Novel objects elicit greater activation in the basolateral complex of the amygdala of wild rats compared with laboratory rats

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ABSTRACT. Wild animals tend to avoid novel objects that do not elicit clear avoidance behaviors in domesticated animals. We previously found that the basolateral complex of the amygdala (BLA) and dorsal bed nucleus of the stria terminals (dBNST) were larger in trapped wild rats compared with laboratory rats. Based on these findings, we hypothesized that the BLA and/or dBNST would be differentially activated when wild and laboratory rats showed different avoidance behaviors towards novel objects. In this study, we placed novel objects at one end of the home cage. We measured the time spent in that half of the cage and expressed the data as a percentage of the time spent in that region with no object placement. We found that this percentage was lower in the wild rats compared with the laboratory rats. These behavioral differences were accompanied by increased Fos expression in the BLA, but not in the dBNST, of the wild rats. These results suggest that wild rats show greater BLA activation compared with laboratory rats in response to novel objects. We also found increased Fos expression in the paraventricular nucleus of the hypothalamus, ventral BNST, and ventromedial hypothalamus, but not in the central amygdala of wild rats. Taken together, our data represent new information regarding differences in behavioral and neural responses towards novel objects in wild vs. laboratory rats.

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

Wild and domesticated animals show distinct behaviors, for instance, in their response to novel objects. Specifically, wild animals tend to avoid novel objects [11, 40, 44] whereas domesticated animals of the same species mostly approach and investigate them [12]. However, little is known about the neural mechanisms that cause differences in avoidance behaviors towards novel objects among wild vs. domesticated animals.

We chose to examine this issue using brown rats (Rattus norvegicus). Wild rats are among the most accessible wild animals because they are common pests in many human societies [31]. To survive against humans’ numerous attempts to eliminate them, wild rats are supposed to show avoidance behaviors towards novel objects. Laboratory rats are a domesticated form of wild rat and show approach behaviors, rather than avoidance behaviors, toward novel objects. Laboratory and wild rats are members of the same species, with the same binomial name and the ability to produce hybrids [4], even though the oldest strain of laboratory rat (Wistar strain) has been kept in laboratories for more than 100 years [30]. Previously, we conducted morphological analyses to identify candidate nuclei that might contribute to differences in avoidance behaviors towards novel objects. We found that the basolateral complex of the amygdala (BLA) and dorsal bed nucleus of the stria terminals (dBNST) were larger in trapped wild rats compared with laboratory rats [24]. Based on these findings, we hypothesized that the BLA and/or dBNST are differentially activated in the two rat types according to differences in avoidance behaviors towards novel objects.

Avoidance behaviors are classified as defensive behaviors towards threats in rats [3, 7]. Therefore, it is possible that the two rat types differentially estimate the threat level of novel objects, and show corresponding differences in avoidance behaviors. Stress is a condition in which an animal’s life is threatened by an uncontrollable and unpredictable stimulus [25]. Activation of the hypothalamic–pituitary–adrenal (HPA) axis is one of the primary stress responses. In the HPA axis response, activation of the paraventricular nucleus of the hypothalamus (PVN) releases a corticotrophin-releasing hormone that stimulates adrenocorticotropic hormone release from the anterior lobe of the pituitary, which in turn stimulates corticosterone release from the adrenal glands.
Therefore, we can infer an animal’s stress level by measuring the magnitude of the HPA axis response through the PVN activities. Based on these facts, we hypothesized that wild rats would show greater PVN activation in situations where they exhibited greater avoidance behaviors towards novel objects compared with laboratory rats.

We assessed these hypotheses in the present study. First, we trapped wild brown rats in downtown Tokyo and housed them individually. Laboratory rats were purchased and housed individually in the same conditions. Then, we placed novel objects at one end of the home cage of each rat and observed avoidance behaviors towards the objects. We also measured Fos expression in the BLA, dBNST, and PVN, as well as in the ventral BNST (vBNST), central nucleus of the amygdala (CeA), and the ventromedial hypothalamus (VMH). This enabled us to assess neural mechanisms associated with the different avoidance behaviors towards novel objects in the two rat types.

