goat antibody to rabbit IgG (BA-1000, Vector Laboratories, Burlingame, CA, USA) for 2 hr at room temperature. The sections were then processed with the avidin-biotinylated peroxidase complex (VECTASTAIN Elite ABC kit, Vector Laboratories) for 2 hr at room temperature and developed using a diaminobenzidine solution with nickel intensification.

Data analysis and statistical procedures

Data were expressed as the mean ± standard error of the mean. P<0.05 was considered to represent significance for all statistical analyses.

Since the novel objects occupied a substantial section of the cage (5 cm), we divided the remaining area equally into close and distal zones (17.5 cm each). The close zones were located adjacent to the novel objects. A researcher recorded the time spent in each zone (the center of the hind paws was used to indicate the location of the rat), the duration of object exploration (e.g., the rat touched the object with its forepaw and/or nose, or sniffed the object within a distance of 1 mm), and the number of transitions between the zones. Microsoft Excel-based Visual Basic software was used to record the duration and number of key presses on a computer keyboard [23, 24]. The differences between the no-object and with-object conditions in each population of subjects (i.e., wild rats and laboratory rats) were analyzed using a paired t-test. To compare the data between the two types of rats, we expressed the time spent in each zone and the number of transitions in the with-object condition as a percentage of that in the no-object condition. The percentage of these behaviors, as well as the duration of exploration, was analyzed using a Student’s t-test. Body weight was analyzed using a Student’s t-test.

For the immunohistochemical analyses, each nucleus was photographed using a microscope equipped with a digital camera (DP30BW, Olympus, Tokyo, Japan). To analyze Fos expression, an experimenter who was blind to the group identity of the subjects counted the number of Fos immunoreactive cells within a 0.5-mm square positioned bilaterally within each nucleus. When the designated area was smaller than the boundaries of the 0.5-mm square, only the cells in the region of interest were counted. Counts were prepared for four sections using ImageJ 1.45 software. The mean number of cells in the LA and BA was considered to be the number of cells in the BLA. We calculated the density of the immunoreactive cells in each nucleus and analyzed the data using a Student’s t-test.

RESULTS

We confirmed that body weight was similar between the wild (205.8 ± 21.8 g) and laboratory rats (202.4 ± 8.9 g).

In the wild rats, the time spent in the close and distal zones in the with-object conditions was similar to that in the no-object condition (Fig. 1A). The number of transitions was similar between the conditions. In the laboratory rats, the time spent in the close and distal zones in the with-object condition was longer (P<0.05) and shorter (P<0.05), respectively, compared to that in the no-object condition. The number of transitions in the with-object condition was less than that in the no-object condition (P<0.01). In addition, the duration of exploration was shorter in the wild rats compared with the laboratory rats (P<0.01).

To directly compare behaviors between the two populations of subjects, we expressed the time spent in each zone and the number of transitions in the with-object condition as percentages with respect to the data obtained in the no-object condition. Statistical analysis showed that the percentage of time spent in the close and distal zones was lower (P<0.05) and higher (P<0.05), respectively, in the wild rats compared with the laboratory rats (Fig. 1B). In contrast, the percentage of transitions was similar between the two rat populations.

After the behavioral test, we analyzed Fos expression in the brain. Statistical analysis revealed that the density of Fos immunoreactive cells in the LA (P<0.05) and BA (P<0.01) was lower in the wild rats compared with the laboratory rats (Fig. 2). When we calculated the density of Fos immunoreactive cells in the BLA, the density was also lower in the wild rats compared with the laboratory rats (P<0.01). In contrast, the densities in the dBNST, vBNST, and VMH were similar between the two rat populations.

DISCUSSION

In the present study, the presence of novel objects did not affect behavioral responses in the wild rats. Conversely, under the same conditions, laboratory rats showed clear approach and exploratory behaviors toward the novel objects, suggesting that the experiment was performed appropriately. Further, compared to the laboratory rats, the lack of behavioral changes in wild rats was accompanied by a lower number of Fos immunoreactive cells in the BLA. The numbers of Fos immunoreactive cells in the vBNST and VMH were similar between the two rat populations. These results demonstrated the existence of wild brown rats that were indifferent to novel objects.

