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Variability in empathic fear response among 11 inbred strains of mice

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Empathy is an important emotional process that involves the ability to recognize and share emotions with others. We have previously developed an observational fear learning (OFL) behavioral assay to measure empathic fear in mice. In the OFL task, a mouse is conditioned for context-dependent fear when it observes a conspecific demonstrator receiving aversive stimuli. In the present study, by comparing 11 different inbred mouse strains that are commonly used in the laboratory, we found that empathic fear response was highly variable between different strains. Five strains – C57BL/6J, C57BL/6NTac, 129S1/SvImJ, 129S4/SvJae and BTBR *T*$^{+}$ *Itpr3*^{tf}/J – showed observational fear (OF) responses, whereas AKR/J, BALB/cByJ, C3H/HeJ, DBA/2J, FVB/NJ and NOD/ShiLtJ mice exhibited low empathic fear response. Importantly, day 2 OF memory was significantly correlated with contextual memory in the classical fear conditioning among the 11 strains. Innate differences in anxiety, locomotor activity, sociability and preference for social novelty were not significantly correlated with OFL. Interestingly, early adolescent C57BL/6J mice exhibited an increase in acquisition of OF. The level of OFL in C57BL/6J strain was not affected by sex or strains of the demonstrator. Taken together, these data strongly suggest that there are naturally occurring OFL-specific genetic variations modulating empathic fear behaviors in mice. The identification of causal genes may uncover novel genetic pathways and underlying neural mechanisms that modulate empathic fear and, ultimately, provide new targets for therapeutic intervention in human mental disorders associated with impaired empathy.

Keywords: Anxiety, conditioned fear, empathy, genetic variability, inbred mouse strain, locomotion, observational fear learning, rodent model of empathic fear, sex and age, sociability, social novelty preference

Empathy, the ability to share and understand the feelings of others, is a crucial component of our social and emotional lives. Deficits in empathy manifest in a variety of disorders such as autism, schizophrenia, alexithymia, as well as psychopathy (Bernhardt & Singer, 2012; Bird *et al.*, 2010; Bora *et al.*, 2009; Frith & Happe, 2005; Lee *et al.*, 2004). Recent imaging studies have significantly contributed to the understanding of the neural networks involved in empathy. In particular, studies using functional magnetic resonance imaging (fMRI) techniques provided compelling evidence that anterior insular and anterior cingulate cortices (ACC) play central roles in vicarious response in pain-related empathy in humans (Danziger *et al.*, 2009; Singer *et al.*, 2004). However, despite recent association studies showing that genetic factors account for both change and continuity in empathy (Ebstein *et al.*, 2010), identification of genes involved in empathic behaviors has been largely limited, as it is difficult to control the social context or manipulate brain functions in humans. By contrast, these same factors can be readily controlled in experiments using animal model systems. We have previously developed a simple behavioral assay to assess observational fear learning (OFL) as a measure of empathy in mice (Jeon *et al.*, 2010; Jeon & Shin, 2011). In this OFL task, instead of receiving direct aversive stimuli, mice are conditioned for context-dependent fear vicariously by observing conspecific others receive repetitive foot shocks. Importantly, the fear response of the observer mouse is positively influenced by the animal's familiarity with the demonstrator (i.e. siblings or long-time mating partners as the demonstrator tend to trigger higher fear response in the observer). Moreover, we have further identified that the affective pain system including the ACC, the midline and intralaminar thalamic nuclei (MITN), in addition to the amygdala plays a crucial role in conditioning of observational fear (OF) (Jeon *et al.*, 2010).

Different inbred mouse strains show different emotional responses to social stress and such differences have been attributed to genetic differences of the strains (Chen *et al.*, 2009; Hovatta *et al.*, 2005; Kulesskaya *et al.*, 2014). In particular, in a tone-based OFL assay, innate response in OFL has been shown to be different between BALB/c and C57BL6J strains (Chen *et al.*, 2009). To further exploit this strain-specific difference, we have extended these observations to 11 inbred mouse strains, where we found a wide range of difference in OFL, providing further evidence that the innate response in empathic fear is under strong genetic

control. The observed phenotypic differences among strains may be attributable to multiple mechanisms, each potentially driven by different neural substrates of OFL behavior. Thus, to further investigate whether the differential levels of OFL between the 11 strains associate with the innate differences in five other behavioral traits: fear conditioning, general locomotor activity, anxiety-like behavior, sociability and preference for social novelty, we attempted to draw correlations between the strain mean values for performance in each of the behavioral tasks and the level of OFL. Lastly, in order to define conditions that may affect the behavior, we have investigated the effects of several factors, including different demonstrator partner strains, age and sex on OFL. The results show evidence for strong genetic diversity underlying empathic fear, but no direct correlation between OFL performance and the other behavioral performance tested among the inbred strains examined.

Materials and methods

Animals

All inbred mouse strains except 129S4/SvJae and C57BL/6NTac strains were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and bred locally from breeding pairs of each strain. Age-matched male mice (13 ± 1 week) for all behavioral experiments except the sex difference experiments. Animals were housed separately by strain, with 4–5 mice per individually ventilated cage and allowed free access to food and water. The housing room was maintained at 25 ± 1°C on a 12-h light/dark cycle and all behavioral experiments were conducted during the light phase of the cycle (between 0800–2000 h). Naïve mice that had no prior experience on any behaviors were used only once for each of the behavioral assays. Each of the behavioral tests was performed as mice became available from breeding, and in no particular order by strain. Animal care and handling were carried out according to the guidelines from the Animal Care and Use Committee of Institute for Basic Science (IBS).

Observational fear learning

Observational fear conditioning was performed as previously described (Jeon *et al.*, 2010; Jeon & Shin, 2011). Briefly, the apparatus for OF conditioning consisted of two identical chambers (each, 18 × 17.5 × 38 cm) containing a transparent Plexiglas partition in the middle and a stainless-steel rod floor (5-mm diameter rods, spaced 1 cm apart), which are modified using a passive avoidance cage (Coulbourn Instruments, Whitehall, PA, USA). Sound and smell could be transmitted between the chambers under the rod floor. For OF conditioning, mice (observer and demonstrator) were individually placed in apparatus chambers for 5 min and then a 2-second foot shock (1 mA) was delivered every 10 seconds for 4 min to one of the mice (demonstrator) via a computer-controlled animal shocker (Coulbourn Instruments). To assess contextual memory, observer mouse was placed alone back into the training context 24 h after training and observed freezing behavior for 4 min. In all experiments, the observer and demonstrator mice were nonsiblings and noncagemates. The behavior of the mice was recorded with the Freezeframe software (V3.32; Coulbourn Instruments) and analyzed with Freezeview software (Coulbourn Instruments). Significant motion pixels (SMP) algorithm in Freezeframe software was employed for automated analysis of activity and fear response. Mice exhibiting strange behaviors (i.e. immobility or jumping) during the habituation were excluded from data analysis. Motionless bouts lasting more than 1 second were considered as freeze and SMP threshold value of 30 was used for all subjects except 4 weeks old mice. For 4 weeks group, threshold value of 6 was used due to the small body size.

Pavlovian fear conditioning

Conventional fear conditioning was performed as previously described (Jeon *et al.*, 2010). On training day, mice were placed in the fear-conditioning chamber (Coulbourn Instruments). After a 5-min exploration period, three foot shocks (0.7 mA/1 s) separated by 1-min intervals were delivered to the mice. The mice remained in training chamber for another 60 seconds before being returned to home cages. To assess contextual learning, we placed the mice back into the chamber 24 h after training. The behavior of the mice was recorded and analyzed with FreezeFrame software as described above.