**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.

**Animals**

Wild rats (5 males) were captured at a park near a train station in downtown Tokyo, Japan and transferred to the laboratory at Ikaru Shodoku Corporation immediately. Upon arrival, they 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 acclimate to the colony room for about 5 weeks. We purchased laboratory rats (6 Wistar male rats, 7 weeks old) 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. They were treated in the same way as the wild rats with the exception that we allowed them about 1 week to acclimate to the colony room. Food (CE-2, Clea Japan, Tokyo, Japan) and water were available ad libitum to all rats. During colony room acclimation, we occasionally observed the subjects and judged their preferred side of the cage.

**Behavioral test**

The behavioral test was conducted in the home cage of each subject between 10:00 to 15:00. 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. There were two conditions, as follows: the with-object and no-object situation. In the with-object situation, two randomly chosen novel objects were placed at the end of the preferred side of the cage (Fig. S1A). The objects were either 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 situation), the cage was returned to the original location in the colony room. Then, we removed the partition and used a video camera (HDR-HC9, SONY, Tokyo, Japan) to record the subject’s behavior during a subsequent 20-min period. Because of the limited number of available wild rats, all subjects underwent the behavioral test once in the no-object situation and once in the with-object situation. The two trials were separated by a 2–3-hr interval. In our pilot study, we found that the behavioral test in the with-object situation established contextual fear conditioning in the wild rats. Thus, to reduce the effects of fear conditioning, all subjects were first tested in the no-object situation and then tested in the with-object situation.

**Immunohistochemistry**

After the behavioral test, the home cage was kept undisturbed for an additional 40 min so that the subjects were exposed to the novel objects for a total of 60 min. Then, each subject was 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, and then placed in 30% sucrose/phosphate buffer for cryoprotection.

We used the avidin-biotin-peroxidase immunohistochemistry method to detect Fos expression, as previously described [20, 23]. We collected 4 successive 40-µm coronal sections containing the dBNST and vBNST (Bregma −0.12 mm), PVN and VMH (Bregma −1.80 mm), and lateral amygdala (LA), basal amygdala (BA), and CeA (Bregma −3.12 mm). After incubation with 0.3% hydrogen peroxide in phosphate buffered saline (PBS) for 30 min, the sections were washed with PBS, PBS containing polyoxyethylene (10) octylphenyl ether (PBST), and PBS again for 10 min each. Then, sections were incubated with normal goat serum (VECTASTAIN Elite ABC kit; Vector Laboratories, Burlingame, CA, U.S.A.) for 30 min, followed by a primary rabbit antibody to c-Fos protein (ABE457; EMD Millipore, Temecula, CA, U.S.A.) diluted 1:7,500 for 65 hr at 4°C. After washing the sections with PBS, PBST, and PBS again for 10 min each, they were incubated with a secondary biotinylated goat antibody to rabbit IgG (VECTASTAIN Elite ABC kit) for 2 hr. Then, the sections were washed with PBS, PBST, and PBS again for 10 min each and processed with the avidin-biotinylated peroxidase complex (VECTASTAIN Elite ABC kit) for 2 hr. After washing the sections twice with sodium acetate buffer for 10 min each, they were developed using a diaminobenzidine solution with nickel intensification for 6 min. Finally, the sections were washed with sodium acetate buffer and PBS for 10 min each and mounted on glass slides. The sections on the glasses were dehydrated in ascending concentrations of ethanol, xylene cleared, and cover slipped.

**Data analyses and statistical procedures**

Because the novel objects occupied a substantial region of the cage (5 cm), we divided the remaining area equally into close...
and distal zones (17.5 cm each) (Fig. S1B). The close zones were located next to the novel objects. A researcher recorded the time spent in each zone (the center of hind paws was considered as the location of the rat), the duration of exploration (the rat touched an object with its forepaw and/or nose or sniffed towards the object within 1 mm of the object), and the number of transitions between the zones. As in our previous studies, we used Microsoft Excel-based Visual Basic software to record the duration and number of key presses on a computer keyboard [17, 22]. The differences between the no-object and with-object situations in each type of the subjects were analyzed using a paired r-test. To compare the data between the two types of the subjects, we expressed the time spent in each zone and the number of transitions in the with-object situation as a percentage of those in the no-object situation. The percentage of these behaviors, as well as the duration of exploration, was analyzed using a Student’s t-test.

