

## ORIGINAL ARTICLE

# Experience of a hierarchical relationship between a pair of mice specifically influences their affective empathy toward each other

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## Abstract

Prior experience of social hierarchy is known to modulate emotional contagion, a basic form of affective empathy. However, it is not known whether this behavioral effect occurs through changes in an individual's traits due to their experience of social hierarchy or specific social interrelationships between the individuals. Groups of four mice with an established in-group hierarchy were used to address this in conjunction with a tube test. The rank-1 and rank-4 mice were designated as the dominant or subordinate groups, respectively. The two individuals in between were designated as the intermediate groups, which were then used as the observers in observational fear learning (OFL) experiments, an assay for emotional contagion. The intermediate observers showed greater OFL responses to the dominant demonstrator than the subordinate demonstrators recruited from the same home-cage. When the demonstrators were strangers from different cages, the intermediate observers did not distinguish between dominant and subordinate, displaying the same level of OFL. In a reverse setting in which the intermediate group was used as the demonstrator, the subordinate observers showed higher OFL responses than the dominant observers, and this occurred only when the demonstrators were cagemates of the observers. Furthermore, the bigger the rank difference between a pair, the higher the OFL level that the observer displayed. Altogether, these results demonstrate that the hierarchical interrelationship established between a given pair of animals is critical for expressing emotional contagion between them rather than any potential changes in intrinsic traits due to the experience of dominant/subordinate hierarchy.

## Practitioner points

- Subordinate observer or dominant demonstrator resulted in higher affective empathic response in familiar pairs but not unfamiliar pairs.

Hee-Sup Shin and Jaeseung Jeong contributed equally to this study.

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- The relative social rank of the observer with respect to the demonstrator had a negative linear correlation with the affective empathic response of the observer in familiar pairs but not unfamiliar pairs.
- The effect of social rank on affective empathy is attributed to the prior social hierarchical interrelationship between them and is not due to intrinsic attributes of an individual based on one's dominance rank.

**KEYWORDS**

affective empathy, emotional contagion, familiarity, hierarchical interrelationship, observational fear learning, phenotypic variability, social dominance

## 1 | INTRODUCTION

Social animals naturally prefer to live together in groups and organize themselves into a dominance hierarchy.<sup>1–3</sup> Social hierarchy formation within a group results in a distinct dominance status (e.g., dominant or subordinate) depending on the individual's attributes. Recent studies have reported that even isogenic individuals may show various phenotypic differences according to their social rank, for example, anxiety, resilience in social defeat stress, aggression, and social motivation.<sup>1,4–6</sup> These hierarchical effects are mainly explained by changes in each individual's intrinsic physiological state (e.g., neuroendocrine system or energy-related metabolites) according to its dominance status through prior hierarchical experiences.<sup>1,4,6,7</sup>

Familiarity is also known to be established and maintained along with social hierarchy formation.<sup>8,9</sup> Familiarity toward the target has been consistently reported to significantly affect diverse empathic behaviors, such as social fear learning, motor mimicry, and prosocial behavior, across various species.<sup>10–13</sup> While the familiarity bias has been consistently demonstrated in a variety of laboratory environments, studies on the hierarchical effects are still controversial.<sup>4–7,14</sup> Furthermore, studies of hierarchical effects on empathy in the context of familiarity remain elusive due to the lack of consistent and sufficient pieces of evidence.<sup>15–17</sup> A recent study reported that subordinate rats showed increased fear responses after interacting with a fear-conditioned familiar dominant rat from their home-cage.<sup>17</sup> As hierarchical effects are generally attributable to the intrinsic traits of an individual with a respective dominance hierarchy of its own,<sup>4,6</sup> the abovementioned phenomenon could also be interpreted as occurring due to differences in the intrinsic characteristics of the subjects. However, as mentioned earlier, it is difficult to completely rule out the possibility that familiarity, such as prior social interrelationships, was reflected in this phenomenon.

In addition to familiarity issue, in a previous study, each dominance rank was determined through qualitative observation from the home-cage under the social condition of three animals in one cage<sup>17</sup>; hence, it was difficult to examine the quantitative relationship between social rank and affective empathy in detail, for example, linear correlation between dominance score and level of empathic response. Also, as each social rank of the observer and the rank of the demonstrator were not controlled independently, it was difficult to

clearly distinguish whether the hierarchical effect was mainly due to the traits of the observer or the demonstrator.

To overcome these limitations, behavioral methodologies that can precisely and robustly quantify the social rank are needed. There are various ways to identify the dominance hierarchy within a cage, such as agonistic behavior, barbering, territorial urine marking, ultrasonic courtship vocalization, food competition, the warm spot test, and the tube test.<sup>1,18,19</sup> Among these, the tube test has been widely used as a robust and straightforward method for identifying dominance hierarchies as it shows not only a stable and linear phenotypic result but also a high correlation with the social rank identified through other abovementioned behavioral assays reflecting various respective traits of social dominance.<sup>4,19,20</sup>

Observational fear learning (OFL) is one of the representative behavioral assays to study affective empathy or social transfer of fear in various species.<sup>11,21,22</sup> In the experimental design, observers who witness the demonstrator suffer from pain or distress show behavioral or physiological responses (e.g., increased heart rate and skin conductance, and decreased freezing) representing the level of emotional transfer in response to others' suffering without the direct experience of the aversive stimuli. As OFL is a pairwise (observer–demonstrator) behavioral paradigm measuring the emotional transfer of fear from a target, each characteristic of the observer and demonstrator that might affect the experimental results should be carefully distinguished and controlled independently. (i) Observer's traits (e.g., a higher level of OFL response could be attributed to the observer's trait related to anxiety) or (ii) demonstrator's stressful responses during the OFL period, such as jumping, running, vocalization, and urination, can also affect OFL responses of the observers.<sup>11,21,23</sup>

This study aimed to verify the relationship between social dominance and emotional empathic response in the context of prior social interrelationship, that is, familiarity within a pair. To this end, the tube test and OFL test were used as primary behavioral methodologies to monitor social dominance and empathic fear response, respectively. We wanted to clarify whether the putative intrinsic attributes that depended on an individual's social rank are sufficient for the hierarchical effect on affective empathic behavior or whether prior social experience within a pair is necessary. Therefore, a subject was paired with a familiar target from his home cage or a stranger target from a different breeding cage for the OFL test.

