Comparative Analysis of the Responses to Intruders with Anxiety-Related Behaviors of Mouse

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Anxiety in mice can be measured by behavioral reactivity to social or non-social stressors. These behaviors were compared by performing the resident-intruder test (social) as well as the light-dark transition and open-field tests (non-social) for the FVB, C57BL/6, and BALB/c lines of mouse. The three inbred lines showed significant differences in their responses to intruder mice. Three factors, accounting for about 68% of the total variance, were extracted from the scores obtained from the three behavioral tests. The first two major factors are primarily associated with the anxiety-related behaviors. One includes anxiety behaviors with a locomotive basis, while the other includes defecation measured in both anxiety tests. The third factor explains the three social behaviors, facial investigation, ano-genital investigation, and following, observed in the resident intruder test, although facial investigation is also moderately associated with the second factor. The results indicate that the behavioral responses to an intruder share a component distinct from anxiety-related behaviors.

Anxiety-like behaviors in mice can be measured by various ethological tests that are based on the approach-avoidance model. The open-field (OF) and light-dark (LD) transition tests have been used to measure anxiety-related behaviors of mice in unconditioned situations. Previously, four factors were extracted from variables of OF test (Carola et al., 2002). These are 'motor activity', 'hesitation', 'anxiogenic area approach-avoidance', and 'risk assessment / exploration'. In addition, anxiety, exploration, risk assessment, and decision-making were suggested to be key components of behaviors from the LD test (Lepicard et al., 2000). The anxiety-related behaviors are significantly affected by genetic background of mice (Crawley et al., 1997; van Gaalen and Steckler 2000). Many loci associated with anxiety-related behaviors have been reported in mice (Gershenfeld and Paul, 1997; Gershenfeld et al., 1997; Turri et al., 2001).

Social interactions of rats in various environmental stimulations have been widely used as indicators of anxiety-related behaviors, which were validated pharmacologically (File and Seth, 2003). The rats treated with diazepam, an anxiolytic drug, showed increased social interaction in high-illumination conditions. In addition, defensive response to natural threat, a social behavior, was suggested to be related to anxiety (Griebel et al., 1996). However, Berton et al. (1997) were unable to extract a common factor shared by social and anxiety-related behaviors. In their test, social interactions were separately scored in neutral and aversive environments, while aggressive behavior (fight) was additionally measured with the resident intruder (RI) test.

Meanwhile, aggressive behavior provoked in social context is associated with anxiety related behavior, observed as time spent in light chamber in LD test (Guillot and Chapouthier, 1996). Moreover, both aggression and anxiety-related behaviors were altered in some null mouse mutants. For example, adenosine A2a null mice show increased anxiety in the elevated plus maze and LD test, while aggression was reduced in the RI test (Ledent et al., 1997). A null mutant of the histamine H1 receptor showed extended transfer latency to the closed arm of the elevated plus maze with reduced attack frequencies and prolonged aggression latency (Yanai et al., 1998). This suggests that genes influencing aggressive and anxiety-related behaviors are related.

In this study, behaviors were scored in the RI test of three inbred strains of mice (FVB/N, C57BL/6N, and BALB/cAnN) and compared with data from the OF and LD tests. These inbred lines were previously tested for their variability in various anxiety behaviors (Kim et al., 2002). The RI test has been employed to measure aggressive behaviors as well as other social behaviors of mice (Bell et al., 1995; Crawley, 2000). Such studies were primarily focused on the aggressive behavior and its association with other types of behavior (Guillot and Chapouthier, 1996; Maxson and Canastar, 2002). Here,

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we intended to describe these mouse behaviors to an intruder and their relationship with the anxiety behaviors. One of the distinct social interactions observed with this test is the investigation of the intruders' body parts, including facial and ano-genital regions, by the resident mouse. These behaviors were scored for their underlying factors and analyzed to establish that the behaviors observed in the RI test form a unique cluster, perhaps associated with social behaviors described previously.