For immunohistochemical analyses, each nucleus was captured using a microscope equipped with a digital camera (DP30BW, Olympus, Tokyo, Japan). The regions of interest were located based on the background staining. To analyze Fos expression in the BLA, 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 in the LA and BA. Counts were made for 4 sections using ImageJ 1.45 software. When the nucleus was smaller than the boundaries of the 0.5-mm square, only the cells in the region of interest were counted. The mean number of cells in the LA and BA was considered to be the number of cells in the BLA. To analyze Fos expression in the dBNST and PVN, we bilaterally counted the number of Fos immunoreactive cells within a 0.5-mm square using the same method. Because the vBNST, CeA, and VMH were much smaller than the boundaries of the 0.5-mm square, the experimenter counted the number of Fos immunoreactive cells within the nucleus, and measured the area of the nucleus. Then, we calculated the density of the immunoreactive cells in all nuclei, and analyzed the data using a Student’s t-test.

RESULTS

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

Results of the behavioral tests were shown in Fig. 1A. In the wild rats, the time spent in the close and distal zones in the with-object situations were shorter (P<0.05) and longer (P<0.05), respectively, compared with those in the no-object situation. The number of transitions was similar between the situations (P=0.09). In contrast, in the laboratory rats, the time spent in the close and distal zones in the with-object situation were longer (P<0.01) and shorter (P<0.01), respectively, compared to those in the no-object situation. The number of transitions in the with-object situation was fewer compared with those in the no-object situation (P<0.01). In addition, the duration of exploration was shorter in the wild rats compared with the laboratory rats (P<0.01). These results

![Fig. 1. Results of the behavioral test. (A) The time spent in the close and distal zones and the number of transitions in each situation and the duration of exploration in the with-object situation (mean ± SEM) of the wild (Wild) and laboratory (Laboratory) rats. (B) The data in the with-object situation was expressed as percentages with respect to the data obtained in the no-object situation. *P<0.05 with paired t-test or Student’s t-test.](http://example.com/fig1.png)
suggest that the wild and laboratory rats showed avoidance and approach behaviors towards the novel objects, respectively.

In order to directly compare behaviors between the two types of the subjects, we expressed the time spent in each zone and the number of transitions in the with-object situation as percentages with respect to the data obtained in the no-object situation. Statistical analyses revealed that the percentage of time spent in the close and distal zones was lower ($P<0.01$) and higher ($P<0.01$), respectively, in the wild rats compared with the laboratory rats (Fig. 1B). In contrast, the percentage of transitions was similar between the two rat types ($P=0.46$) (Fig. 1B).

Although we obtained lighter laboratory rat subjects one week before (150–200 g), the wild rats were lighter than the laboratory rats at the time of testing (wild rats: $218.6 \pm 19.3$ g; laboratory rats: $275.2 \pm 3.7$ g; $P<0.05$, Student’s t-test). Therefore, it is possible that the differences in avoidance behaviors towards novel objects were due to the difference in their body weight, rather than in the rat type. To clarify this, we assessed the correlation (Pearson’s correlation) between body weight and behavioral measures in the with-object situation. We found no correlation within either the wild and laboratory rats. Therefore, it seems unlikely that the differences in body weight caused the differences in avoidance behaviors towards novel objects.

After the behavioral test, we analyzed Fos expression in the brain (Fig. 2). The density of Fos immunoreactive cells was shown in Fig. 3. Statistical analyses revealed that the density of Fos immunoreactive cells in the LA ($P<0.01$) and BA ($P<0.01$) was higher in the wild rats compared with the laboratory rats. When we calculate the density of Fos immunoreactive cells in the BLA, the density was also higher in the wild rats compared with the laboratory rats ($P<0.01$). However, the density was similar between the two types of the subjects in the CeA ($P=0.39$). In the BNST, the density of Fos immunoreactive cells in the vBNST ($P<0.05$), but not in the dBNST ($P=0.08$), was higher in the wild rats compared with the laboratory rats. Furthermore, we found that the density of Fos immunoreactive cells in the PVN ($P<0.01$) and VMH ($P<0.01$) was higher in the wild rats compared with the laboratory rats.