Open field test

Exploratory activity in a novel environment was assessed in one 30-min test in an open field box. Individual mouse was placed in the periphery of the field and the paths of the freely exploring animals were recorded for 30 min by a video camera. Center time was calculated as the percent of time spent in the center 18% of the field, and distance traveled was measured in total cm covered. The open field box ($50 \times 45 \times 40 cm^3$) was made of gray plastic wall and the center was defined as a square area (center, $20 \times 20\ cm^2$). The videos were analyzed using a custom made software based on MATLAB.

Elevated plus maze

Mice were given one 5-min trial on the plus maze, which had two white open arms (25 × 8 × 20 cm), two black enclosed arms (25 × 8 × 20 cm) and a central platform (8 × 8 × 8 cm) in the form of a cross. The maze was placed 50 cm above the floor. Mice were individually placed on the center section with their heads directed toward one of the closed arms. The total time spent in each arm or center, and total number of entries into each arm was analyzed by video monitoring for 5 min. Percent open arm time was calculated as 100 × (time spent on the open arms/(time in the open arms + time in the closed arms)).

Social behavior

Sociability and preference for social novelty were tested in an automated three-chambered social approach apparatus using methods previously described (Moy *et al.*, 2007). The test animals were placed in an opaque-white walled Plexiglas arena ($60 \times 40 \times 22\ cm^3$) divided into a center chamber and two side chambers. Every group of mice was naïve to this task and all other tasks, and had not been exposed to the arena prior to testing. Retractable doors built into the two dividing walls allowed access to the side chambers. The subject mouse was acclimated to the apparatus before sociability testing with a 10-min habituation session for all three empty chambers. The subject was then briefly confined to the center chamber while a novel object (inverted steel-wire cage) was placed in one side chamber and a novel mouse (stranger 1) contained inside of an identical inverted wire cage was placed in the other side chamber. After both wire cages were positioned, the two side doors were lifted and the subject mouse was allowed access to all three chambers for 10 minutes. At the end of the 10-min sociability test, each mouse was further tested in a third 10-min session to quantify preference to spend time with a new stranger (stranger 2). Mice used as the novel unfamiliar strangers (stranger 1 and 2) were age- and sex-matched mice of the same strain as the subject mice. The movement of test mouse was video-recorded and the amount of time spent in each chamber was analyzed using the EthoVision XT software Version 9 (Noldus, Wageningen, Netherlands).

Statistical analysis

Data from each strain were analyzed separately, using within-strain comparisons of behavioral parameter(s) relevant to the specific task. Data presented are means ± SEM. Behavioral data were analyzed with Sigma plot 12 (StatSoft, Inc., Tulsa, OK, USA), using a Student's *t*-test, a two-way ANOVA or a two-way repeated measure (RM) ANOVA, as appropriate. In case either normality (Shapiro–Wilk) or equal variance test failed, Mann–Whitney rank sum test or Kruskal–Wallis

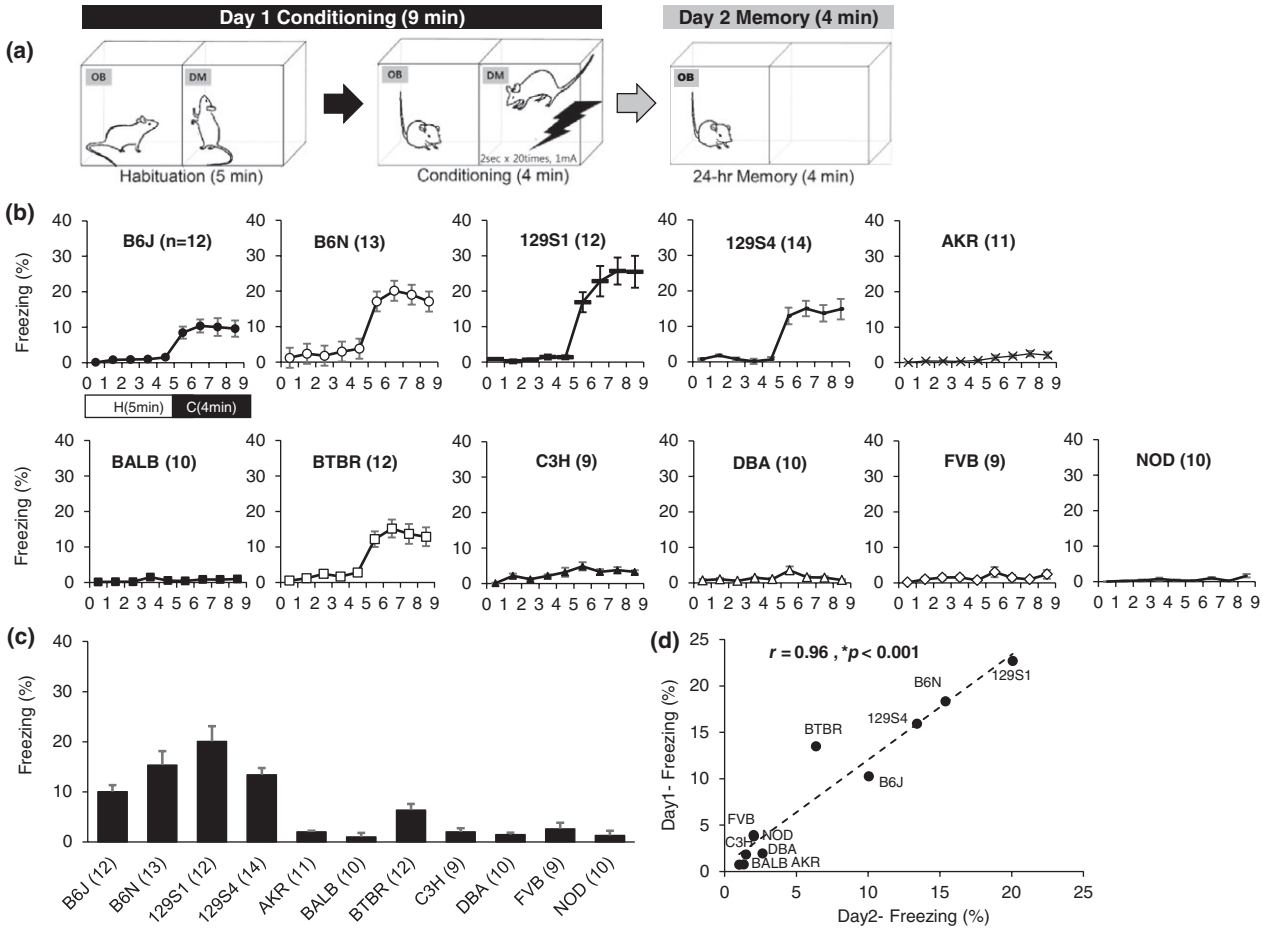

Figure 1: Wide phenotypic variation in OFL among 11 inbred mouse strains. (a) Diagram of OFL chamber and scheme of the behavioral assay. (b) Survey of OFL across 11 inbred strains. Strain B6J, B6N, 129S1, 129S4 and BTBR mice showed a significant increase in OF during the 4-min conditioning period. H, Habituation; C, conditioning. Data indicate means ± SEM. (c) Distribution of 24 h contextual memory for OF across 11 inbred strains. (d) A significant correlation between strain mean values for day 1 OF and day 2 OF memory ($r = 0.96$ and $P < 000.1$). Correlation coefficient (r) and $^{*}P$-value by Pearson's r analysis indicated within inset in bold. Values represent mean ± SEM. The number of mice for each strain tested is given in parentheses.

one-way ANOVA were used. The mean values for freezing on day 2 OF memory for 11 strains, and age and sex in B6J experiments were not normally distributed. Heritability (H^2) was calculated by comparing genetic (interstrain) variance with environmental (intrastrain) variance using one-way ANOVA. Correlations between average percent freezing during OFL and other behavioral data were performed using the Pearson's correlation coefficient test. The threshold for statistical significance was set at $P < 0.05$.