To consider the social rank of the observer or demonstrator one at a time as an independent variable, intermediate rank groups were employed. In the tetrad cage condition, the rank-1 and rank-4 mice were designated as the dominant or subordinate groups, respectively. The two individuals in between were designated as the intermediate groups. To investigate whether the demonstrator's social rank could affect the observer's OFL response, two groups of demonstrators with distinct social rank (dominant, subordinate) were paired with intermediate observers. In a reverse condition, two groups of observers with distinct social rank were paired with intermediate demonstrators. To investigate the qualitative relationship between the phenotypic variations of OFL responses and the degree of relative social hierarchy difference within a pair, David's score<sup>24,25</sup> was used to calculate the relative cardinal dominance score of the observer with respect to the demonstrator.

## 2 | METHODS AND MATERIALS

### 2.1 | Animals

We followed the ethical guidelines of the Institutional Animal Care and Use Committee of the Institute for Basic Science (IBS) and the Korea Advanced Institute of Science and Technology (KAIST). Animals were maintained in an individually ventilated cage (IVC) at room temperature ( $22^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ ) in a specific pathogen-free facility. Food and water were available ad libitum under a 12:12-h light–dark cycle (8 AM to 8 PM lights on). C57BL/6J strain mice (*Mus musculus*) were initially purchased from Jackson Laboratory (JAX, Bar Harbor, USA) and bred within their colony at the IBS/KAIST facility. Male pups were obtained from the mating cages during the weaning period, cohoused with conspecific siblings, and maintained. Male adult (11–20 weeks old) mice were used for the experiment. Mice were housed under the IVC rack in cages (MT-005 MVCS CAGE; Three-shine Inc, Daejeon, South Korea) [ $200 \times 320 \times 145$  mm, material: Polysulfone (PSF)].

The social conditions of all mice were controlled as a total of 3–5 mice per cage immediately after weaning (about 3 weeks after birth) and maintained until the end of all experiments except for the conditions of isolation/congested environment. The four mice per cage (tetrad) condition was mainly used for the tube test followed by OFL. All subjects were naïve before the experiments. Cagemates were used as demonstrators during OFL in the case of familiar pair conditions. Thus, 90 mice from 36 cages and 212 mice from 53 cages (tetrad) were used in the experiment with social housing conditions and the rest of the experiments (tube test followed by OFL), respectively. Based on the result 3.4 that observers did not show differences in OFL responses according to the nonfamiliar demonstrator's social rank, some demonstrators (6/4 mice per dominant/subordinate group; from 7 cages) of unknown rank (naïve to tube test) were paired with observers in the nonfamiliar OFL experiment result 3.5. Animals with physical abnormalities [low body weight ( $<20$  g) or severe injuries] were excluded from the experiments or analysis.

## 2.2 | Behavioral experiments

All behavioral experiments were conducted in a soundproof chamber with a ventilation system under a dim light (4–10 lux) during the light cycle. The home-cages with subjects were moved to a behavior room for 30 min before the experiments for room habituation. Room temperature was maintained at  $23^{\circ}\text{C}$ , and a white noise generator (approximately 95 dB near the speaker and 65 dB at the center of the chamber) was activated to dilute the unwanted background acoustic noise. In addition, 70% ethanol and distilled water were used to clean the apparatus after each test.

### 2.2.1 | Observational fear learning

OFL behavior methodology was used to measure the level of emotional contagion of fear in mice.<sup>11,26,27</sup> A passive avoidance cage (Coulbourn Instruments, Whitehall, PA, USA) was modified to be used as the apparatus for OFL. A transparent Plexiglas partition was put in the middle of the cage to separate two chambers ( $180 \times 175 \times 380$  mm, each)—for the observer mouse and demonstrator mouse. The cage was dimly illuminated (4–10 lux) using a light-emitting diode from the center of the ceiling. A stainless-steel rod floor (5-mm diameter, spaced 10-mm apart) was put under both chambers to deliver an electric shock. Although the rod floor was at the bottom of both chambers, the shock was delivered only to the demonstrator side. The apparatus was designed to allow for perception of major sensory modalities for social information transfer, including visual, auditory, and olfactory signals through vacant spaces at the bottom of the grid and a transparent partition.

Behaviors of the observer and demonstrator were recorded at 15 Hz using two digital cameras (Sentech, Berlin, Germany) fitted at each ceiling. The observer's behavior was mainly analyzed to study empathy, and the demonstrator's behavior was used to analyze fear-related behavior. Electric shock was generated using a precision animal shocker (Coulbourn Instruments). Trigger of signal for shock delivery and data analysis was conducted using ACT-711 USB interface (Coulbourn Instruments) and FreezeFrame3 software (Actimetrics; Coulbourn Instruments).

Under the experimental condition, the observer and demonstrator mice were located on the grid at each side divided by the transparent partition. After a 5-min acclimation period (for acclimation to the apparatus), electric shock (1 mA) was delivered to the demonstrator mouse for 2 s every 10 s during 4-min training. The protocol parameters were optimized to elicit sufficient OFL responses from observer mice witnessing the demonstrator's painful reaction, such as vocalizing and jumping behavior.<sup>11</sup>

### 2.2.2 | Tube test

Social dominance within a cage was measured using a modified version of the tube test.<sup>20,28</sup> The tube test apparatus was mainly

composed of a 300-mm Plexiglass tube (26-mm diameter) and two acrylic chambers (100 × 100 × 100 mm each) attached to the tube to help subjects get accustomed to entering into the tube. The subjects were given an adaption period of 3 days before the primary tube test. Then, each mouse was gently put into the acrylic box, which was connected to the tube, and trained to run through the tube 2–3 times.

