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Physiology

Enhanced social reward response and anxiety-like behavior with downregulation of nucleus accumbens glucocorticoid receptor in BALB/c mice

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ABSTRACT. Social anhedonia is a psychological state with difficulty in experiencing pleasure from social interactions and is observed in various diseases, such as depressive disorders. Although the relationships between social reward responses and anxiety- and depression-like behaviors have remained unclear, a social reward conditioned place preference (SCPP) test can be used to analyze the rewarding nature of social interactions. To elucidate these relationships, we used 5-week-old male mice of AKR, BALB/c, and C57BL/6J strains and conducted behavioral tests in the following order: elevated plus-maze test (EPM), open field test (OFT), SCPP, saccharin preference test (SPT), and passive avoidance test. The nucleus accumbens of these mice were collected 24 hr after these behavioral tests and were used for western blotting to determine the levels of receptors for brain-derived neurotrophic factors and glucocorticoids. BALB/c mice displayed the highest levels of anxiety-like behavior in EPM and OFT as well as physical anhedonia-like behaviors in SPT. They also showed increased responses to social rewards and huddling behaviors in SCPP, with downregulated glucocorticoid receptor (GR). Regression analysis results revealed positive influences of anxiety- and physical anhedonia-like behaviors and expressions of GR on social reward responses. Collectively, temperament associated with anxiety and physical anhedonia may affect social reward responses, which possibly is influenced by the expression of GR that can modify these psychological traits.

KEYWORDS: anhedonia, anxiety, depression, glucocorticoid receptor, nucleus accumbens, social reward

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Reduced social interaction, especially in the form of a diminished capacity to experience pleasure by social interactions (social anhedonia), is found in mental disorders, including major depressive disorder (MDD) [6, 44], schizophrenia [6, 13, 30, 44], and autism spectrum disorders [14]. Although anhedonia, recognized in social and physical phenotypes, is one of the core symptoms of MDD, this symptom is difficult to treat using selective serotonin reuptake inhibitors, the first-line treatment for MDD [51]. Knutson *et al.* (1998) showed that paroxetine treatment did not alter positive affect [27]. Moreover, the presence of anhedonia is a risk factor for the poor outcome of treatments for MDD [47]. One of the plausible causes of anhedonia is a reduced reward response, because attenuated reward response was reported in morphine-induced conditioned place preference (CPP) in MDD rat models [42]. Therefore, we focused anhedonia, especially social type, in this study and aimed to reveal the basis of hedonic behaviors required to discover novel therapies for MDD and/or anhedonia.

To date, several methods have been used for evaluating physical anhedonia, including sucrose or saccharin preference tests, in psychiatric disease animal models. However, MDD is known to be a heterogeneous disorder, which means that an astonishing diversity is found in pathophysiology and treatment responsiveness among patients with MDD [20]; therefore useful methods for evaluating social anhedonia are required, as models' validity may be underestimated if only physical anhedonia is evaluated in MDD animal models. For the analysis of social reward, several methods have been used including CPP test [11]. Previous studies have suggested

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that CPP is formed in rodents paired with a possible reward induced by social interactions with same-sex conspecifics. Calcagnetti and Schechter (1992) showed a rewarding aspect of rats' social play behaviors by using SCPP test [11]. The effectiveness of social-reward CPP (SCPP) in rats has been confirmed repeatedly over the last 10 years [19, 43, 48, 52, 53]. SCPP development has also been reported in mice [28, 31, 40, 41]. Although the SCPP test seems to be a beneficial method for measuring the rewarding nature of social interactions in rodents, social anhedonia and other anxiety- and depression-like behaviors have not been directly compared. In the present study, these relationships were investigated because we hypothesized that the pathological reinforcement of psychological traits, including depressive mood and anxiety, can influence social anhedonia. Inbred strains of mice were used to test this hypothesis, since genetic factor is thought to significantly influence the temperament that underlies the expression of anxiety- and depression-like behaviors. Because each inbred strain has each stable genetic and temperament background, comparisons among these strains will benefit the examination of the interaction between the social reward and trait anxiety and depression. Therefore, we compared social reward response, anxiety, depression, and learning/memory behaviors in AKR, C57BL/6J strains as well as BALB/c strain that was reported to display higher level of anxiety-like behavior than other strains [26].