Result

Variability in OFL among 11 inbred mouse strains

In the OFL test, a mouse (observer) can learn fear when it observes another mouse (demonstrator) receive aversive foot shocks (Fig. 1a). The level of fear of the observer mouse was measured by the level of freezing behavior. To explore the naturally occurring genetic variation in empathic fear, we conducted OFL test on 11 common inbred mouse strains: C57BL/6J (B6J), C57BL/6NTac (B6N), 129S1/SvImJ (129S1), 129S4/SvJae (129S4) and BTBR *T*$^{+}$ *Itpr3*^{tf}/J (BTBR), AKR/J (AKR), BALB/cByJ (BALB), C3H/HeJ (C3H), DBA/2J (DBA), FVB/NJ (FVB) and NOD/ShiLtJ (NOD), representing the strains used for whole genome sequencing (Keane *et al.*, 2011). We observed a significant variability in day 1 OF among the 11 strains (ANOVA, $F_{10,111} = 19.3$, $P < 0.001$, Fig. 1b). OF responses were highly reproducible among individual animals of each inbred strain. For clarity, the freezing levels of each of the 11 strains are separately illustrated in Fig. 1b. Strains B6J, B6N, 129S1, 129S4 and BTBR exhibited increase in freezing response in the OFL training. By contrast, we found low level of OF in strains AKR, BALB, C3H, DBA, FVB and NOD mice, showing no increase in freezing responses during the 4-min training (see Movie S1, Supporting Information). To measure the contextual memory 24 h after the training, we then placed the observer mouse alone back to the

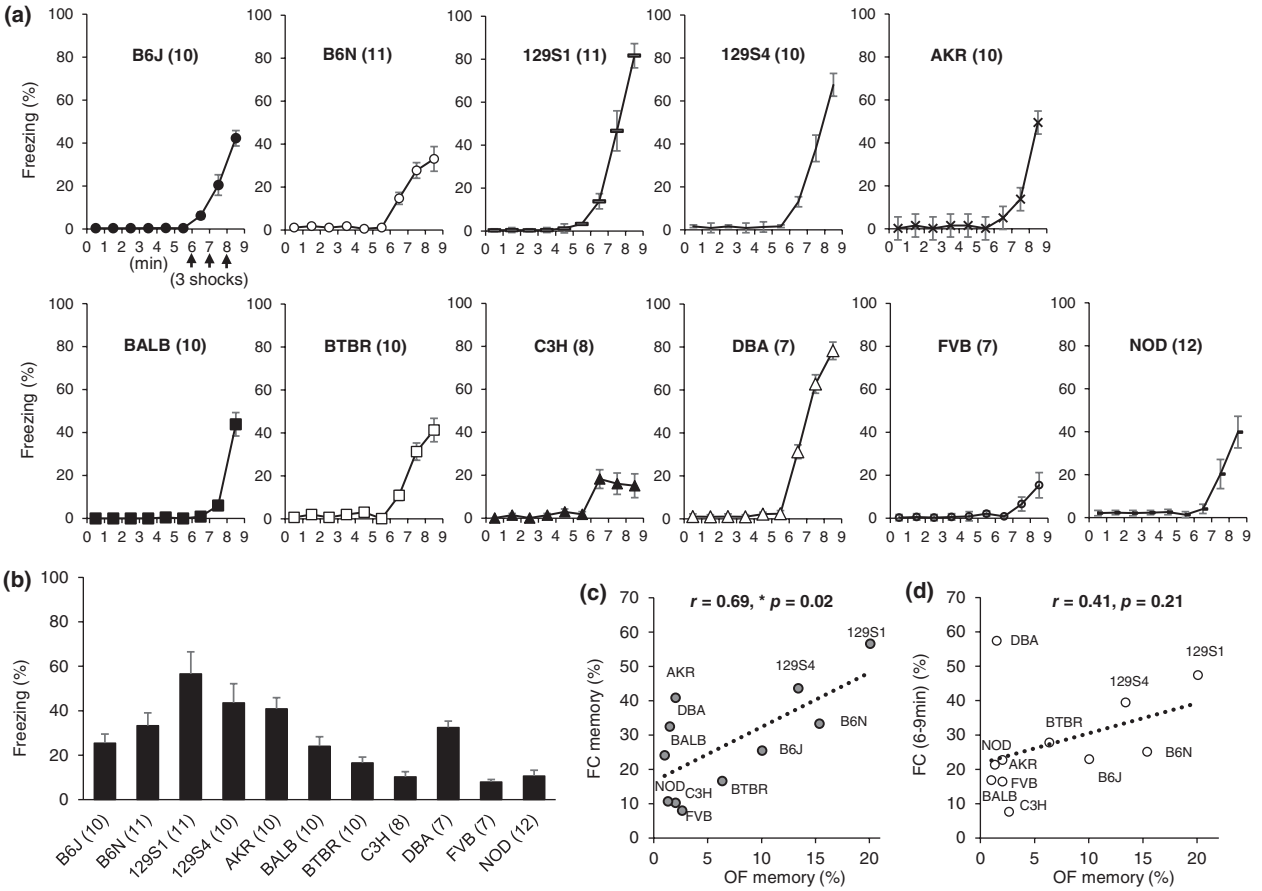

Figure 2: Strain-specific difference in Pavlovian fear conditioning. (a) Survey of fear conditioning performance across 11 inbred mouse strains. All strains except strains C3H and FVB showed a significant increase in conditioned fear over trials and reached freezing response of ~40% after the third shock. $n = 10–15$ per strain. (b) Distribution of 24 h contextual memory for conditioned fear across 11 inbred strains. (c) A significant correlation between strain mean values for 24 h OF memory and 24 h contextual fear memory. (d) No significant correlations between strain mean values for 6–9 min conditioned fear and day 2 OF memory. Correlation coefficient (r) and P-value by Pearson's r analysis indicated within inset in bold. Values represent mean ± SEM. The number of mice for each strain tested is given in parentheses.

same chamber. Strain-specific differences in day 2 OF memory across the 11 strains were also found (Kruskal–Wallis ANOVA, $P < 0.0001$, Fig. 1c). Strains 129S1, 129S4, B6J and B6N mice demonstrated significant levels of contextual freezing with means of 15–20% of the time. Strains AKR, BALB, C3H, DBA, FVB and NOD mice that showed no freezing behaviors during the training on day 1 displayed also low levels of fear memory, confirming that there was impairment in acquisition of OF in these inbred strains. We searched for a correlation between strain mean values for day 1 OF and day 2 OF memory. A highly significant correlation ($r = 0.96$ and $P < 000.1$) was found among the 11 mouse strains (Fig. 1d). We have calculated heritability by comparing genetic (interstrain) variance with environmental (intrastrain) variance using one-way ANOVA. The heritability (H^2) for strain mean values of OF memory was estimated to be 0.54, indicating that the strain-specific difference in OFL is under genetic control.