The tube test experiment requires a pair of subjects at a time, as does OFL. Each mouse was gently placed into the acrylic boxes connected to each end of a transparent cylindrical acrylic tube. The experimenter simultaneously opened each door between the box and tube, allowing both mice to approach the center of the tube. They push each other, and the one who is pushed out of the tube is considered defeated. The pairwise match was terminated after one of the two subjects had won more than two times and was performed for at least 3 days for all pairwise combinations of the mice from a cage in a round-robin fashion.

## 2.3 | Analysis

### 2.3.1 | Automated analysis of freezing behavior

The emotional contagion of the fear response of the observer during OFL is measured by freezing behavior as in the general fear conditioning behavioral paradigm.<sup>29</sup> The significant motion pixels (SMP) algorithm implemented in the FreezeFrame software was used for the automated analysis of freezing behavior.<sup>30</sup> A subject's degree of movement was calculated as a pixel difference between two consecutive frames from the recorded video. This yielded raw data of the activity level by SMP as the motion index of the subject. Activity level below the threshold of 30 SMP was considered as an immobile or putative freezing status. To distinguish fragments of immobile status from actual freezing responses, a minimum 1-s freezing bout threshold was given; immobility maintained for a ≥ 1-s time bout was considered as an excellent freezing bout.

The sum of the freezing bout durations (s) was averaged (%) in a 1-min time-bin (e.g., nine bins for 9-min data). Average freezing (%) during baseline (5-min acclimation) and OFL (4-min test) was obtained by averaging the five time-bin and the four time-bin, respectively; baseline (%) and OFL (%). A relative increase in the freezing level in the OFL period compared with the baseline was calculated (OFL – baseline) and used for all statistical analyses. Observers or demonstrators that showed abnormal behavior, such as excessive freezing, were excluded based on the following criteria: excessive average freezing during baseline >10% and excessive time-bin (1-min) freezing during baseline >15%. Some of the demonstrators' data were excluded due to problems during the recording process (criteria: OFL – baseline <5%).

### 2.3.2 | David's score

David's score is one of the appropriate metrics to calculate the cardinal social dominance score.<sup>14,24,25</sup> Unlike the simple calculation of

ordinal rank by comparing the total accumulated number of wins per subject within a group, David's score considers all pairwise agonistic outcomes (ratio of wins/losses) of an individual with regard to its targets' outcomes iteratively within a group (all combinations).<sup>24,25</sup> Therefore, it can reflect more detailed information on the hierarchical social interrelationship.

David's score was calculated based on the results of the tube test. Daily David's scores were computed for each individual within a cage to yield daily traces of social rank and determine rank stability. To calculate a final David's score and social rank, the final 3-day pairwise agonistic outcomes of an individual were accumulated as an interaction matrix. The weighted averages of the agonistic outcomes were calculated with a discount rate of 0.9.

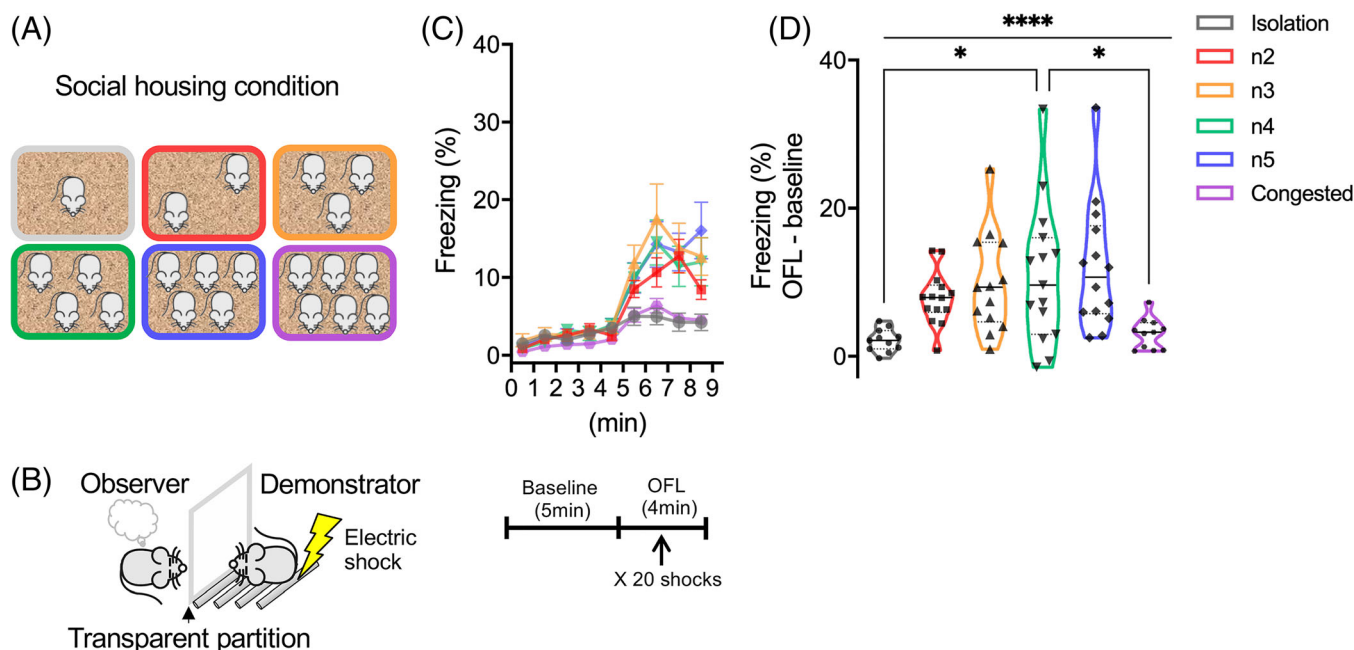
$$O_j = \sum_{i=1}^3 o_i * \gamma^{3-i}; j = \{\text{win, lose}\}, i: \text{day} = \{1, 2, 3\}, \gamma = \text{discount rate},$$

where the  $O_j$  represents the total agonistic outcomes (win, lose, respectively), and the  $o_i$  represents the agonistic outcomes of the day ( $i$ ). Therefore, the past was relatively attenuated concerning the recent outcomes.