**DISCUSSION**

In the present study, we found that the wild rats showed lower percentage of time spent in the close zones compared with the laboratory rats. This was accompanied by increased Fos expression in the BLA, but not in the dBNST, of the wild rats. These results suggest that novel objects elicited greater activation in the BLA of wild rats compared with laboratory rats. Such differences in avoidance behaviors were accompanied by increased Fos expression in the PVN, vBNST, and VMH, but not in the CeA, of the wild rats. These results suggest that the seemingly harmless novel objects served as more threatening stimuli for wild rats. Taken together, these data represent new information regarding the differences in behavioral and neural responses towards novel objects between wild and laboratory rats.

To the best of our knowledge, this is the first study to report that wild rats, on an individual level, show greater avoidance behaviors towards novel objects than do laboratory rats. Although avoidance behaviors have been observed in groups of wild rats [6, 21, 32], previous attempts to observe avoidance behaviors in individual wild rats were not successful. In these studies, novel object(s) were simply placed in one of three compartments in a familiar test apparatus. However, the presence of the novel objects did not decrease the time spent in the compartment [33, 41]. One reason for the discrepancy between the present findings and those of previous studies may be that while we placed the novel objects in the preferred side of the home cage, the objects were placed in a neutral compartment in the previous studies. The rats may have had a lack of motivation to approach the area where the novel objects were placed, thus decreasing the likeliness of avoidance behaviors. This interpretation is supported by previous findings that avoidance behaviors in individual wild rats were successfully observed using indirect measures, such as a reduction of food or water consumption, after changing objects in the home cage related to feeding or drinking [1, 6]. In these studies, rats were motivated to approach the objects to engage in eating and drinking.

In the present study, we sampled the brain of the subjects that were tested both in the no-object and with-object situations due to several limitations associated with the usage of wild rats. Therefore, we acknowledged the possibility that Fos expression caused by the test in the no-object situation might have prevented us from evaluating the differences in Fos expression in response to the novel objects. Nonetheless, we have conducted this study because, even in this schedule, the difference in Fos expression would suggest the possible contribution of the focal nucleus.

We found that differences in avoidance behaviors were accompanied by differences in Fos expression in the BLA, but not in the dBNST. These results suggest that novel objects elicited greater activation in the BLA of wild rats compared with laboratory rats. However, it is not yet clear why the novel objects elicited differences in BLA activation, with the observed differences in avoidance behaviors as a possible consequence. One possibility is that the BLA is generally more reactive to stimuli in wild rats compared with laboratory rats. In mice, serotonin 1A receptor knockout mice showed greater avoidance behaviors toward novel objects, as well as increased anxiety behaviors in the open field test and elevated zero maze test, compared with wild-type mice [16]. Although the receptor exists in a wide variety of the brain regions [43, 47], the changes in the knockout mice could be ascribed to the lack of serotonin 1A receptor in the BLA. For example, the suppression of serotonin 1A receptor expression in the BLA, together with the suppression in the CeA, increased anxiety behaviors in the elevated plus maze test and open-field test in mice [29]. In contrast, an intra-BLA injection of serotonin 1A receptor agonist reduced anxiety behaviors in the elevated T-maze in rats [39]. When serotonin 1A receptor was compared between wild and laboratory rats, its bindings were different in a variety of brain regions, although the differences in the BLA were not investigated in that study [15]. In addition, administration of serotonin 1A agonist reduced behavioral reactivity in wild roof rats to several stimuli when they were placed in an inescapable situation [2]. Therefore, differences in the function of the serotonin 1A system may be responsible for the unique BLA activity in the two rat types. An alternative possibility is that the BLA in wild rats is more reactive to specific stimuli, including novel objects. For example, Rho-
GAP interacting CIP4 homolog 2 (Rich2) is a protein that is highly enriched in the postsynaptic density of excitatory synapses and participates in the maintenance and elongation of spines by actin polymerization. In the BLA of Rich2 knockout mice, the number of mature spines is decreased while the spine density is similar to that of wild-type mice. Compared with wild-type mice, Rich2 knockout mice show greater avoidance behaviors towards novel objects with increased Fos expression in the BLA [35]. However, these knockout mice showed similar levels of sociability and a similar preference for novel conspecifics compared with wild-type mice [36]. Furthermore, knockout and wild-type mice show similar nesting behaviors, as well as behaviors in the open field test, elevated plus maze test, and forced swim test, although knockout mice also show excessive self-grooming [36]. Therefore, it is possible that differences in the ratio of mature synapses in the BLA are responsible for the variance in BLA activity in response to specific stimuli, including novel objects, between the two rat types. A more detailed characterization of the BLA in wild rats would