Fear conditioning

Different inbred mouse strains show differences in freezing response in the classical Pavlovian fear conditioning (Balogh & Wehner, 2003; Bolivar *et al.*, 2001; Bothe *et al.*, 2005; Hefner *et al.*, 2008; Owen *et al.*, 1997). Because OF is expressed by freezing response, we next determined whether the differential level of OF among the 11 strains was due to the difference in freezing response to conventional conditioned fear. As shown in Fig. 2a, there were strain-specific differences in fear conditioning, generally consistent with the literature (Balogh & Wehner, 2003; Bolivar *et al.*, 2001; Bothe *et al.*, 2005; Hefner *et al.*, 2008; Owen *et al.*, 1997). Strains 129S1, 129S4, AKR, B6J, B6N, BALB, BTBR, DBA and NOD mice exhibited an increase in freezing across trials (~40% after third shock), whereas strains C3H and FVB mice had poor performance in acquisition of conditioned fear, showing freezing levels lower than 20% after the third foot shock. Wide range of phenotypic difference in

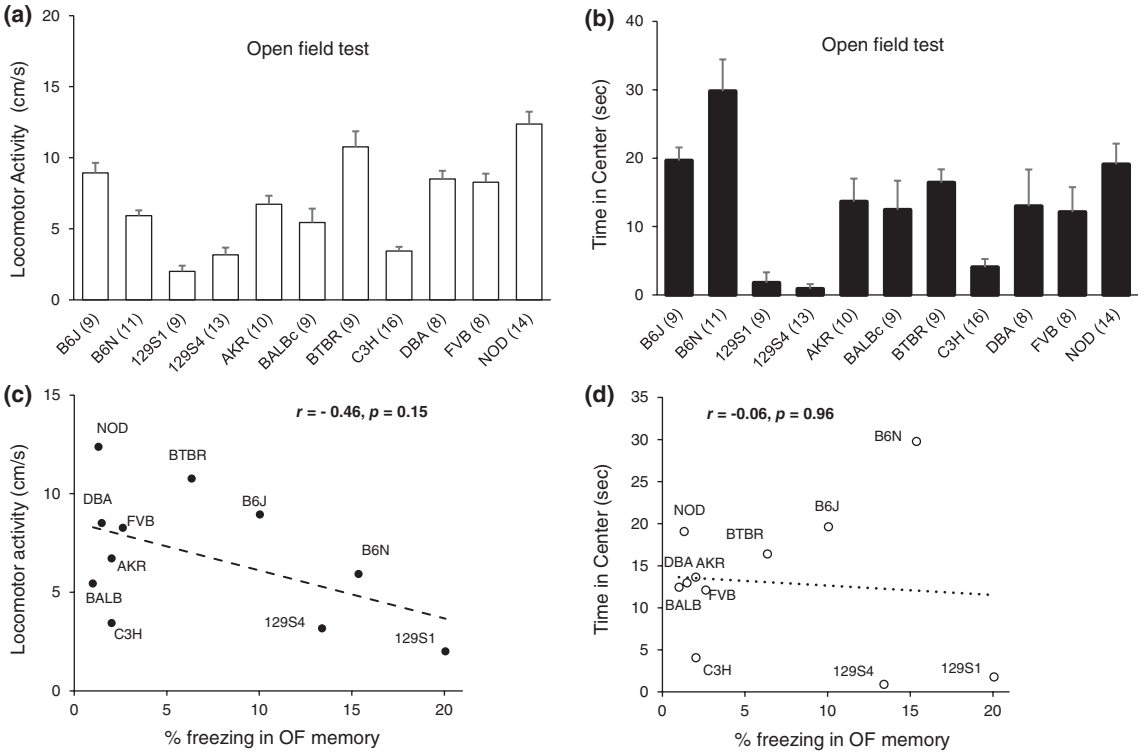

Figure 3: Strain mean correlations between activities in the novel open field and OFL. Distribution of (a) locomotor activity and (b) center time in open field test across 11 inbred mouse strains. Values represent mean ± SEM. The number of mice for each strain tested is given in parentheses. No significant correlations between strain mean values for locomotor activity (c) or center time (d) and day 2 OF memory. Correlation coefficient (*r*) and *P*-value by Pearson's *r* analysis indicated within inset in bold.

24 h contextual fear memory among the 11 strains were also observed (ANOVA, $F_{10,106} = 12.16$, $P < 0.001$, Fig. 2b). Strains 129S1, 129S4 and AKR mice displayed the highest level of contextual fear memory with mean freezing of 40–50% of the time. By contrast, C3H and FVB strains exhibited low level of freezing, only 7–10% of the time. Mean percent freezing for BALB and BTBR mice were approximately 20%, showing an intermediate level of freezing in the context conditioning, consistent with previous reports of lower fear conditioning in BTBR strains compared to B6 mice (Yang *et al.*, 2012).

It should be noted that the low OF learner strains, C3H and FVB, (Fig. 1b) exhibited low levels of freezing in the classical fear conditioning, thus suggesting that the low level of OF in these strains is likely due to their poor ability in conditioned fear. By contrast, despite low freezing in OFL, the level of conditioned fear in strains AKR, BALB, DBA and NOD were similar to the high OF learner strains (B6J, B6N, 129S1, 129S4 and BTBR). We analyzed the correlation between strain mean values for the freezing levels of 24 h contextual fear memory and those of the OFL test. Interestingly, a statistically significant correlation was found between OF memory and FC memory among the 11 mouse strains ($r = 0.69$ and $P = 0.02$). Because differences in hippocampal formation, sensory and motor systems and motivation between inbred mouse strains has been known to contribute to the strain-specific difference in 24 h contextual fear memory (Balogh & Wehner, 2003; Bolivar *et al.*, 2001; Bothe *et al.*, 2005; Hefner *et al.*, 2008; Owen *et al.*, 1997), some of the same brain nuclei and neurochemical systems might also be involved in the OF memory retrieval.

Open field test

To examine whether locomotor activity or anxiety-like behavior contribute to the differential level of OFL, the 11 mouse strains were evaluated for exploration in a novel environment in the open field test. We found strain-specific differences in open field test, similar to previous reports (Miller *et al.*, 2010; Moy *et al.*, 2007, 2008). Strain distributions for locomotor activity and the percent of time spent in the center (18% of the field) are shown in Fig. 3. Strain NOD mice had the highest level of locomotor activity, whereas strains 129S1 and129S4 mice showed the lowest levels of activity among the 11 strains. Overall, most strains preferred the periphery to the center during the first 5 minutes (Fig. 3b). Strain B6N mice exhibited the highest level of center time in the open field, while 129S1 and 129S4 strains showed extremely low levels of center time in comparison to all the other strains, suggesting that these strains show anxiety-like behavior. To determine whether this difference in locomotor activity or the center time has a relationship with the level of OFL, we next analyzed the correlation between strain mean values for the