The nucleus accumbens (NAC) is a part of the reward circuitry where the expression of various genes is changed after the formation of reward dependencies [56]. A possible contribution of a brain-derived neurotrophic factor (BDNF) and its receptor in NAC to reward dependence formation has been shown. While acute cocaine administration induced the expression of BDNF4 mRNA, a BDNF variant composed of exon IV or VIII in the ventral striatum [32], chronic cocaine administration did not alter this gene. Bahi *et al*. (2008) manipulated the expression of BDNF and tropomyosin receptor kinase B (TrkB; one of the receptors for BDNF) in NAC using a lentiviral system and found that the overexpression of these genes enhanced CPP caused by repetitive cocaine applications [2]. Stress and glucocorticoid (one of the stress hormones) are other modulators reward dependence formation. An enhanced conditioning effect of cocaine in CPP was observed in rats that received chronic unpredictable stress [21] or social defeat stress [36]. Glucocorticoid may mediate an influence of stress on drug dependence, as adrenalectomy or glucocorticoid receptor (GR) antagonists (metyrapone and aminoglutethimide) prevent the enhancement of morphine-induced CPP by uncontrollable foot shock stress in rats [17]. The self-administration of propofol was reportedly enhanced by intra-NAC corticosterone administration [55]. Overall, these reports suggest that glucocorticoid plays some roles in reward responses. In addition, systemic administration of mifepristone—another GR antagonist—inhibited memory reconsolidation of SCPP, but the mineralocorticoid receptor (MR) antagonist did not influence this effect [1]. Notably, BDNF [10, 12] and glucocorticoids [35, 50] are involved in the pathogenesis of depressive disorders. Our previous studies demonstrated a molecular interaction between TrkB and GR [37–39], while revealing the influence of stress/glucocorticoid on BDNF functions [15, 38]. Here, we investigated changes in the protein expression of receptors for BDNF (TrkB and p75) as well as GR and MR in NAC in three inbred strains of mice to elucidate the molecular factors that influence social reward responses.

MATERIALS AND METHODS

Animals

Three inbred strains of male mice (AKR, BALB/c, and C57BL/6J) were purchased from Tokyo Laboratory Animals Science Co. (Tokyo, Japan). Four-week-old animals were introduced to the laboratory from breeders, and we started the experiments 1 week later. Two or three conspecific animals were housed in a polycarbonate cage (30 × 18 × 13 cm for mice) with free access to food (MF, Oriental Yeast Co., Tokyo, Japan) and water. The temperature, humidity, and light-dark cycle of the room were conditioned to 22.5 ± 2°C, 55 ± 20% and 12 hr/12 hr (light on at 0800 hr), respectively. All experimental procedures were approved by the Animal Care Committee of Musashino University (permit number: 13001) and followed the institutional guidelines with every effort to minimize the number of animals used for each experiment and their suffering.

Experimental design

The mice were subjected to a sequence of behavioral tests (Fig. 1). Elevated plus-maze (EPM) and open field test (OFT) were performed on days 1 and 2, respectively. Acclimatization to the testing apparatus and a pretest trial of SCPP were conducted on day 3, followed by a resting period on days 4 and 5. Conditioning was then performed from days 6 to 9, and a preference test was followed on day 10. A saccharin preference test (SPT) began at the start of the dark-phase (lights off at 2000) on day 10 until 1200 on day 13. The training trial of the passive avoidance test (PAT) was performed during the afternoon of day 13, followed by a retention test 24 hr

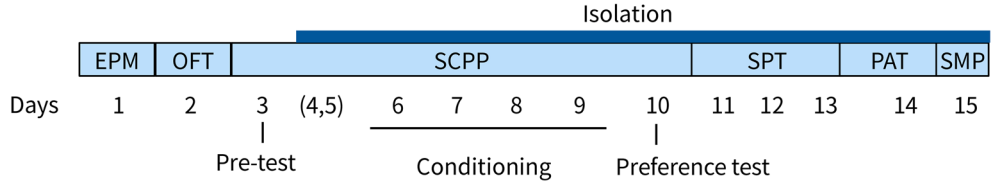

Fig. 1. Schema showing the sequence of behavioral tests for the mouse study. The light blue bar represents the duration of each test performed. The dark blue bar indicates the period of social isolation. EPM: elevated plus-maze test, OFT: open field test, PAT: passive avoidance test, SCPP: social reward conditioned place preference test, SMP: sampling of nucleus accumbens, SPT: saccharin preference test.

after the training. The mice were decapitated under the anesthesia conditions [mixture of medetomidine (0.75 mg/kg BW), midazolam (4 mg/kg BW), and butorphanol (5 mg/kg BW)] and brain tissues were collected on day 15. Social isolation was started after the pretest of SCPP (day 3) and continued to the end of the study (day 15). The behavioral experiments were conducted at light-phase (from 1000 hr to 1800 hr).

A brain matrix (BRC Co., Nagoya, Japan) was used to make 1.0-mm-thickness slices from the collected brain. The NAC was punched out from the brain slice (−1.65 to −0.65 mm from Bregma) using a 1.5-mm diameter biopsy needle (BPP-15F, Kai Co., Tokyo, Japan). The sample was pushed into 1.5-mL tubes, frozen on dry-ice, and stored at −80°C until use.

Behavioral tests

SCPP: A three chamber apparatus was made from gray vinyl chloride walls and floors. The partitions of chambers were comprised of transparent vinyl chloride walls with gates (10 × 10 cm) so that animals could pass through. The left and right chambers (25 × 40 × 40 cm) were attached with wallpapers that had white lines with a black background (left) or black dots with a white background (right). The ambient brightness was set between 25 and 35 lx in the center of every chamber during all procedures. Between every trial, the chambers were cleaned with 70% ethanol.

The SCPP method used in this study followed Thiel *et al*.'s [48] experimental procedure. In this experiment, all mice were used as subjects during the evaluation of place preference in the pretest at day 3 and the preference test at day 10. On the first day, a group of mice (n=4–5 mice in each trial) were introduced into the left or right chambers with the gates open and allowed to move freely for 10 min in order to acclimatize them to the apparatus. Then, the same group of animals was introduced into the opposite chamber and subjected to another 10-min acclimatization. The group consisted of conspecific mice picked up from two to three cages.