The final David's score was used for all analyses related to the relative David's score of the observer with respect to the demonstrator. When the pairwise agonistic outcome between an observer and a demonstrator was unstable (criteria; the number of days of perfect win or loss for the observer/demonstrator <1 day), it was excluded from the pair-specific behavioral analysis (familiar pair OFL).

### 2.3.3 | Statistical analysis

Statistical analyses were performed using Prism 9.0 (GraphPad). Normality was assessed using Shapiro–Wilk test. Welch's<sup>31</sup>  $t$ -test was used to compare two independent groups if the dataset had a normal distribution. Welch's one-way analysis of variance (ANOVA) was used to compare more than two groups, followed by Dunnett's T3 multiple comparisons test (post-hoc). When the dataset had non-normal distribution, the outliers were excluded based on the interquartile range (IQR)<sup>32</sup> calculation, followed by a retest for normality. Nonparametric tests were prepared for the dataset still had a non-normal distribution: Mann–Whitney  $U$  test to compare two groups and Kruskal–Wallis  $H$  test (one-way ANOVA by ranks) followed by Dunn's multiple comparisons test (post-hoc) to compare more than two groups. As all of the final data had normal distribution, nonparametric tests were not used. Before linear regression analysis, normality and homoscedasticity of residuals were confirmed by Shapiro–Wilk test and fitted value versus residual plot, respectively. Pearson's correlation coefficient  $R$  was calculated through linear regression analysis to determine whether two variables were significantly correlated: relative David's score (Obs – Dem) as an independent variable and OFL response (OFL – baseline) as a dependent variable (both cardinal). Significance levels  $\alpha$  were set at 0.05 (5%), and asterisks representing  $p$ -values were as follows: \* $p$  < 0.05; \*\* $p$  < 0.01; \*\*\* $p$  < 0.001, and \*\*\*\* $p$  < 0.0001. All the data were presented as means



**FIGURE 1** Empathic fear response is decreased in abnormal social housing conditions. (A) Illustration of six groups depending on the social housing condition. Each group of home-cage borders is distinctly color labeled; gray: n1 (isolation), red: n2, orange: n3, green: n4, blue: n5, purple: n6 (congested) mice per cage. (B) Schematic illustration of the observational fear learning (OFL) test as a behavioral paradigm to measure the emotional contagion of fear. After 5 min of acclimation, electric foot shock is given to the demonstrator mouse during 4 min of training at regular intervals (2-s duration  $\times$  20, 10-s interval). (C, D) Freezing behavior (%) of the observer mouse witnessing the demonstrator's reactions is measured. Six different color labels depending on the social housing condition; n1 (isolation) ( $n = 11$ ), n2 ( $n = 14$ ), n3 ( $n = 13$ ), n4 ( $n = 15$ ), n5 ( $n = 14$ ), and n6 (congested) ( $n = 11$ ). (D) Significant difference among the six groups. The individual housing and congested housing groups showed lower freezing compared with the n4 (4 mice per cage) group. No statistical differences were found in the rest (2, 3, 4, or 5 mice per cage) groups

$\pm$  standard error of the means. Percent freezing was employed as a dependent variable reflecting the subject's affective empathic response, except for the results Section 3.2. In results Section 3.2, contingency tables of integer counts with respect to two categorical variables were statistically analyzed; social rank, and stability condition. Two-sided Fisher's exact test and Chi-square test were applied to  $2 \times 2$  or arbitrary dimensions of contingency tables, respectively.

## 2.4 | Graphical illustrations

All graphs, including time-series, scatter-bar, regression, and stacked histogram, were plotted using Prism 9.0 (GraphPad). Schematic illustrations were prepared using Microsoft PowerPoint (for Mac, v16.54) (Microsoft). Finally, mice were illustrated using the AutoDraw tool from JamBoard (Google).