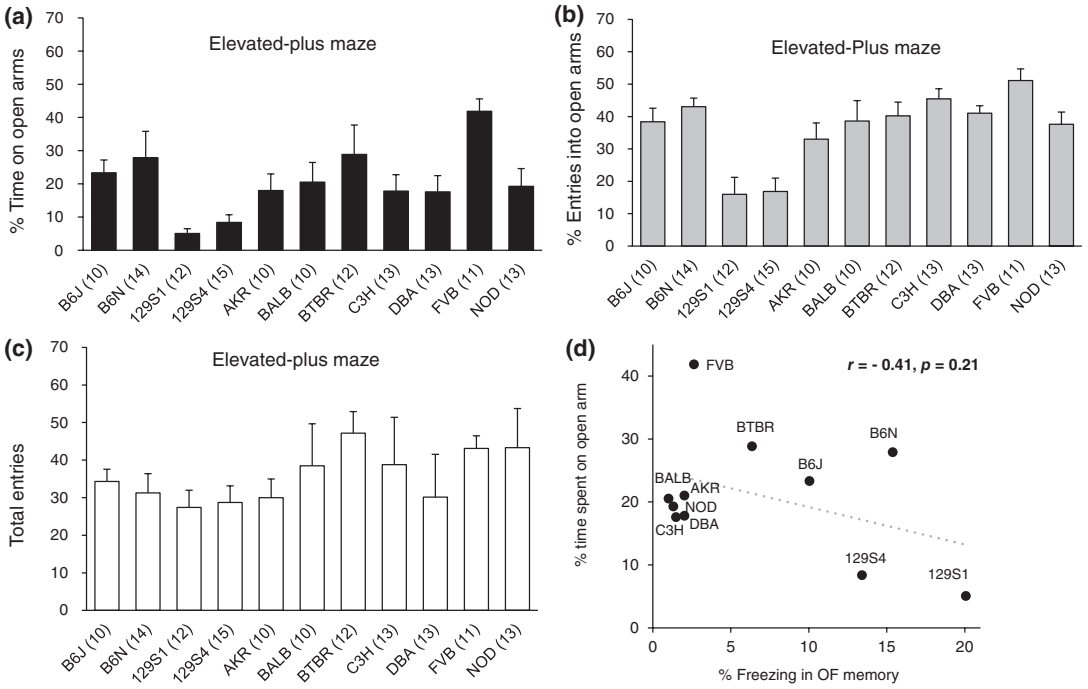

Figure 4: Strain mean correlations between anxiety-like behavior in the elevated plus maze and OFL. Distribution of (a) percent time spent on open arms, (b) percent entries into open arms and (c) total arm entries across 11 inbred mouse strains. Values represent mean ± SEM. The number of mice for each strain tested is given in parentheses. (d) No significant correlation between strain mean values for open arm time and day 2 OF memory. Correlation coefficient (*r*) and *P*-value by Pearson's *r* analysis indicated within inset in bold.

behaviors of open field test and those of the OF memory. Neither the locomotor activity nor the center time attained a statistically significant correlation with the level of OFL in these strains. (Fig. 3c,d).

Elevated plus maze

To further determine correlation between the strain difference in anxiety-like behavior and OFL, we carried out the elevated plus maze (EPM) test. We observed a strain-specific difference in all variables in EPM, generally consistent with the previous literature (Moy *et al.*, 2007, 2008). Figure 4 shows the percentage of session time spent on the open arms, and the percentage of the total entries to the open arms, as well as the total number of entries, for the 11 inbred strains. While strain FVB mice showed the highest level of time spent on the open arms and percent entries into open arms, 129S1 and 129S4 strain had the lowest level of open arm time and percent entries, again indicating an anxiety-like behavioral phenotype. No significant strain mean correlation between the open arm time and the level of day 2 OF memory was found among the 11 strains (Fig. 4d), suggesting that the anxiety-like behavior is not a major factor determining the differential levels of OFL between the strains.

Three-chamber social approach test

To determine whether the level of OFL is associated with sociability or preference for social novelty, we performed the three-chamber behavioral assay on the 11 strains. Strain-specific difference in sociability was observed among these 11 strains. Consistent with previous literature (Moy *et al.*, 2007, 2008), we have found that strains B6J ($F_{1,22} = 14.8$, $P < 0.05$), B6N ($F_{1,20} = 13.7$, $P < 0.05$), 129S4 ($F_{1,22} = 7.8$, $P < 0.05$), AKR ($F_{1,22} = 12.9$, $P < 0.05$), C3H ($F_{1,24} = 16.4$, $P < 0.05$), DBA ($F_{1,20} = 20.1$, $P < 0.001$) and FVB ($F_{1,24} = 23.0$, $P < 0.001$) mice spent more time in the chamber containing an unfamiliar stranger 1 mouse compared with the side containing an empty wire cage (within-group RM ANOVA, $P < 0.05$). By contrast, 129S1, BALB, BTBR and NOD mice failed to demonstrate significant sociability to the stranger mouse. On preference for social novelty, only B6J and DBA mice exhibited significant preferences for spending time in the chamber containing a new unfamiliar stranger 2 compared with the familiar stranger 1 mouse (within-group RM ANOVA, $P < 0.05$, Fig. 5b). Strains B6N, 129S1, 129S4, AKR, BALB, BTBR, C3H, FVB and NOD mice failed to show a preference to the stranger 2 mouse.

Sociability or social novelty preference showed no significant correlation with the level of OFL (Fig. 5c,d). Strains BALB and NOD mice displayed deficits both in sociability and OFL. Intriguingly, strain BTBR that has been well known for deficit in sociability exhibited a significant level of OFL (Fig. 1b). The strain BTBR mice showed no significant difference in day 1 OF response (ANOVA, $F_{1,22} = 3.2$, $P = 0.08$,) and day 2 OF memory (ANOVA, $F_{1,22} = 4.2$, $P = 0.07$) when compared with