Materials and Methods

Subjects

Four inbred strains of mice, FVB/N (F), C57BL/6N (B6), BALB/cAnN (BC), and C3H/HeN (C3H) were purchased from Daehan Biolink. A total of 80 male mice were used in this study (F; n=26, B6; n=28, and BC; n=26). All mice were raised in groups of five per mouse cage (27×22×13 cm) in temperature controlled facilities at 22°C under 12 h of light-dark cycle with lights on at 07:00. Humidity was maintained at 55% with food and water freely available. The food contained protein (24%), fat (4.5%), fiber (3.5%), moisture (11.5%), ash (7%), phosphorus (0.88%), calcium (1%), lysine (1.3%), methionine (0.5%), cystine (0.35%), vitamin A (45 IU/g), vitamin D (4.5 IU/g), vitamin E (66 mg/kg), and vitamin K (20 mg/kg). All experiments followed the NIH Guideline for the Care and Use of Laboratory Animals.

Non-social anxiety-related behavior test

Behavioral tests were performed under white lighting between 08:00 to 13:00. The sequential procedure of the behavioral testing involved the LD, OF, and then the RI test. For LD, inbred mice at 11 wk of age were used, with the OF test following at 12 wk of age. The testing apparatus and protocols of the two anxiety-related tests have been described previously (Kim et al., 2002).

Five behaviors were measured in the LD. The number of transitions (TRANS), time spent in the light chamber (DUR, sec), stretch-attend posture (SAP), defecation (LDD), and time spent in the far light chamber (DFL, sec) were scored over 5 min. The criteria for TRANS, SAP, and LDD have been described previously (Grewal et al, 1997; Kim et al., 2002). For measuring DFL, the light chamber was divided into two equal parts by an imaginary line (Adriaan and Paylor, 2002). The far light region was defined as a region far from the dark chamber. The following phenotypes were recorded in the OF test: distance traveled (ambulation, OFA); defecation score (faecal boli/session, OFD); the total number of vertical movements (rearing and leaning activities, VM); the number of center circle crossings (OFC); and time spent in the center circle (DCC, sec). The center circle was defined as an imaginary circular arena (10 cm diameter) in the central part of the apparatus. For measuring TRANS, DUR, DFL, OFC, and DCC, only those cases in which all four paws of mice crossed to the defined region were counted.

Resident-intruder test

After finishing LD and OF tests, mice were housed individually for 7 d. The cage bedding was changed 1 d prior to the RI test. Five individuals of male C3H mice (n=28) were housed per cage, and the last three animals were housed in one cage. The male C3H mice at 7 wk of age were used as intruders once per day. After the first 10 min test, resident mice were re-isolated for one wk and retested.

One aggressive and three investigative behaviors were observed in the RI test. ATK was determined as the total bouts of aggression such as bite and kick. FAC and AG were determined as the total bouts of resident's sniffing intruder's face and ano-genital regions, respectively. FOL was defined as total bouts of chasing the intruder after contact.

Seven d elapsed between each test in order to reduce any influence from previous tests. When data obtained from two different RI tests was compared, similar attack proportions of the naïve and retested BALB/c mice were obtained (P=0.878). In this case, 20 mice were used in each test with the ICR strain as intruders. This indicates that previous anxiety test does not affect the aggressive behavior of the following RI test.

Statistical analysis

The Shapiro-Wilk test was used to determine the method of analysis. Only the OFA behavioral variance of the three inbred lines of mice followed a normal distribution ($P<0.05$). Consequently, nonparametric analyses were adopted for statistical analysis. The nonparametric Kruskal-Wallis test and one-way ANOVA followed by a Bonferroni-Dunn test were used for comparative analysis across strains (Sankoh et al. 1997). For social investigative behaviors, FAC, AG, and FOL, we used data from the first RI test. The phenotypic correlation coefficients were calculated using all the data (N=80). The Spearman rank correlation analysis was carried out. As we performed multiple comparison, Bonferroni corrected ($P<0.05/91=0.000549$) criteria was adopted.