## 3 | RESULTS

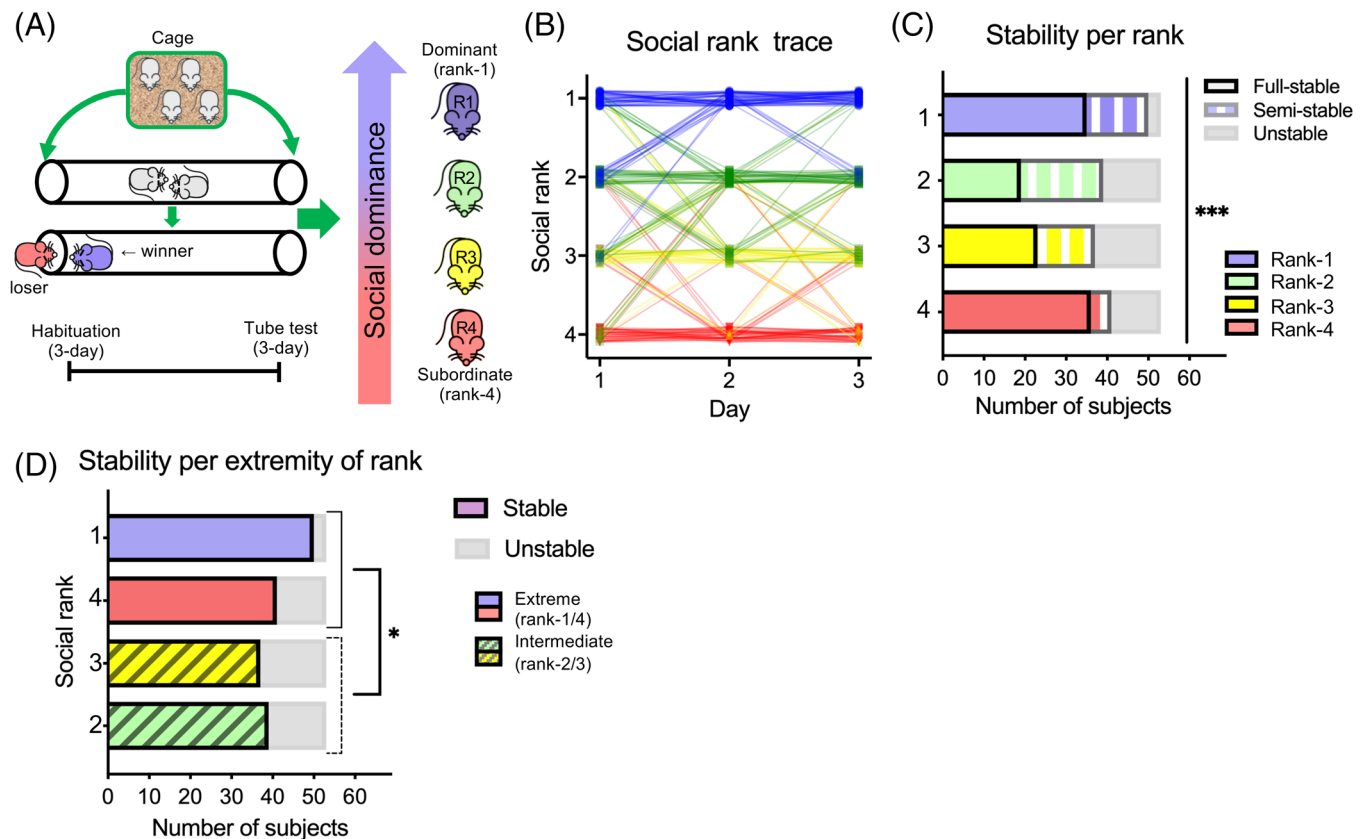
### 3.1 | Empathic fear response is modulated by social housing condition

As rodents are social animals, they are usually cohoused with a proper number of conspecific cagemates for more than a few weeks before

the behavioral experiments to ensure appropriate social environments under laboratory conditions.<sup>33–35</sup> Abnormal social density conditions, such as social isolation or congested housing, have been reported to significantly affect the experimental results of social behavior.<sup>4,34,36,37</sup>

OFL behavioral assay was conducted on six groups depending on the social housing condition (the number of mice per cage) of the subjects to investigate the effect of social housing condition as a putative influential social factor on affective empathy. Percent freezing was employed as a dependent variable reflecting the subject's affective empathic response. The social housing condition (independent variable) was determined based on the number of mice in a cage: 1 mouse per cage (individual housing/social isolation;  $n = 11$ ), 2 ( $n = 14$ ), 3 ( $n = 13$ ), 4 ( $n = 15$ ), 5 ( $n = 14$ ), and 6 (congested housing;  $n = 11$ ). Results indicated that the social housing condition is an influential factor in emotional empathic response, as evident from the statistical difference among the six groups. (Figure 1D;  $F_{[5,32,96]} = 11.77$ ,  $p < 0.0001$ , Welch's one-way ANOVA). Specifically, the isolation group and the congested group showed significant decreases compared with most of the remaining groups (Figure S1A; Isolation versus others; isolation versus n2: \*\*\* $p = 0.0007$ , isolation versus n3: \* $p = 0.0198$ , isolation versus n4: \* $p = 0.0383$ , isolation versus n5: \* $p = 0.0117$ . Congested versus others; congested versus n2: \*\* $p = 0.0063$ , congested versus n3: \* $p = 0.0458$ , congested versus n4:  $p = 0.0806$ , congested versus n5: \* $p = 0.0247$ . No statistical significance in the rest of the pairs. Dunnett's T3 multiple comparisons for post-hoc). No statistical difference was found in the groups





**FIGURE 2** Stability of social rank is higher in extreme ranks. (A) Diagram of the tube test apparatus and experimental scheme to measure social hierarchy. Round-robin pairwise match within a cage of four mice during the 3-day tube test. (B) Rank trace to show the stability of social rank during the 3-day period per each rank ( $n = 43$  tetrads). Each color indicates a mouse of a specific social rank (blue: rank 1, green: rank 2, yellow: rank 3, and red: rank 4). (C) Stacked histograms were plotted according to three stability criteria (full-stable: maintained the same rank for 3 days, semi-stable: maintained the same rank for 2 days with a total rank change of 1, unstable: the rest; stripe pattern: semi-stable, Gray: unstable) per each rank. A significant difference was observed in the stability depending on four different social ranks. (D) Compared with the individuals of intermediate ranks (ranks 2 and 3; diagonal stripe pattern), a higher number of those of extreme ranks (ranks 1 and 4) maintained their own social hierarchy rank (full-stable and semi-stable)

with 2, 3, 4, or 5 mice per cage (Figure S1B;  $F_{[3,26.62]} = 1.282$ ,  $p = 0.3010$ , Welch's one-way ANOVA). The result implies that the social condition beyond the range of 2–5 mice per cage should be considered inappropriate for social behavioral experiments. The post-hoc analysis also revealed that the individual housing and congested housing groups particularly showed a lack of empathic fear response compared with the control group (4 mice per cage; social condition used in rest of the experiments) (Figure 1D;  $p = 0.0140$ ,  $p = 0.0303$ , respectively, Dunnett's multiple comparisons test). These results confirm that social housing with social deficiency and housing with excessive social density in a confined space could harm the individual's empathic fear response.