In the pretest, a single mouse was introduced into the neutral chamber and their behavior was recorded for 10 min using a charge-coupled device camera mounted over the chamber (CCD-camera; Wat-1000, Watec Co., Tsuruoka, Japan), a personal computer (Toshiba, Tokyo, Japan), and ANY-maze software (Stoelting Co., Wood Dale, IL, USA). The total time spent in each chamber was measured by ANY-maze using the centroid as the mouse position and used as the baseline preference. The partner for pairing was chosen from mice that had strain and preferred chamber (left or right) in common, and similar body weights.

Two days after the pretest, conditioning was started and repeated for 4 days. The gates in the partitions were closed during conditioning. Animals spent 10 min alone in the chamber where they preferred to stay during the pretest (control chamber) and then spent 10 min in the opposite chamber (conditioned chamber) with the partner. The animal behaviors were recorded using a CCD-camera and hard disk drive (HDD) recorder (RD-E1005K, Toshiba).

One day after the last conditioning, the partition gates were opened, and the preference of chambers was remeasured as was in the pretest for 10 min.

Observation of social interaction: Two observers who were blind to the strains analyzed the social behavior on the first day of conditioning of SCPP. Observers were trained to identify behaviors typically observable during social interactions including anogenital sniffing, huddling, nape attacks, boxing, and pinning. The start and end times of these behaviors were manually recorded. The data collection of social behavior was performed using following definitions. Anogenital sniffing: the subject brought its nose near its partner's tail route and moved his whiskers (sniffing). Huddling: two mice stayed inactively and closely enough touching their bodies. Nape attacks: one animal brought its nose into contact or near contact with its partner's nape. Boxing: the both mice reared oppositely with each other moving their front paws against pear. Pinning: one animal was lying on its back while its partner was standing over him.

EPM: This apparatus had two open arms (25 × 6-cm with 5-mm ridges, north and south direction) and two closed arms (25 × 6 ×8 cm, east and west direction) connected by a center area (6 × 6 cm). The maze was elevated 30 cm from the floor. The mice were introduced into the closed arm from the center area and allowed to move freely for 10 min. Their behaviors were analyzed using ANY-maze software. The ambient brightness was set to 30 lx in the center area. Between every trial, the maze was cleaned with 70% ethanol. Measured parameters were total distance traveled, stay time and entry in open and closed arms. Percentage of entry into open arms was calculated by dividing the number of entries into open arms by sum of that into open and closed arms and multiplying this by 100.

OFT: This apparatus was a gray vinyl cuboid (66 × 40 × 40 cm) without a ceiling. The mice were introduced into the right-near side of the field and their behavior was recorded using a CCD-camera and HDD-recorder for 10 min. The distance traveled and the percentage of time spent in center during the test was analyzed using ANY-maze. The ambient brightness was set to 30 lx in the field center. Between every trial, the maze was cleaned with 70% ethanol.

SPT: A bottle of distilled water (DW) was set on the right hole, and a bottle containing 0.1% saccharin (Kanto Chemical Co., Tokyo, Japan) solution was set on the left hole in the stainless cover of the cage. The bottle weight was measured at 0, 24, and 64 hr after the start of the test. After the measurement at 24 hr, the bottle's place was changed to prevent the development of a preference for bottle's place. Preference was expressed as the percentage saccharin water consumption (g) of total fluid (DW plus saccharin water) consumption (g).

PAT: We used an apparatus that had a light box (5 × 12.5 × 9 cm) connected to a dark box (5 × 12.5 × 9 cm). The mice were introduced into the light box, and the latency of entry into the dark box was measured. After the entry, the gate between the light and dark box was closed and a foot shock (50 V, 100–200 μA) was given for 20 sec. The latency of entry into the dark box was remeasured again 24 hr after the foot shock as a retention test. If the mouse did not enter the dark box for more than 5 min, the result was recorded as 300 sec. Both boxes were cleaned with 70% ethanol between every trial.

Western blotting

To detect the expression of stress-related molecules, we performed immunoblotting as previously reported (Numakawa *et al*., 2009). Briefly, brain tissues punched out from the NAC were collected and lysed in sodium dodecyl sulfate lysis buffer (1 mM phenylmethylsulfonyl fluoride, 20 mM Tris-HCl, 10 mM NaF, 5 mM EDTA, 2 mM Na_3VO_4, 0.5 mM phenylarsine oxide, and 1% sodium dodecyl sulfate). After determining total protein concentration for each sample, equal protein loadings were used for each immunoblot. For primary antibodies, anti-βactin (control protein, 1:2,500, Sigma, Burlington, MA, USA), anti-GR (1:500, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-MR (1:1,000, Santa Cruz Biotechnology Inc.), anti-TrkB (1:200, BD Biosciences, Franklin Lakes, NJ, USA), and anti-p75 antibodies (1:1,000, Promega, Madison, WI, USA) were applied. Band intensity reflecting immunoreactivity was analyzed using Lane & Spot Analyzer software (ATTO Corp., Tokyo, Japan).