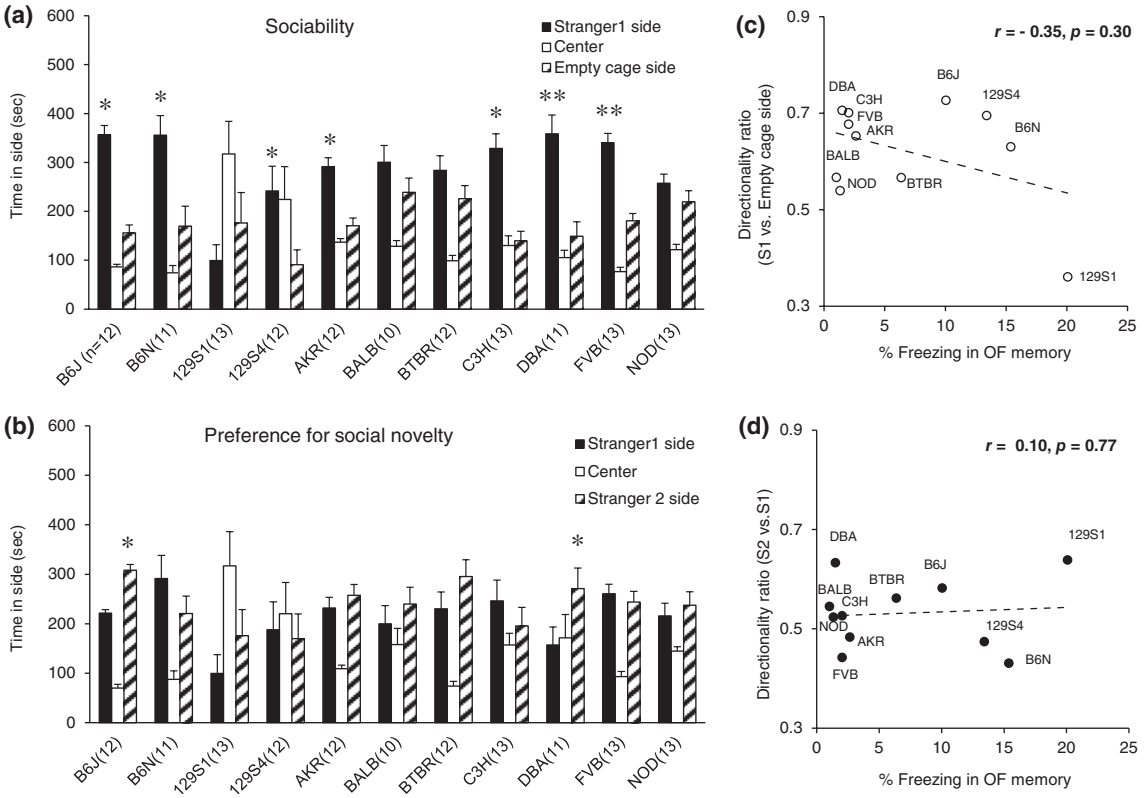

Figure 5: Strain mean correlations between social approach behaviors in three-chamber test and OFL. Duration of time spent in each chamber during the test for (a) sociability and (b) preference for social novelty in 11 inbred mouse strains. Data shown are mean ± SEM for each strain for 10 min. $*P < 0.05$, $**P < 0.001$. The number of mice for each strain tested is given in parentheses. No significant correlation between strain mean values for (c) directionality to stranger 1 mouse (S1) against empty cage side or (d) directionality to unfamiliar stranger 2 (S2) against familiar stranger 1 (S1) and day 2 OF memory. Correlation coefficient (r) and P-value by Pearson's r analysis indicated within inset in bold.

B6J mice. On the contrary, the low OF learner strains AKR, C3H, DBA, and FVB mice demonstrated significant sociability. Thus, these data suggest that the difference in social recognition and approach is not responsible for the differential response of OFL in mice.

B6J strain demonstrates OFL toward different demonstrator strains

We have previously observed that B6J demonstrator mice showed more homogeneous responses displaying all behavioral reactions to 1 mA foot shocks (i.e. running, vocalization and/or jumping). When lower 0.7 mA foot shocks were given, B6J observers displayed lower freezing response in the OFL test (Jeon *et al.*, 2010). Additionally, as it is possible that different inbred strains display different visual, olfactory or auditory cues while receiving foot shocks, we have examined whether these potential behavioral difference between different demonstrator strains affect the levels of freezing in B6J observer mice. To determine whether the extent of the observer's freezing can be different between in-group and out-group demonstrators, we evaluated the level of OFL in B6J mice toward different partner strains. We have chosen a high OF learner strain (129S1) and a low OF learner strain (FVB) as out-group demonstrators. Strain 129S1 has an agouti coat color, high anxiety-like phenotype and low locomotor activity. Strain FVB mouse shows a white coat color, aggressive behavior and high sociability. Intriguingly, despite the different responses to foot shocks among the three demonstrator strains (two-way RM ANOVA, $F_{2,27} = 28.09$, $P < 0.001$, Fig. 6a), B6J observer mice paired with out-group 129S1, or FVB demonstrators showed similar levels of OFL as compared with the in-group B6J-B6J pairs (two-way RM ANOVA, $F_{2,24} = 0.94$, $P = 0.41$, Fig. 6b). On day 2 OF memory, we also found no difference between B6J → B6J, B6J → 129S1 and B6J → FVB groups (Fig. 6c).

Effect of age on OFL behavior in B6J mice

Because adolescent mice differ from adults on levels of anxiety and stress-related behaviors (Adriani & Laviola, 2004; Hefner & Holmes, 2007), we have attempted to evaluate OFL in male B6J mice aged 4 (early adolescent), 8 (early adult), 12 (adult) and 16 (adult) weeks of age. Interestingly, although there were no significant differences in overall freezing levels among four groups (RM ANOVA, $F_{3,36} = 2.18$, $P = 0.11$), in

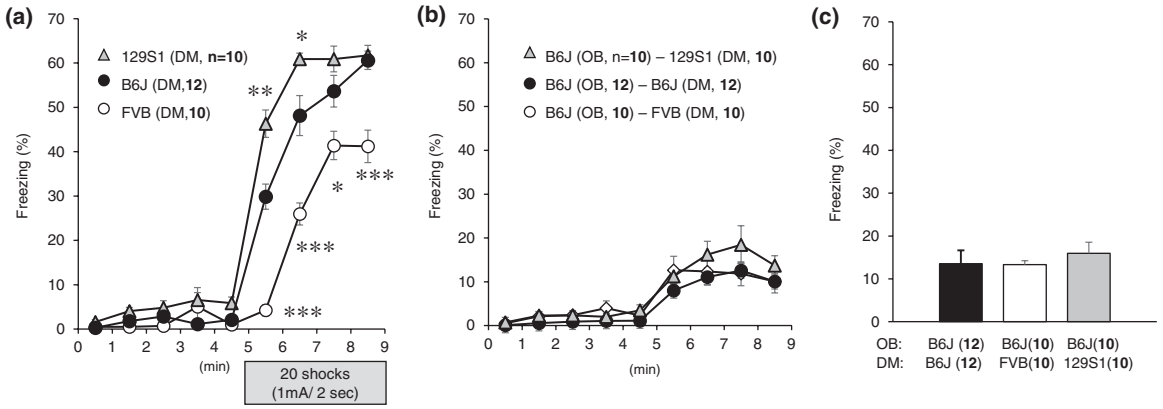

Figure 6: OF response in B6J mice paired with different partner demonstrator strains. B6J mice demonstrate OFL even for out-group demonstrator strains. (a) Difference in conditioned fear of demonstrator strains 129S1, B6J and FVB mice. All strains showed a significant increase in conditioned fear during the 20 foot shocks. No significant difference in (b) day 1 OF and (c) day 2 OF memory of B6J observer (OB) mice to B6J, 129S1 or FVB demonstrators (DM). Values in (a) indicate mean ± SEM. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. The number of mice for each strain tested is given in parentheses.

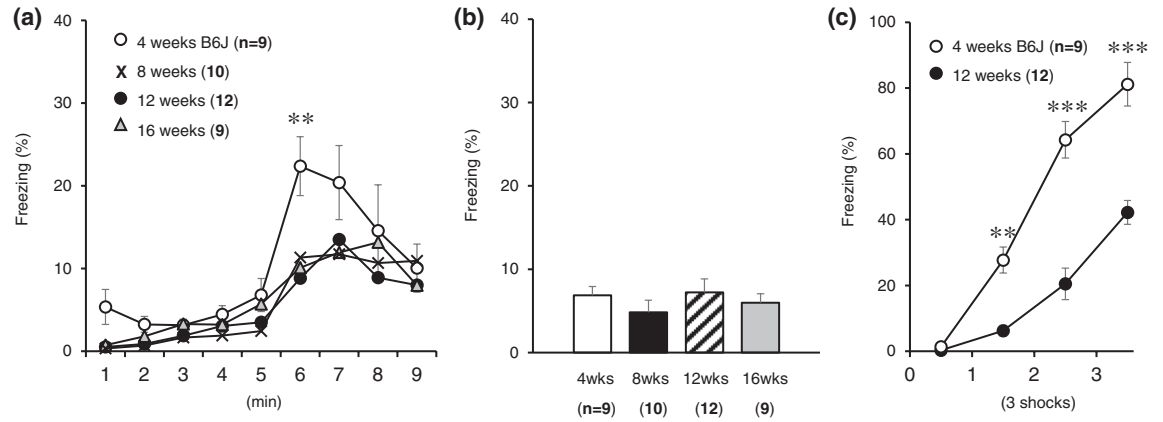

Figure 7: OF and Pavlovian fear conditionings across different age-groups of B6J mice. (a) Early adolescent (4-week-old) mice showed more freezing during OFL than adults. $**P < 0.01$; 4-week vs. 8-week, $P < 0.01$; 4-week vs. 12-week, $P < 0.01$; 4-week vs. 16-week B6J mice. No significant difference in (b) 24 h memory between the four different age groups. (c) In Pavlovian fear conditioning, 4 weeks old mice froze more than older mice. $**P < 0.01$, $***P < 0.001$; 4-week vs. 12-week B6J mice. The number of mice for each strain tested is given in parentheses.