Fig. 2. Representative photomicrographs showing Fos immunoreactive cells and schematic diagrams of the location of the analyzed areas in the lateral (LA), basal (BA), and central amygdala (CeA), dorsal (dBNST) and ventral nucleus of the stria terminalis (vBNST), paraventricular nucleus of the hypothalamus (PVN), and ventromedial hypothalamus (VMH) of the wild (Wild) and laboratory (Laboratory) rats. The number of Fos immunoreactive cells was counted within a 0.25-mm² square (open square) or in the entire nucleus (open square containing an *). The numbers in the diagrams indicate the distance from the Bregma (mm). Horizontal bar indicates 500 μm.
clarify this point.

Consistent with our hypothesis, we found that Fos expression in the PVN was higher in the wild rats compared with the laboratory rats. To the best of our knowledge, this is the first study to assess the HPA axis response to acute stimuli in wild rats. Our results suggest that the novel objects served as a stronger threat for wild rats compared with laboratory rats. The increased Fos expression observed in the BLA, vBNST, and VMH of the wild rats supports this interpretation. The BLA is known to play a pivotal role in avoidance behaviors towards unconditioned [19] and conditioned threats [28]. Although the role of the vBNST in responding to threats remains controversial [14, 34], it has been found to play an important role in freezing in response to predator odor (2,3,5-trimethyl-3-thiazoline, a component of fox odor) [10] and in the HPA axis response to restraint [5] and air puff stimuli [38]. The mouse VMH has been found to regulate defensive behaviors towards a predator (rat), including avoidance behaviors [37]. That we found no difference in Fos expression in the CeA between the two rat types further supports this interpretation. For example, in contrast to its established roles in passive defensive responses including freezing [18, 27] and enhancement of the startle reflex [45], the CeA was recently found to be suppressed by the BLA when rats and humans showed active defensive responses towards electrical shocks, including avoidance behaviors [42]. Taken together, these data regarding HPA axis activity and Fos expression patterns in response to the novel objects suggest that wild rats regarded the seemingly harmless novel objects as a threat.

Based on the anatomical connections of each nucleus [13, 14], we hypothesized the following neural mechanisms. When wild rats encounter novel objects, their BLA is activated. This activation is transmitted to the vBNST [45] and then to the PVN [8, 9]. As a result, the HPA axis response is elicited. The BLA activation is simultaneously transmitted to the basomedial nucleus of the amygdala [26] and then to the VMH [48]. The VMH activation is further transmitted to the anterior hypothalamus [46] and induces avoidance behavior.

In summary, we found that the differences in avoidance behaviors towards the novel objects were associated with increased Fos expression in the BLA, but not in the dBNST, of the wild rats. These results suggest that novel objects elicited greater activation in the BLA of wild rats compared with laboratory rats. We also found that Fos expression in the wild rats was increased in the PVN, vBNST, and VMH, but not in the CeA. Taken together, our findings represent new information regarding the differences in behavioral and neural responses towards novel objects in wild vs. laboratory rats. Because previous analyses of the effects of domestication have generally focused on morphological differences, little is known about corresponding changes in brain function. We believe that our findings illuminate new elements in the effort to characterize the effects of animal domestication.

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