Statistical analysis

The preference score in SCPP followed Bjorness and Greene's [5] method and was calculated as follows: preference score (sec)= $(T_{post\_conditioned}-T_{post\_control})-(T_{pre\_conditioned}-T_{pre\_control})$, where $T_{post\_conditioned}$ and $T_{post\_control}$ are stay times in the conditioned and control chambers during the preference test and $T_{pre\_conditioned}$ and $T_{pre\_control}$ are those during the pretest, respectively.

Data concerning behavior and protein expression were analyzed by Kolmogorov-Smirnov test and their normality was confirmed. Multiple comparisons using Holm's method were followed after one-way or factorial analysis of variance (ANOVA) as an omnibus test. These data were also used in Pearson's product-moment correlation analysis when the correlation of two behavioral parameters was of interest. The preference score in SCPP was also analyzed using one sample *t*-test adjusted by Holm's method. As, latency into the darkbox of PAT did not satisfy normality (D=0.440, *P*=0.019 in AKR day 2), nonparametric alternatives were used: Kruskal–Wallis and pairwise Wilcoxson tests adjusted by Holm's method.

The relationships between social reward response and other behavioral parameters, as well as protein expression were analyzed using multiple linear regression (MLR). An optimal model for this analysis was chosen using stepwise model selection based on the Akaike information criterion. Some behavioral parameters were combined, and their average value was entered into the model to satisfy tolerance for multicollinearity using a variance inflation factor test (<10) and the independence of each parameter using the Durbin–Watson test (*P*>0.05). A parameter combination was performed by considering the correlation of two variables and the modality (behaviors or molecular expressions) of the parameters in each selection. Saccharin preferences (first 24 hr and last 40 hr), percentage of entry and time in (to) open arms of EPM, and percentage of time in center of OFT were combined and multiplied by −1 as anxiety- and physical-anhedonia-like behaviors and used in the final model.

Data analysis was conducted using R software (R Foundation for Statistical Computing, Vienna, Austria). *P*-values or adjusted p-values by Holm's method were considered statistically significant when they were less than 0.05. Values are expressed as mean ± standard error of the mean (SEM).

RESULTS

Strain differences in social reward dependence in mice

To investigate the possible contribution of genetic background in the social reward responses, the difference between strains for the amount of time spent in the chambers was measured before and after social reward conditioning in SCPP. Figure 2 shows the preference score results calculated by the stay time in each chamber, and Table 1 lists the ANOVA results. We observed positive figures of scores in all strains. Notably, one sample *t*-test demonstrated significant increases in scores of BALB/c (*P*=0.028) and C57BL/6J (*P*=0.012) versus the hypothetical value without change (i.e. mean=0), suggesting increased preference for the chamber paired with social interactions after conditioning. A similar trend was also found in AKR (*P*=0.076). In this test, the effect of strain was significant, according to the ANOVA results [F(2,28)=4.271, *P*=0.0241]. BALB/c mice displayed the largest preference that satisfied statistical significance toward AKR (*P*=0.043) and C57BL/6J (*P*=0.043).

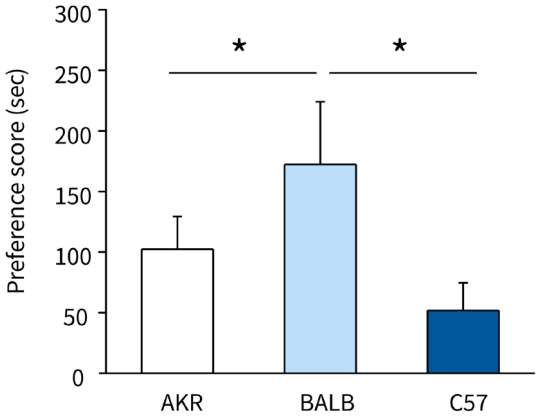

Fig. 2. The preference score in the social-reward conditioned place preference test in BALB/c exceeded those in other strains. BALB: BALB/c, C57: C57BL/6J, Columns and bars represent mean and SEM. N=12 (AKR), 11 (C57BL/6J), and 8 (BALB/c). *, *P*<0.05.

Strain differences in anxiety- and physical anhedonia-like behaviors as well as learning/memory

We examined anxiety- and physical anhedonia-like behaviors as well as the learning behavior of inbred mice. BALB/c mice displayed the smallest value in the percentage of entry into open arms [Fig. 2A; F(2, 28)=4.366, *P*=0.022; *P*=0.358 AKR vs. BALB/c; *P*=0.068 AKR vs. C57BL/6J; *P*=0.017, BALB/c vs. C57BL/6J] as well as that of stay time in the open arms of EPM [Supplementary Fig. 2A; F(2, 28)=5.493, *P*=0.009; *P*=0.170, AKR vs. BALB/c; *P*=0. 113, AKR vs. C57BL/6J, *P*<0.008, BALB/c vs. C57BL/6J]. The percentage of stay time in the center of the OFT was also the smallest in BALB/c [Fig. 3B; F(2, 27)=9.228, *P*<0.001; *P*<0.001, AKR vs. BALB/c; *P*=0.043, AKR vs.