### 3.2 | Stability of dominance rank varies depending on the social rank within a cage

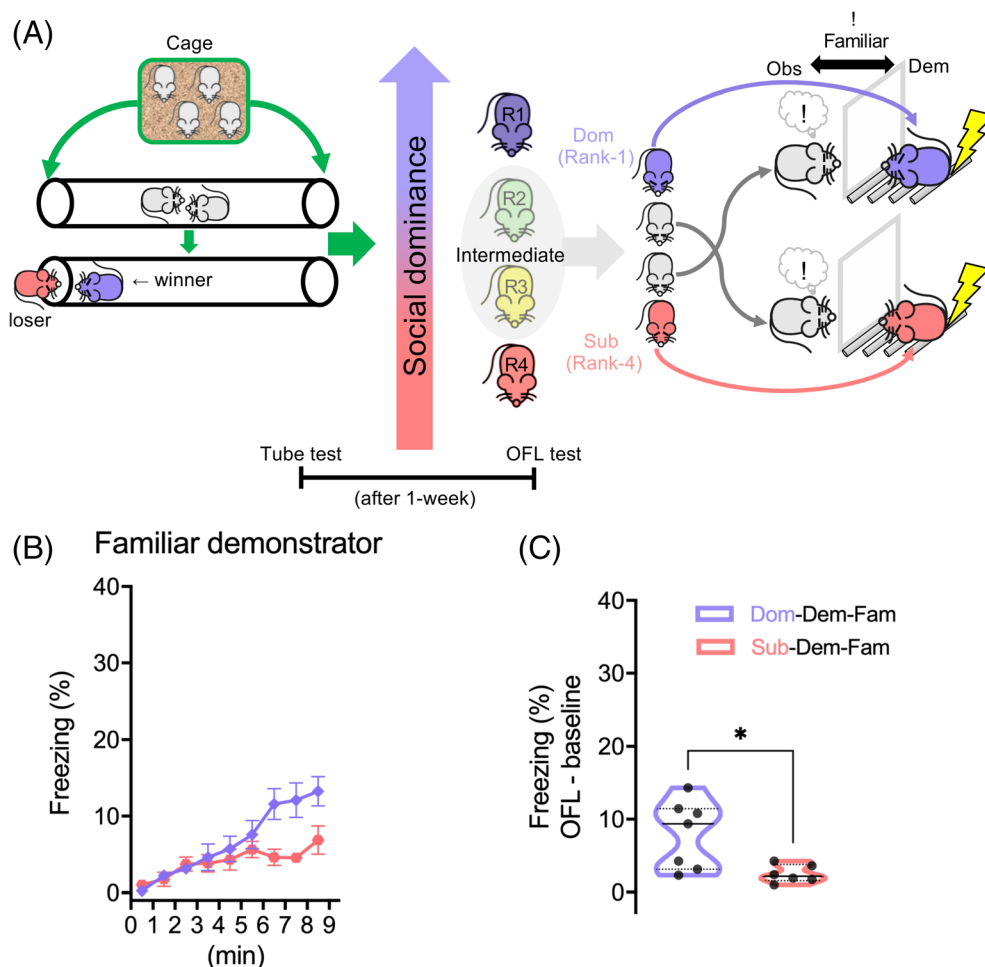
Rodents that have been cohoused with conspecific cagemates for more than a few weeks in a cage of a fixed social environment tend to establish a social hierarchy, which is known to be one of the influential

factors that induce behavioral phenotypic variability in isogenic individuals.<sup>4,14</sup>

The tube test was performed to observe the social rank within a cage. First, the social rank based on dominance within a cage was determined in the order of the highest value (total dominance score) measured from the test; the individual with the highest value was assigned rank 1, whereas the individual with the lowest value was assigned rank 4 (tetrad; 4 mice in a cage). Second, to examine the stability of the social rank, an individual's daily social rank was also line-plotted with four different color labels depending on the final social rank during the 3 days (Figure 2B; blue: rank 1, green: rank 2, yellow: rank 3, and red: rank 4). The individual traces qualitatively showed that the stability varied depending on the social rank (Figure 2C, D).

Three different social rank stability criteria were applied for each of the four different social ranks to analyze the social rank stability more quantitatively. Subjects who maintained the same rank for 3 days were classified as the “full-stable” group, and the ones who maintained the same rank for 2 days with a minimal change of rank (only total rank change of 1 was allowed) were classified as the

**FIGURE 3** Empathic fear response of an observer is elevated toward a familiar dominant demonstrator. (A) Illustration of the behavioral analysis. The tube test was followed by the OFL test. Based on the results of the tube test, two demonstrator groups were established: dominant (Dom-Dem-Fam;  $n = 7$ ) and subordinate (Sub-Dem-Fam;  $n = 6$ ). Familiar cagemate demonstrators are paired with observers. (B, C) The observer of intermediate ranks showed higher freezing toward the dominant demonstrator over the subordinate demonstrator