Combined with the findings of our previous study [30], we propose that rats from different locations exhibit different responses to novel objects. Therefore, large-scale ecological analyses are crucial to assess whether the observed phenotype is shared among wild rats. Wild rats are widely considered to be a valuable tool for investigating and understanding phenotypes that are not present in laboratory rats. One such phenotype is the resistance to anticoagulant agents used to control rodent pests. Analyses in wild rats revealed that mutations in the vitamin K epoxide reductase complex subunit 1 gene yield this phenotype [21]. Neophobia could be another representative phenotype that laboratory rats do not have. The importance of BLA was suggested based on our previous studies using wild rats [30]. We consider that examining the underlying physiological and/or neural mechanisms of the phenotypes

J. Vet. Med. Sci. 83(1): 78–83, 2021
would be valuable because wild rats showing the same phenotypes would share the same underlying mechanisms. However, such analyses provide no information regarding how widespread these phenotypes are in wild populations. Indeed, while neophobia was observed in rats trapped at the park in our previous study, no neophobia was observed in rats trapped at the market in this study. We should therefore be careful when extrapolating characteristics from a small number of wild rats as being representative of all wild rats.

As opposed to our assumption, compared to the wild rats, the laboratory rats showed higher levels of Fos expression in the BLA. One possible reason for this could be that the BLA plays an important role in exploratory behavior. It has been demonstrated that the exploration of novel objects is rewarding for laboratory rats [4]. For example, when given a choice, rats prefer to stay in areas where they had explored a novel object previously [3]. This novel-object conditioned place preference has been shown to share the same neural mechanisms as when the conditioned place preference has been established with addictive drugs [5]. It has also been reported that an anticipation of access to novel objects can produce a conditioned increase in activity [5] in a way that is similar to food and psychomotor stimulants [33, 35]. Recently, it was clarified that the BLA plays important roles in behavioral responses, not only to negative valence stimuli, but also to positive valence stimuli. For example, two spatially segregated populations of neurons in the BLA have been shown to be specifically activated by negative valence stimuli (e.g., shocks, predator odor, and bitterness) and positive valence stimuli (e.g., females, peanuts, and sweetness), respectively [22]. In addition, the activation of each population of neurons plays an important role in a wide variety of behavioral responses to each of the valence stimuli [22]. It is therefore possible that exploratory behavior towards novel objects was elicited by the activation of positive valence-specific neurons in the BLA.

Based on the findings of the present and previous studies [30], we hypothesize that an individual’s response towards novel objects is determined by two variables. Most studies in the literature have attempted to explain the response towards novel objects using only one variable. For example, it was proposed that the degree of novelty determines the response towards novel objects. Thus, intense novelty may elicit an avoidance response while mild novelty may elicit an approach response in wild rats [19]. Another variable that has been proposed is the stress status of the animal. Wild rats in states of high or low stress may show avoidance or approach responses towards novel objects, respectively [37]. However, to the best of our knowledge, no attempts have been made to explicitly test these hypotheses. In addition, these explanations cannot account for the indifference observed in the wild rats of the present study. Specifically, we have demonstrated that wild rats kept and tested in the same condition showed different behaviors towards the same objects. Therefore, we consider that it would be more appropriate to hypothesize that two variables affect an individual’s response towards novel objects. Specifically, we propose that an individual’s response towards novel objects can be expressed as an integration of vertically connected vectors for the repellent and appetitive drives [13]. Under such a scenario, an individual exhibits an avoidance or approach response when the direction of the summation vector is closer to the repellent or appetitive vector, respectively. In addition, the length of the summation vector represents the intensity of the response.
For example, wild rats in our previous study exhibited neophobia because they had a long repellent vector and a short appetitive vector. Similarly, wild rats in the present study were indifferent to novel objects because both of the vectors were short. Conversely, laboratory rats approached novel objects because they had a short repellent vector and a long appetitive vector. The additional advantage of this model is that it can be used to illustrate the intensity of the conflict between the repellent and appetitive drives, which can be represented as the area between the vectors [13]. However, since little is currently known about wild rats that are indifferent to novel objects, further research is required in order to verify this hypothesis.

In summary, we found that rats that had been trapped in a metropolitan central wholesale market in Tokyo were indifferent to novel objects. The results show that rats from different locations exhibit different responses to novel objects. Analyses of individuals enabled us to obtain detailed information regarding the phenotype of wild rats. However, as we demonstrated in the present study, the existence/intensity of phenotypes in individuals can vary among populations. In contrast, ecological studies could identify environmental factors that contribute to the establishment of different phenotypes and/or clarify the prevalence of the target phenotype within a population. Therefore, the combination of both types of studies (i.e., analyses of individuals and of populations) is considered necessary to better understand the behavior of wild rats.

POTENTIAL CONFLICTS OF INTEREST. The authors have nothing to disclose.

ACKNOWLEDGMENT. This study was supported by JSPS KAKENHI (20H03160 and 20H04766).