In order to test suitability of data for principal components analysis (PCA), Kaiser-Meyer-Olkin (KMO) and Bartlett's sphericity test were carried out using the SPSS package (SPSS Inc.). Principal components analysis was performed for all variables except ATK and SAP. As squared multiple correlation (SMC) are low (<0.4), the two indices, ATK, and SAP were excluded in PCA. In PCA, orthogonal rotation was done by the varimax procedure. For factor matrix, Kaiser criteria (eigenvalue≥1) were used. All data was transformed into rank. Statistical analyses except KMO and Bartlett's sphericity tests were performed with STATISTICA program (StatSoft Inc.).

Results

Strain differences in anxiety-related and social behaviors

The OF, LD, and RI tests performed on F, B6, and BC lines showed a broad spectrum of anxiety levels in a previous study (Kim et al., 2002). As shown in Table 1, significant effects on anxiety-related indices were found across the inbred lines as analyzed by nonparametric test [OFA, H=53.4; OFD, H=31.1; VM, H=60.4; OFC, H=25.4; TRANS, H=41.1; LDD, H=31.5; SAP, H=28.5; DUR H=48.0, and DFL, H=30.0, all $P<0.0001$, DCC, H=11.7, $P=0.0029$]. In the RI test, an aggressive behavior, ATK, and three investigative behaviors, FAC (investigation of the intruder's face), AG (investigation of the intruder's ano-genital region) and FOL (following the intruder's movement after investigation) were scored. The Kruskal-Wallis analysis indicated significant differences in the incidences of ATK (H=31.2, $P<0.0001$), FAC (H=24.2, $P<0.0001$), AG (H=6.7, $P=0.036$) and FOL (H=13.1, $P=0.001$) when using C3H as intruders. Significant differences of RI behaviors between strains were obtained by one-way ANOVA followed by Bonferroni-Dunn test (Table 1). Overall activities of RI behaviors are higher in FVB and lower in BL/6 with BALB/C in the middle, except for FAC.

Correlation between various behaviors

The phenotypic correlations between behaviors were obtained by Spearman rank correlation test with Bonferroni corrected P value ($P<0.05/91=0.000549$, Table 2). Higher levels of significance were obtained not only between scores of the same anxiety test, but also between scores of different tests: OFA with ones from the LD test, TRANS (R=0.714, $P<0.000001$) and DFL (R= 0.526, $P<0.000001$); TRANS with ones from the OF test, OFA (R=0.714, $P<0.000001$), OFD (R=-0.528, $P<0.000001$), VM (R=0.689, $P<0.000001$), and OFC (R=0.52, $P<0.000001$). The RI behaviors were also significantly correlated with anxiety-related behaviors. The non-aggressive behavior of FAC was positively correlated with VM (R=0.386, $P<0.00041$), while FOL with OFA (R=0.379, $P<0.00052$).

Relationship between the social and anxiety-related behaviors

The principal factor analysis revealed a common factor for the indices of RI and anxiety-related behaviors. The KMO value of 0.72 and the Bartlett's sphericity 0.000 (χ^2 =560.427, df=66, $P<0.0001$) indicated adequacy of the data to the analysis. Based on Scree plot, three factors were obtained (Table 3), each explaining 37.1%, 18.5%, and 12.7% of the variance. Six indices, OFA, VM, OFC,