the 5- to 6-min period of the training session, the freezing response in the 4-week old mice was significantly higher than those of older mice (*post hoc* Student–Newman–Keul, $P < 0.01$; 4-week vs. 8-week, $P < 0.01$; 4-week vs. 12-week, $P < 0.01$; 4-week vs. 16-week, Fig. 7a). There was also no difference in day 2 OF memory among the four groups (Kruskal–Wallis test, $P = 0.395$, Fig. 7b). To further determine whether the high level of OF in early adolescent mice is due to difference in the expressivity of conditioned fear, we examined a separate cohort of 4 weeks old B6J mice on conditioned fear. As consistent with a previous report (Hefner & Holmes, 2007), 4 weeks old mice froze more than adult mice in the classical fear conditioning (RM ANOVA, $F_{1,19} = 29.34$, $P < 0.001$, Fig. 7c). Thus, these data suggest that the enhanced response in conditioned fear may have contributed to the high level of OF in early adolescent B6J mice.

Effect of sex on OFL behavior in B6J mice

Because sex difference in conditioned fear and emotional contagion in rodents were reported (Baran *et al.*, 2010; Bolivar *et al.*, 2001; Langford *et al.*, 2006; Wiesenfeld-Hallin, 2005) and women frequently score higher on standard tests of empathy, social sensitivity and emotion recognition than men (Schulte-Ruther *et al.*, 2008; Singer *et al.*, 2006), we attempted to investigate the effect of sex on OFL. To examine sex-specific effect, we evaluated OF of male and female B6J observer mice toward same or different sex demonstrator partners. The mice in each pair were not littermates or cage mates. As shown in Fig. 8, there was no significant difference

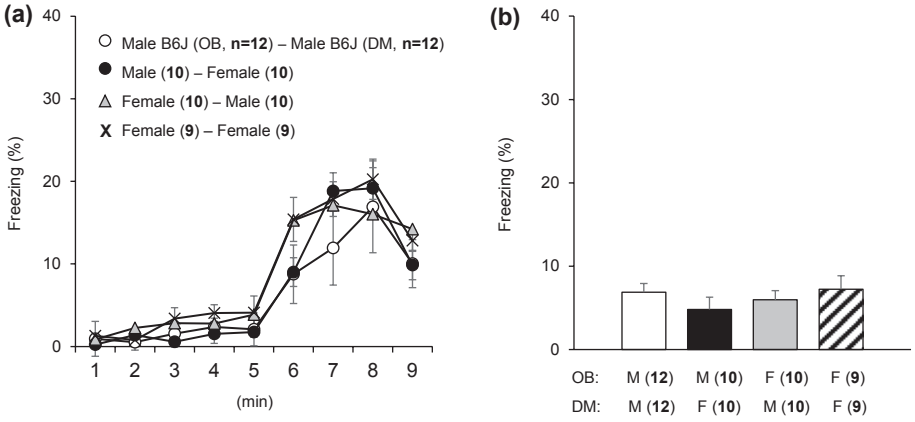

Figure 8: Effect of sex on OFL in B6J mice. (a) No significant difference in OFL between male or female observer (OB) mice toward either male or female demonstrator (DM). Both male and female observer mice showed a significant increase in freezing during (a) day 1 OF and (b) day 2 OF memory, which was independent of the sex of partner demonstrator mice. Values indicate mean ± SEM. The number of mice for each strain tested is given in parentheses.

in the levels of day 1 OF (ANOVA, $F_{3,37} = 0.76$, $P = 0.53$) and day 2 OF memory (Kruskal–Wallis test, $P = 0.63$) across four different pair groups: male (observer) → male (demonstrator), male → female, female → male and female → female groups.

Discussion

In this study, we have shown that different inbred mouse strains differ in the OFL, suggesting that empathic fear behavior is under strong genetic control. Male mice from five inbred strains, B6J, B6N, 129S1, 129S4 and BTBR, demonstrated significant level of empathic fear response whereas strains AKR, BALB, C3H, DBA, FVB and NOD mice did not exhibit freezing in OFL behavior. Of these low OF learner strains, the C3H and FVB mice also showed poor performance in the classical fear conditioning task. However, despite showing similar level of conditioned fear when compared with the high OF learner strains (B6J, B6N and BTBR), strains AKR, BALB, DBA and NOD mice displayed low OFL, indicating that the low level of empathic fear in these three strains may not have resulted from abnormalities in fear expression or context conditioning ability, but from impairment in the neural function regulating empathic fear in mice.

Importantly, our previous published data demonstrated that the fear response of the observer mouse was positively influenced by the animal's familiarity with the demonstrator (Jeon *et al.*, 2010). Because empathy is broadly defined as affective behaviors focused on the response of the observers and familiarity is considered as a factor increasing empathy in observers for the state of the demonstrators, our behavioral assay for OFL could be reasonably matched to the empathic fear shown in higher primates and humans. The freezing response itself during OF conditioning in our study seems to be consistent with emotional contagion because freezing of the demonstrator and observer mice occurred at the same time. However, when the observer mouse was placed alone back in the same chamber next day, the mouse showed freezing response (contextual fear memory), indicating that an association has been made between the distress state of conspecifics and the specific environment where the event happened. As discussed in the literature (Panksepp & Lahvis, 2011), this subsequent effect is distinct from emotional contagion because day 2 freezing behavior expressed by the observer took place long after its exposure to the distressed conspecific and the observer had never experienced the foot shocks. Therefore, our finding indicates the social transfer of an emotional state from one mouse to another.

Because we have previously shown that visual cues influence the observer mouse in OFL (Jeon *et al.*, 2010; Jeon & Shin, 2011), it is possible that behaviors for OFL is confounded by abnormalities in the sensory ability. For instance, two of the strains selected for the present study have been known for their vision deficiency. The genomes of the C3H and FVB mice include the gene for retinal degeneration (*Pde6brd1*), which leads to blindness by the age of weaning (Gimenez & Montoliu, 2001; Pittler & Baehr, 1991). Thus, in addition to their low levels of freezing in fear conditioning, poor visual ability may also contribute to the low OFL in C3H and FVB strains. By contrast, strains AKR and DBA mice have been reported to exhibit a good vision (Brown & Wong, 2007), suggesting that the low OFL in these two strains is not associated with their visual ability.

It has been well demonstrated that fear or social stress can be influenced by anxiety-like behaviors. Although we found no significant correlation between the level of OFL and innate difference in anxiety-like traits among these 11 strains, we do not exclude the possibility that individual difference in anxiety could affect phenotypic variation in OFL in some strains. The strain 129S1 is significantly less 'social' than the other strains and exhibits high % freezing both in OFL and in the classical fear conditioning, so it may not be unreasonable that the high anxiety-like traits affect the phenotypes in OFL and sociability in 129S1 strain. However, we have observed that the complexity of the genetic variation in OFL and in sociability across the 11 strains precludes a simple answer to this question. For example, despite the fact that another 129sv strain, 129S4, shows extremely low exploratory activities, high anxiety and high % of conditioned fear, which are not significantly different from 129S1, we have found that this strain exhibits lower level of OFL than that of 129S1 and no deficits in sociability. Conversely, strain C3H mice showed high anxiety-like phenotype in open field test, but they showed no freezing response in OFL and no deficit in sociability. Therefore, anxiety could be an important factor in

determining variation in OFL in some strains, but we surmise that the high level of OFL and social deficits in 129S1 strain may not have resulted from high levels of anxiety or freezing.