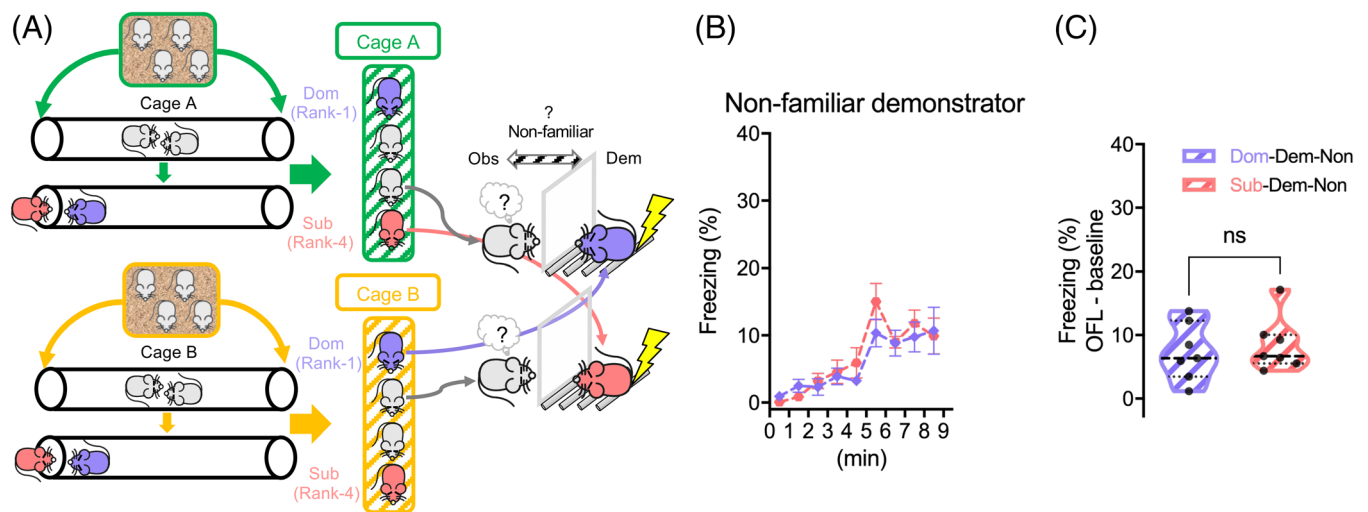


“semi-stable” group. The rest were classified as “unstable.” The number of individuals corresponding to each criterion was determined and expressed as a histogram. The result revealed a significant difference in the contingency table (4: rank  $\times$  3: stability condition) (Figure 2C;  $\chi^2_{(6)} = 25.19$ ,  $p = 0.0003$ , Chi-square test). The tendency to maintain social rank was found to vary depending on the extremity of the social rank (2: binary rank extremity  $\times$  2: binary stability). Compared with the individuals of intermediate ranks (ranks 2 and 3), a higher number of those of extreme ranks (ranks 1 and 4) maintained their social rank (full-stable and semi-stable) (Figure 2D;  $p = 0.0181$ , Fisher's exact test, two-sided,  $n = 212$ ). This suggests that not all mice seem to be created equal in their tendency to maintain their social rank (i.e., the proportion of individuals who maintained a highly stable social rank in a given social environment). Mice of extreme social ranks were more likely to maintain their stable social rank than mice of intermediate social ranks, a finding that was consistent with the results of a previous study.<sup>4</sup> No significant difference was observed in the contingency table of dominant-subordinate grouping [2: binary social rank (dominant: rank-1, rank-2; subordinate: rank-3, rank-4)  $\times$  2: binary stability] (Figure S2B;  $n = 212$ ,  $p = 0.0923$ , Fisher's exact test, two-sided,  $n = 212$ ), which was used in a previous study.<sup>6</sup> Thus, only stable rank-1 and rank-4 mice were classified as “dominant (Dom)” and

“subordinate (Sub)” groups, respectively, and the remaining as “intermediate” groups for the remaining phases of the experiments.

### 3.3 | Empathic fear response of an observer is elevated with a familiar dominant demonstrator

After assessing the stable social rank through the tube test, OFL was performed as the primary behavioral assay. The social rank of the demonstrator was considered as an independent variable with a putative effect on OFL. Therefore, the observers were grouped into intermediate social rank groups, whereas the demonstrators were divided into two groups: dominant (Dom) and subordinate (Sub). Behavioral assay results showed that the observers of intermediate social rank elicited significantly higher OFL to the “Dom” demonstrator from the same breeding cage (Dom-Dem-Fam;  $n = 7$ ) than to the familiar “Sub” demonstrator (Sub-Dem-Fam;  $n = 6$ ) (Figure 3C;  $T_{(6,940)} = 2.965$ ,  $p = 0.0212$ , Welch's  $t$ -test, two-tailed). No difference was noted in the freezing responses of the observers of differing social ranks during the acclimation period (Figure S4A;  $T_{(8,394)} = 0.3586$ ,  $p = 0.7288$ , Welch's  $t$ -test, two-tailed), suggesting that the effect of the demonstrator's social rank on the observer's freezing response is specific to the emotional responses of the social partner given an electric shock.



**FIGURE 4** No difference in the empathic fear response of an observer toward a nonfamiliar demonstrator. (A) Illustration of the behavioral analysis. Similar to Figure 3A, except that the paired demonstrators were nonfamiliar conspecifics from different cages (reverse pair setting). Based on the results of the tube test, two demonstrator groups were established: dominant (Dom-Dem-Non;  $n = 8$ ) and subordinate (Sub-Dem-Non;  $n = 5$ ). Nonfamiliar stranger demonstrators from different cages are paired with observers. (B, C) There was no significant difference in the observer's freezing toward a nonfamiliar demonstrator

Further, there was no difference in freezing responses between the two different social rank groups of demonstrators (Figure S3B;  $T_{[9,081]} = 1.646$ ,  $p = 0.1340$ , Welch's  $t$ -test, two-tailed).

### 3.4 | Dominance of a demonstrator is not a sufficient condition to modulate the empathic fear response

The effect of the demonstrator's social rank on the observer's OFL response might be naively interpreted as the result of the changes in the demonstrator's characteristics, as mentioned earlier. However, suppose these variations in the individual intrinsic characteristics of the demonstrators according to their social rank are necessary and sufficient conditions to induce different levels of empathic fear responses from observers. In that case, similar group differences should still be observed depending on the social rank regardless of the familiarity or prior social experience (e.g., the interrelationship between demonstrator and observer).