Table 1. Summary of results from analysis of variance

Paradigm	Dependent variables	Independent variables	DF	*F*-value	*P*-value
SCPP	Preference score	Strain	2, 28	4.271	0.024 *
EPM	Percentage of stay time in OAs	Strain	2, 28	6.062	0.007 *
		Test order	1, 28	2.186	0.150
		Strain: Test order	2, 28	2.012	0.153
	Percentage of stay time in CAs	Strain	2, 30	2.263	0.122
	Percentage of entry into OAs	Strain	2, 28	4.366	0.022 *
		Test order	1, 28	4.605	0.041 *
		Strain: Test order	2, 28	3.458	0.046 *
	Distance traveled	Strain	2, 30	5.505	0.009 *
	Number of entries into OA/CAs	Strain	2, 30	1.735	0.193
OFT	Distance traveled	Strain	2, 30	22.19	<0.001 *
	Stay time in center	Strain	2, 27	9.228	<0.001 *
		Test order	1, 27	2.040	0.165
		Strain: Test order	2, 27	0.208	0.814
SPT	Preference in first 24 hr	Strain	2, 30	32.72	<0.001 *
	Preference in last 40 hr	Strain	2, 30	17.25	<0.001 *
SPB	Huddling	Strain	2, 18	3.751	0.044 *
	Anogenital sniffing	Strain	2, 18	2.986	0.076
WB	Glucocorticoid receptor	Strain	2, 12	13.29	<0.001 *
	Mineralocorticoid receptor	Strain	2, 12	1.239	0.324
	Tropomyosin receptor kinase B	Strain	2, 12	1.618	0.239
	P75	Strain	2, 12	0.712	0.510

CA: closed arm, DF: degree of freedom, EPM: elevated-plus maze test, OA: open arm, OFT: open field test, SCPP: social-reward conditioned-place preference, SPB: social play behaviors, SPT: saccharin preference test, WB: western blotting. *, $P<0.05$.

BALB/c; $P<0.001$, BALB/c vs. C57BL/6J]. Although the total number of entry into open/closed arms did not differ among strains [Supplementary Fig. 2C; F(2,30)=1.735, $P=0.193$], BALB/c mice displayed the least distance traveled during EPM [Supplementary Fig. 2D; F(2, 30)=5.505; $P=0.009$, AKR vs. BALB/c; $P=0.27$, AKR vs. C57BL/6J; $P=0.062$, BALB/c vs. C57BL/6J] and OFT [Supplementary Fig. 3; F(2, 30)=22.19, $P<0.001$; $P<0.001$, AKR vs. BALB/c; $P<0.001$, BALB/c vs. C57BL/6J] among the three stains. Because the order of introduction into the experimental apparatus can bias the results of anxiety-like behaviors [7], the effect of test order on anxiety-like behavior was also examined. The effect of test order was significant in the percentage of open arms entry [F(1, 28)=4.605, $P=0.041$], and the interaction between test order and strain was significant [F(2, 28)=3.458, $P=0.046$] in EPM. The correlation coefficients between the percentage of open arms entry and test order were −0.30 in AKR, 0.64 in BALB/c, and 0.50 in C57BL/6J, although they were not statistically significant (Supplementary Fig. 4). No significant effect of test order and its interaction with strain were observed in either the percentage of stay time in open arms of EPM or the center in OFT (Table 1). BALB/c mice also displayed the lowest preferences for saccharin water in the first 24 hr [Fig. 3C left; F(2, 30)=32.72, $P<0.001$; $P<0.001$, AKR vs. BALB/c; $P<0.001$ BALB/c vs. C57BL/6J] and in the last 40 hr [Fig. 3C right; F(2, 30)=24.95; $P<0.001$; $P<0.001$, BALB/c vs. AKR; $P<0.001$, BALB/c vs. C57BL/6J] in SPT.

The difference in learning and/or memory among groups (strain and experimental day) was observed in PAT [Fig. 3D; $\chi^2(5)=20.64$, $P<0.001$]. Although latencies of entry into the dark box were comparable among strains on day 1 ($P=1$, Wilcoxon test), those on day 2 were significantly increased in BALB/c and C57BL/6J mice compared to that of day 1 ($P=0.042$ in BALB/c; $P=0.010$ in C57BL/6J). The trends for shorter latency on day 2 were shown in the AKR strain compared to the BALB/c or C57BL6/J strains, although these differences did not reach statistical significance. During the retention test, the numbers of mice that refused to enter into the darkbox were 1 in AKR, 7 in BALB/c, and 3 in C57BL/6J.

We found anogenital sniffing, huddling, and nape-attack behaviors while analyzing social behaviors during the conditioning of day 6 (SCPP). The reliability of nape-attack behavior was low due to a small sample size (two pairs of AKR and one pair of C57BL/6J), and this behavior was removed from the statistical analysis. The strain difference in the total time of huddling behavior was large [Supplementary Fig. 1A; F(2,18)=3.751, $P=0.044$], and a significant difference was found between BALB/c and C57BL/6J by Holm's multicomparison test ($P=0.041$). Relatively small differences were found between species in anogenital sniffing [Supplementary Fig. 1B; F(2,18)=2.986, $P=0.076$].