Table 1. Kruskal-Wallis tests for behaviors obtained with three inbred strains

	Behavior	FVB (N=26)	C57BL/6 (N=28)	BALB/C (N=26)	H-value	P-value
Resident-intruder	ATK	38.0± 6.4	1.7± 1.2#	15.2± 3.8#	31.2	<0.0001
	FAC	16.2± 1.3	15.9± 1.2	8.8± 0.9#*	24.2	<0.0001
	AG	19.5± 2.4	12.6± 1.0#	13.5± 1.5#	6.7	0.036
	FOL	6.0± 1.0	2.1± 0.3#	4.1± 0.7	13.1	0.001
Open field	OFA	3304.2±90.3	1885.7±55.2#	1688.9±86.8#	53.4	<0.0001
	OFD	1.7± 0.5	2.6± 0.3	6.3± 0.6#*	31.1	<0.0001
	VM	53.7± 2.6	20.6± 1.4#	9.1± 1.5#*	60.4	<0.0001
	OFC	5.2± 0.5	2.4± 0.3#	2.0± 0.4#	25.4	<0.0001
	DCC	5.3± 0.7	3.5± 0.8	2.5± 0.5#	11.7	0.0029
Light-dark transition	TRANS	19.5± 1.2	11.9± 0.7#	7.2± 0.9#*	41.1	<0.0001
	LDD	3.7± 0.6	1.3± 0.4#	6.7± 0.6#*	31.5	<0.0001
	SAP	0.4± 0.1	0.3± 0.1	1.7± 0.3#*	28.5	<0.0001
	DUR	169.6± 8.1	110.6± 5.0#	209.9± 8.5#*	48.0	<0.0001
	DFL	71.9± 7.7	44.9± 2.4#	40.4± 4.2#	30.0	<0.0001

The marks (#) represent a data significantly different from that of FVB, and the marks (*) with data significantly different from that of C57BL/6 as a result of post hoc analysis of Bonferroni ($P<0.0167$). ATK: the total bouts of aggression. FAC: the total bouts of residents sniffing to intruder's face. AG: the bouts of resident's sniffing to intruder's ano-genital region. FOL: the total bouts of chasing the intruder after contact. OFA: distance traveled. OFD: the total number of faecal boli. VM: the total number of vertical movement. OFC: the total number of center circle crossing. DCC: time spent in the center circle. TRANS: the total number of transition. LDD: the total number of faecal boli. SAP: the total bouts of stretch-attend posture. DUR: time spent in the light chamber. DFL: time spent in the far light chamber. Mean±SE.

Table 2. Correlation coefficients between various indices from the RI and anxiety-related tests

Behavior	Resident-intruder				Open field					Light-dark transition				
	ATK	FAC	AG	FOL	OFA	OFD	VM	OFC	DCC	TRANS	LDD	SAP	DUR	DFL
ATK														
FAC	−.142													
AG	−.036	.374												
FOL	.176	.208	**.703**											
OFA	.290	.339	.331	**.379**										
OFD	.040	−.341	−.100	.012	**−.383**									
VM	.212	**.386**	.241	.299	**.897**	**−.411**								
OFC	.240	.200	.291	.281	**.679**	−.231	**.638**							
DCC	.143	.122	.192	.142	**.508**	−.156	**.468**	**.744**						
TRANS	.197	.170	.211	.087	**.714**	**−.528**	**.689**	**.520**	.349					
LDD	.203	−.264	.079	.153	.023	**.643**	−.130	.030	.013	−.142				
SAP	.195	−.319	−.137	−.016	−.308	.244	**−.422**	−.131	−.077	−.265	.286			
DUR	.284	−.310	−.004	.117	−.010	**.410**	−.171	−.014	−.031	−.196	**.557**	.301		
DFL	.256	.097	.050	.144	**.526**	−.200	**.552**	.308	.216	.327	−.045	−.291	.177	

The *n* for all calculation was 80. The coefficients more than 0.378 ($P<.000549$) are marked with bold characters. Critical values for *r* can be assessed with $P<0.05/91=.000549$.

DCC, TRANS, and DFL, were positively loaded onto the factor one. The index of FAC was negatively loaded on the factor two, so do positively the three non-social indices of OFD, LDD, and DUR, from the OF and LD tests. The third factor comprises only three RI behaviors (FAC, AG, and FOL). The inbred mice tested here display significant strain variations (Fig. 1). Although FVB generally exhibits higher reactivities to most of the behaviors, especially ones associated with factor I, activities of factor II associated behavior are more pronounced in BALB/C, which is consistent with the previous data (Beuzen and Belzung, 1995; Mineur and Crusio, 2002). Considering the lower locomotive activity of BALB/C strain than that of FVB, factor III behaviors, AG and FOL, appear to be prominent in BALB/C mouse. Therefore, the behaviors of FVB and BALB/C mice are polarized in different directions.