Patients with autism spectrum disorder (ASD) show impaired emotional processing with deficit of social recognition and empathy (Bird *et al.*, 2010; Frith & Happe, 2005; Lee *et al.*, 2004; Lombardo *et al.*, 2007; Minio-Paluello *et al.*, 2009). Similar to previous studies (Brodkin, 2007; Moy *et al.*, 2007, 2008), we also observed a large strain variability in sociability and preference for social novelty among our 11 inbred mouse strains. However, the variability in sociability was not significantly correlated with the differential levels of empathic fear among these strains. Seven strains –B6J, B6N, 129S4, AKR, C3H, DBA and FVB – demonstrated significant sociability. However, of these strains, only 129S4, B6J and B6N mice showed OF responses. Strain BALB and NOD mice displayed deficit in sociability and low OFL. Surprisingly, BTBR strain, an inbred mouse strain well-known for its prominent behavioral phenotypes of autism (Silverman *et al.*, 2010), exhibited a similar level of OFL when compared with B6J mice. Our finding in mice is congruent with a previous report that no deficit in emotional empathy was found in a cohort of autistic individuals (Hadjikhani *et al.*, 2014). We have also found no correlation between social novelty preference and level of empathic fear among these 11 strains. Preference for social novelty was detected only in B6J and DBA strains but all the other strains failed to spend more time in the chamber with the new stranger 2 mouse than in the chamber with the more familiar stranger 1. Taken together, these data indicate that difference in empathic fear and in these two social recognition behaviors (sociability and social novelty preference) may be modulated by different background genes within the inbred strains used in this study.

Although strain-specific difference in sociability reported in literature was well replicated in the current study, it should be noted that preference for social novelty for several inbred strains in our study is not consistent with previous studies. We observed significant preference for social novelty only in B6J and DBA strains, but strains BALB, BTBR and FVB mice in this study failed to show significant preference for social novelty. As a previous study demonstrated the different social approach-avoidance or communicative behaviors between different inbred strains (Brodkin *et al.*, 2004), we surmise that this discrepancy might be caused by different strains used for stranger mice in the wire cages. In the present study, we used mice of the same strain as the unfamiliar stranger mice, whereas the previous studies used only B6J mice as strangers when examining different strains both for sociability and social novelty preference (Moy *et al.*, 2007, 2008). In addition, it is possible that different testing procedures utilized between the two studies might result in the difference in preference for social novelty for several inbred strains. Because of the potential confounding effects of repeated testing and natural intraindividual variations in behaviors over time, it has been suggested that subjecting mice to a succession of multiple behavioral tests is not ideal (Fraser *et al.*, 2010; Paylor *et al.*, 2006; Voikar *et al.*, 2004). In the present study, all the mice that were tested for the three-chamber task had no prior experience of any behavioral tests. But previous studies have performed open field locomotion and rotarod tasks before the same animals were tested on three-chamber assay (Moy *et al.*, 2007, 2008). Thus, the different result in social novelty preference might be due to prior test experience of mice. However, because it has been well known that results from behavioral assays can significantly differ between laboratories, even when procedures, animal source and other environmental factors are carefully controlled and standardized (Crabbe *et al.*, 1999; Wahlsten *et al.*, 2003, 2006), we cannot exclude the possibility of unknown environmental variations.

In humans, OF and safety learnings from a racial in-group demonstrator have been reported to be more potent than learning from a racial out-group demonstrator (Golkar *et al.*, 2015). In earlier work, using a mouse model of cue-conditioned fear, Chen *et al.* (2009) reported that the level of OF in B6J mice was higher than that of BALB strain when exposed to the tone alone. The author further demonstrated that vocal communication played an important role in the social transfer of fear among mice. On the basis of this finding, we investigated contribution of various behavioral reactions of different out-group demonstrator strains to foot shocks (i.e. jumping, freezing, running or vocalization) in triggering the differential level of empathic freezing in B6J observer mice. Despite such potential behavioral difference between demonstrator strains, we have found that B6J observer mice demonstrated similar level of OFL toward different out-group demonstrator strains (129S1and FVB). Because we did not measure distress vocalization during the training, it is not sure whether the three demonstrator strains vocalized differently when receiving foot shocks. However, even if demonstrator's vocalization or social cues during the training differ in the details of expression and characteristics between these three inbred strains, these difference might not significantly affect the level of OFL in B6J mouse strain. Although B6J mouse strains demonstrated empathic fear even for out-group demonstrator strains in the OFL behavior, it should be noted that this finding may not be the same if we test different observer strains. We cannot rule out the possibility that other mouse strains might show OFL differently when paired with these three demonstrator strains. Because it is not certain which particular aspect of demonstrator's behavior triggers vicarious freezing in observer mice, B6J strain might respond differently if other demonstrator strains are paired.

Difference in age or sex did not significantly affect the level of OFL in B6J mice in this study. Although we have observed that 4 weeks old adolescent male mice showed more observational freezing than older mice, their level of freezing in fear conditioning was also higher than that of adult mice. Consistent with our findings, a previous study also demonstrated that mice at the early adolescent stage acquired and expressed conditioned fear response to a greater degree as compared to adults (Hefner & Holmes, 2007). Thus, it is likely that the increased level of OF in 4 weeks old mice might be due to enhanced acquisition of conditioned fear. In a mouse model of empathic pain, ICR (CD-1) outbred mice showed difference in writhing pain behaviors toward different sex partners (Langford *et al.*, 2006). In the present study, sex difference did not significantly affect the level of OFL in B6J mice.

Similarly, in earlier study no difference in empathic fear was found between male and female mice both in C57BL/6J and BALB/cJ strains (Chen *et al.*, 2009).

In conclusion, the data from present study provide a valuable reference when choosing strain background and critical environmental factors for future studies using genetic mouse models of mental disorders associated with empathic fear. More importantly, our study strongly suggests that there are naturally occurring genetic variations that regulate differential empathic behaviors among inbred mouse strains. Although familiarity, social isolation, stress, prior shock experience and the strength of the US delivered to demonstrator have been known to influence the degree of behavioral response to distress in others (Atsak *et al.*, 2011; Gonzalez-Liencres *et al.*, 2014; Jeon *et al.*, 2010; Panksepp & Lahvis, 2011; Sanders *et al.*, 2005; Watanabe, 2011), many questions regarding factors that trigger the empathic fear response in rodents still remain. We have previously identified that CaV1.2 calcium channel gene (*Cacna1c*) is involved in OFL (Jeon *et al.*, 2010), but the genetic factors regulating OFL in mice still largely unexplored. Therefore, the identification of causal genes may uncover novel genetic pathways and underlying neural mechanisms that modulate empathy and, ultimately, provide new targets for therapeutic intervention in human mental disorders.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Movie S1: Two video clips of a C57BL/6J (high OF learner) and an AKR/J (low OF learner) observer mice that contain the last 1 min (4–5 min) of habituation and the first 1 min (5–6 min) of training.