Strain differences in protein expression in NAC

To elucidate the potential molecular mechanism behind these behavioral changes, western blotting was used to determine the expression levels of corticoid- and BDNF-related molecules. Figure 4 shows that the GR protein expression level in BALB/c

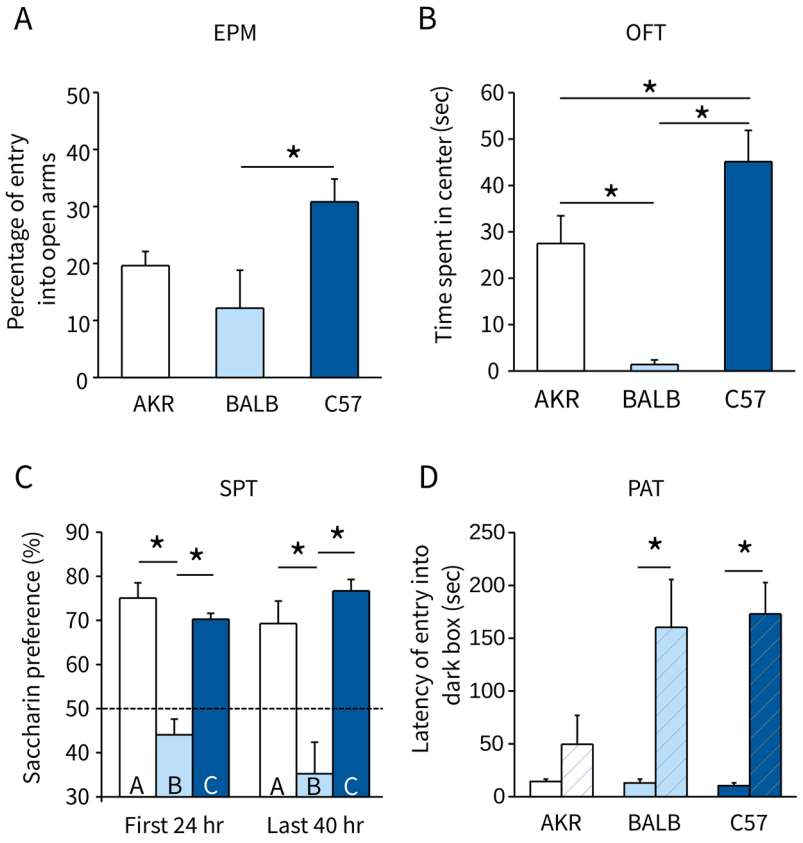

Fig. 3. Strain differences in the anxiety- and depression-like behavior and memory/learning in inbred mice. Percentage of time spent in open arms of elevated-plus maze test (EPM; **A**) and that in center of open field test (OFT; **B**) was the shortest in BALB/c, followed by AKR and C57BL/6J. Saccharin preference was lower in BALB/c mice than in the other strains during the first 24 hr (left) and the next 40 hr (right) in saccharin preference test (SPT; **C**). The latency of entry into the dark box on day 2 was shorter for the AKR strain than for the other strains although the difference was not statistically significant (**D**). Open columns: day 1 (conditioning); columns with diagonal lines: day 2 (retention test). A: AKR, B or BALB: BALB/c, C or C57: C57BL/6J. Data represent mean and SEM. N=12 (AKR), 8 (BALB/c), and 11 (C57BL/6J). *, $P<0.05$.

[F(2,12)=13.29, $P<0.001$] was below those of AKR ($P=0.019$) and C57BL/6J ($P=0.002$). When comparing the MR levels between these strains, no significant difference was confirmed [F(2, 12)=1.239, $P=0.324$]. Altered levels of both TrkB and p75 between these mice strains also did not reach statistical significance [F(2, 12)=1.618, $P=0.239$ in TrkB; F(2, 12)=0.712, $P=0.51$ in p75].

Relationships between social reward responses to behavioral and molecular parameters

The relationships between parameters observed in this study were analyzed using MLR. The preference score in the SCPP was entered as a response variable, whereas other behavioral and molecular parameters were entered as explanatory variables in the full model. Two explanatory variables were selected by stepwise model selection: anxiety- and physical anhedonia-like behavior (combined variable) and GR expression. Table 2 lists the summary of the MLR results. In this model, the adjusted R^2 was 0.518 ($P=0.005$) and the estimated coefficients were 11.29 for anxiety- and physical anhedonia-like behavior (t=4.13, $P=0.001$) and 3.00 for GR (t=2.46, $P=0.030$). Judging from standardized coefficients (z-score), the major contributor to prediction was anxiety- and physical anhedonia-like behavior (0.908) compared with GR (0.248).