Table 3. Principal components analyses for the resident-intruder, open field, and light-dark transition tests

Behavior	Factor 1	Factor 2	Factor 3
Resident-intruder test			
FAC	.173	**−.502**	**.508**
AG	.156	−.016	**.896**
FOL	.186	.162	**.847**
Open field test			
OFA	**.897**	−.059	.259
OFD	−.387	**.755**	.019
VM	**.871**	−.207	.183
OFC	**.805**	.046	.193
DCC	**.681**	.068	.074
Light-dark transition test			
TRANS	**.757**	−.280	.011
LDD	−.004	**.853**	.130
DUR	−.035	**.800**	−.015
DFL	**.641**	.087	−.077
Eigenvalue	4.455	2.219	1.527
% Variance	37.1	18.5	12.7

Factor loading values exceeding 0.4 or 0.7 are considered as moderate (italic) or high (bold), respectively.

Discussion

The OF and LD tests are known to measure anxiety-like behaviors of mice, while the RI test is commonly used to detect aggressive behavior. We carried out these tests to examine a relationship between behavioral reactivity to resident intruder and to anxiety situations. According to the present data, the behaviors in the RI, except for the aggressive one, form a separate cluster, although FAC exhibits a minor association with anxiety. In previous report (Guillot and Chapouthier, 1996), aggression was shown to be correlated genetically with anxiety of mouse. However, we were unable to demonstrate its correlation with the duration (DUR) of the LD test. This might be due to the differences in the experimental and analytical details from the previous one. While Guillot and Chapouthier (1996) used attack proportions for comparison with anxiety in ten inbred strains of mice, we used attack frequencies for three inbred strains. In addition, Guillot used A/JOrl as an opponent mouse,