DISCUSSION

In the present study, we found that BALB/c mice displayed a significant higher preference score for the chamber paired with social interaction compared to AKR and C57BL/6J mice in SCPP. Increases in the preference score of SCPP suggest increased preference for the conditioned chamber compared to the control chamber after conditioning. Although a positive response to social interaction is common in various strains of mice, marked differences between BALB/c and the other inbred strains were also found, thus genetics may have contributed to these differences. Unlike our study, Panksepp and Lahvis (2007) compared the behavior of SCPP using four strains of inbred mice (A/J, BALB/cJ, C57BL/6J, and DBA/2J) and observed that BALB/cJ mice displayed the shortest exploration time in the environment associated with social contact [40]. A stronger influence of social motivation was also reported in C57BL/6J mice compared to BALB/cJ mice in morphine CPP response [25]. Differences in rearing environment between breeders as well as

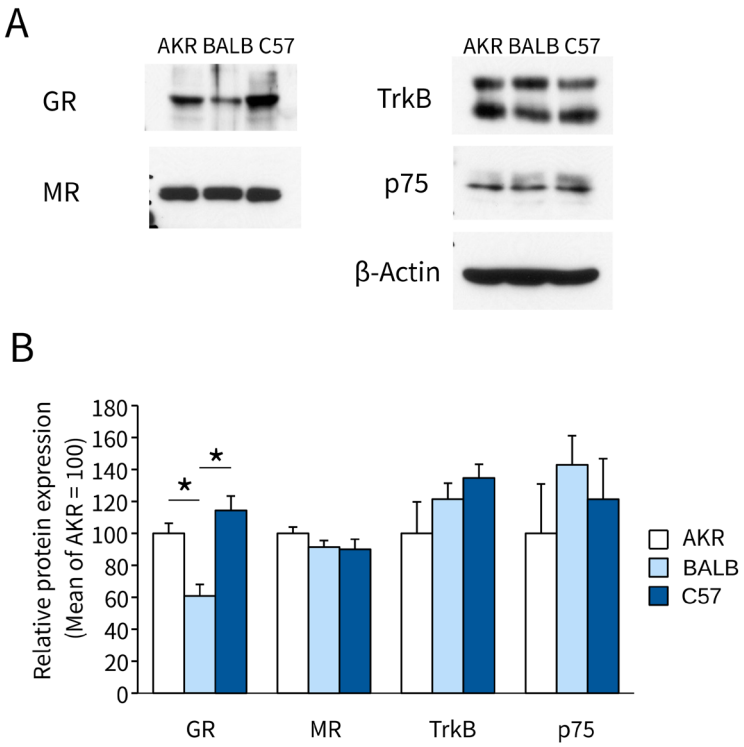

Fig. 4. Low expression of glucocorticoid receptor (GR) in the nucleus accumbens of BALB/c mice. Representative image of the band obtained by western blotting of GR, mineralocorticoid receptor (MR), tropomyosin receptor kinase B (TrkB), p75, and β-actin in the three inbred strains of mice (**A**). Quantitative data with densitometry are shown in (**B**). BALB: BALB/c, C57: C57BL/6J. Data represent mean and SEM. N=5. *, $P<0.05$.

Table 2. Summary of results from multiple liner regression analysis

Effects	Estimate	SE	LL	UL	z
APALB	11.29 *	2.74	5.33	17.25	0.908
GR	3.00 *	1.22	0.34	5.67	0.248
Residuals	–227.08 *	97.38	14.92	439.23	–0.101

Adjusted R^2=0.518 [F(3, 11)=8.519, P=0.005 *], APALB: anxiety- and physical anhedonia-like behaviors, GR: glucocorticoid receptor, LL: lower limit of 95% interval, SE: standard error, UL: upper limit of 95% confidence interval, z: standardized coefficient. *, $P<0.05$.

genetic differences caused by accumulation of point mutation are possible factors explaining this contradiction. Another possibility is the differences in methodologies, especially in experimental conditioning. Our experimental procedure comprised 10-min social and 10-min isolated conditioning trials in a day for 4 consecutive days, whereas Panksepp and Lahvis used five sets of 24-hr social conditioning followed by 24-hr isolated conditioning before the preference test [40]. Furthermore, the floor materials of the experimental apparatus were used for environmental cues in their study, whereas the patterns of the wall were used as cues in our present study. The effects of these methodological differences require further investigation.

The percentage of time spent in and that of entry into open arms in EPM as well as that of time spent in the center of OFT were the lowest in BALB/c among these three strains. EPM and OFT are widely used for investigating rodent anxiety-like behaviors, and the reduction in the percentages of time spent in the open arms of EPM and the center of OFT is thought to be related with anxiety [22]. We also found a low preference for the saccharin solutions in BALB/c mice. Preference for sweetness (e.g., 1% sucrose and 0.1% saccharin solution) is used for judging the state of the physical anhedonia of animals [54]. Notably, anhedonia is associated with psychiatric disorders including MDD [23] and anxiety [45]. Thus, the reduced preference for sweetness supports the tendency of BALB/c mice to exhibit anxiety-like behaviors. One caveat is that anxiety-like behavior in EPM and OFT could be influenced by activity level, because total distance traveled decreased in BALB/c. However, the difference in total distance traveled may have resulted from strain differences in immobility time during the tests, because the total number of entries into open and closed arms in EPM, another measure of activity, did not differ among strains; therefore, this data supports the trait anxiety hypothesis in BALB/c mice mentioned above. The ANOVA results revealed that the percentage of open arms entry (anxiety-like behavior) was significantly affected by the test order of mice in EPM, although its biological meaning was limited by the significant interaction between the

effect of strain and test order. The correlation coefficient between open arms entry and test order was negative in AKR, whereas the opposite moderate values were found in BALB/c and C57BL/6J, although these were not statistically significant. The results except for AKR in EPM were not consistent with a previous report that used only Swiss mice [7]. Therefore, the details of the test order effect, especially its sensitivity among strains, need further study.