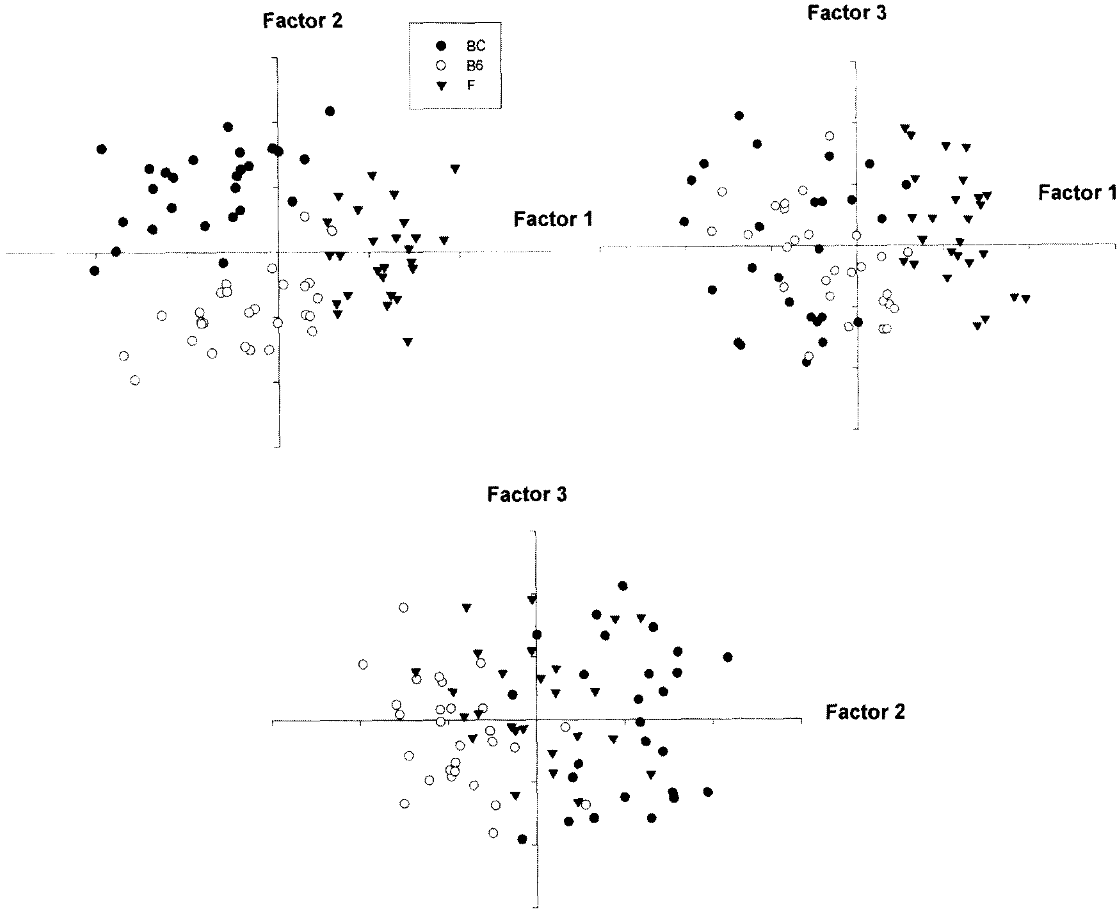

Fig. 1. Distribution of mice along the factors extracted from the principal components analysis. The factor 1 accounts for 37.1% of the total variance, while the factors 2 and 3 account for 18.5 and 12.7%, respectively. Behaviors associated with each factor are described in the Table 3.

while we used C3H as intruder. It is known that the type of opponent can influence aggressive behaviors of resident mice (Brain and Hui, 2003). In fact, we obtained different results with different intruder mice, C3H and ICR (data not shown).

The behaviors primarily associated with the factor 1 (OFA, VM, OFC, DCC, TRANS) seem to reflect their inherent locomotive basis. The previous studies (Liu and Gershenfeld, 2003) including ours (Kim et al., 2002) repeatedly indicated a clustering of these behaviors and described them as 'exploratory fear' or 'anxiety based on motor activity', etc. The second factor explains defecations in both OF and LD tests, in addition to DUR of the LD test. OFD has been weakly associated with locomotion-based anxiety, and thus regarded as a behavior related to stress (Liu and Gershenfeld, 2003). Since this factor also explains DUR, an anxiety behavior validated with diazepam (Lepicard et al., 2000), these behaviors appear to share underlying component of anxiety. However, the third factor is strictly devoted to the behaviors observed in the RI test. These non-aggressive behaviors are expected to be related with other behaviors previously observed in social context (Bell et al., 1995). A considerable association of FAC with both factors (two and three) may indicate an underlying complexity of these behaviors.

Berton et al. (1997) were unable to draw a common factor for aggressive and anxiety related behaviors measured with the OF and plus-maze tests, which is coincided with our analysis. For the same reason, SAP was excluded. Although their analysis of non-aggressive social interactions exhibited no association with anxiety related behaviors, a weak connection between them with one form of social investigative behavior (FAC) was found. This might be due to the present subdivision of investigative behavior into ones for facial or ano-genital regions (Bell et al., 1995), which were perhaps combined in the behavior previously measured as 'sniffing' (Berton et al., 1997). Although the exact nature of these behavioral differences are not clear, it was reported that pheromone complex contained in urine and genital secretions regulate social behaviors of rodent such as individual recognition and copulation (Dulac and Torello, 2003). In aggressive behaviors, objects for attack are reported to be different by the types of aggression in rodent (Blanchard et al., 2003); offensive attacks are directed towards back and flanks of the opponent, whereas defensive attacks are primarily for head and snout of the attacker.

When strain backgrounds on the behaviors were analyzed, some discrepancies between the current data and our previous studies (Kim et al., 2002) were noted. The C57BL/6N line was used instead of C57BL/6J, although the procedures for behavioral measurement were the same. In addition, the age of mice for the LD test was 11 weeks here rather than seven weeks used earlier. Thus, it is possible that emotionality of mouse is manifested differently during development as in rat (Boguszewski and Zagrodzka, 2002).

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