In the MLR analysis, anxiety- and physical anhedonia-like behavior most strongly explained the preference score in SCPP, followed by GR. The coefficient of anxiety- and physical anhedonia -like behavior was positive; therefore, the subject with a higher level of this behavior exhibited stronger responses to social reward. Although the causal relationship between these behaviors and social reward responses was not clarified, we speculate that the temperament that produces anxiety- and physical anhedonia-like behavior intensifies the social reward response and that huddling is one of the mediators between them. Recent studies have shown that huddling increases in response to predatory odor, which activates anxiety-related brain regions (e.g., paraventricular nucleus, central nucleus of the amygdala, and the shell of NAC) [8, 24], and have also suggested that huddling is a defensive response of animals [8]. In addition, some groups have reported that physical contact—a key component of huddling behavior—is important for increasing positive social reward responses in rats [28, 43]. These reports support the present observation that the longest huddling time during the first conditioning of SCPP was found in most anxious BALB/c and also our speculation that a propensity toward anxiety- and physical anhedonia positively influences the behavior that drives to seek social reward via increased huddling during conditioning. One of the limitations of this hypothesis is that the possibility of strain differences in learning/memory capability cannot be completely excluded because only PAT was used to test this ability. Learning in PAT involves an association between the environment and foot shock that depends on tactile sensation; thus, strain differences in this sensation can bias PAT results.

The molecule that influences the social reward response also became clear using regression analysis, namely GR expression in NAC. Notably, the GR expression level in BALB/c was downregulated compared to the other tested strains. GR is a receptor for glucocorticoids, a stress hormone, and is suggested to be implicated in affective disorders, including anxiety and depressive disorders [35, 50]. Importantly, heterozygote mice with a disrupted GR gene displayed increased anxiety- and depression-like behaviors compared with wild-type animals [46], suggesting a preventive role of GR against the anxiety- and depression-like behaviors. We demonstrated that both cortical GR expression and BDNF-dependent release of glutamate, an excitatory neurotransmitter, were repressed in an animal model of depression caused by chronic stress exposure [15]. The roles of glucocorticoids and stress in social interaction and reward dependence have become clearer. For example, pair bond formation of female prairie voles is potentiated by treatment with RU486 (a GR antagonist) [16]. Barik *et al*. (2013) revealed that the conditional deletion of GR in dopamine D_1 receptor-expressing neurons attenuated social aversion caused by chronic social defeat stress, whereas such a phenomenon did not appear after GR deletion in dopamine transporter-expressing cells [3]. This result raises the possibility that GR in dopaminoceptive neurons is involved in the social reward shown in this study. However, using the same transgenic lines of mice, Barik *et al*. revealed that GR deletion in dopaminoceptive neurons inhibits cocaine-induced CPP acquisition and the expression of locomotor sensitization, whereas morphine dependence is intact [4]. This study supports previous studies that demonstrated an increased place preference when cocaine is paired with chronic unpredictable stress [21, 36], enhanced cocaine self-administration by brief social defeat stress [34, 49] or by treatment with corticosterone (major glucocorticoids in rodents) [18, 33]. Therefore, the role of GR in the acquisition of reward dependence may be influenced by the results of the complex interaction between duration/type of stressor and the substance/object of dependence. In this study, the mice were anesthetized before collecting their brains, and this procedure might have affected the expression levels of GR. Brinks *et al*. (2007) reported that the concentration of corticosterone was significantly higher in BALB/c compared to C57BL6/J when they were exposed to a mild stressor [9]. The expression of GR is suppressed by stress exposure [15]; therefore, higher HPA response to anesthesia can be one of the explanations for the low-level expression of GR in BALB/c.

In the present study, we observed differences in the response to social rewards as well as anxiety- and physical anhedonia-like behaviors between strains of mice. Significant influences of GR, anxiety- and physical anhedonia-like behaviors on social reward responses were also observed, although this study did not investigate the causal relationships between these factors. The enhancement in social reward responses may be due to increased huddling or physical contacts that is reported to be induced by anxiety [8], because this social behavior is known to evoke a social reward response [29, 43]. Interestingly, as shown in this study, one of the putative factors affecting social reward is GR, which was supported by the report of the GR-deficient mice that displays anxiety- and depression-like phenotypes and reduced social aversion [3, 46]. The MLR results indicated a positive influence of GR expression on social reward responses; therefore, this protein may play protective roles in stressor load and modify anxiety- and depression-like behaviors and/or social reward response. Existing reports support this hypothesis; around 90% of neurons, especially primary neurons, express GR [57], and intra-NAC glucocorticoid injection stimulates an addictive behavior [55]. The present findings may benefit the search for new treatments for brain disorders showing impaired social interaction, particularly social anhedonia.

CONFLICT OF INTEREST. The authors declare no conflicts of interest.

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