Unfamiliar demonstrators from different cages were paired with observers of intermediate social rank to analyze the issue in the context of prior social relationships or familiarity. Except that the previous social experiences between observers and demonstrators were excluded by employing unfamiliar pairs from different cages, all of the other experimental conditions were the same as before. Two groups of demonstrators with different social ranks were paired with observers of intermediate social rank: nonfamiliar dominant demonstrator (Dom-Dem-Non;  $n = 7$ ) or subordinate demonstrator (Sub-Dem-Non;  $n = 7$ ) from different cages. Unlike the results of the behavioral assay in the familiar pair condition, there was no difference in the observers' empathic fear responses per the demonstrators' social rank when there

were no prior social experiences with one another (Figure 4C;  $T_{[11,97]} = 0.4890$ ,  $p = 0.6337$ , Welch's  $t$ -test, two-tailed). This result indicates that the dominant social rank of the demonstrator itself is not a sufficient condition to induce a higher level of empathic response from the observer. There was no difference in freezing response between the two different social rank groups of demonstrators (Figure S3D;  $T_{[11,00]} = 1.411$ ,  $p = 0.1859$ , Welch's  $t$ -test, two-tailed).

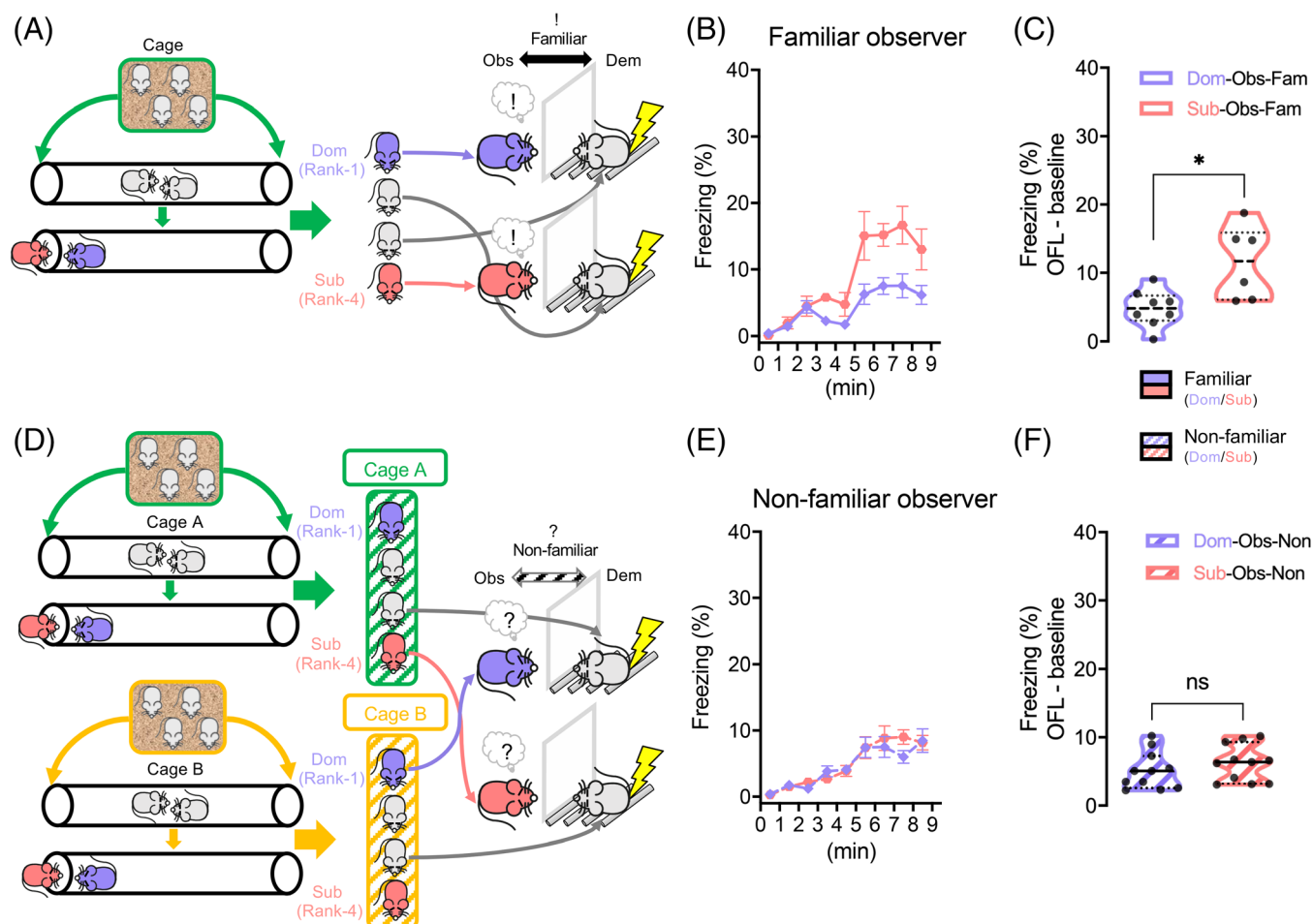
### 3.5 | Empathic fear response is modulated by the social rank of an observer only in the context of familiarity

Observers of intermediate social ranks distinguished the two groups of demonstrators of different social ranks (Sub/Dom) by showing significant differences in empathic responses to each group, and this was only valid for familiar cagemate pairs with prior social experiences (e.g., home-cage and tube test). As with the demonstrator's social rank, the observer's social rank could be relevant to the level of emotional empathic response as aforementioned.

Two different groups of "Dom" and "Sub" mice were used as observers and paired with the demonstrators of an intermediate social rank from the same cage to determine the effect of the observer's social rank on the OFL response. Compared with the "Sub" group observers, the "Dom" group observers (Dom-Obs-Fam;  $n = 8$ ) showed significantly deficient empathic responses to the familiar demonstrators of the intermediate social rank from the same cage (Sub-Obs-Fam;  $n = 6$ ) (Figure 5C;  $T_{[6,888]} = 2.813$ ,  $p = 0.0265$ , Welch's  $t$ -test).

Demonstrators of an intermediate rank group from different cages were paired with an observer from two groups of "Dom" (Dom-Obs-Non;  $n = 11$ ) or "Sub" (Sub-Obs-Non;  $n = 11$ ) to exclude prior social





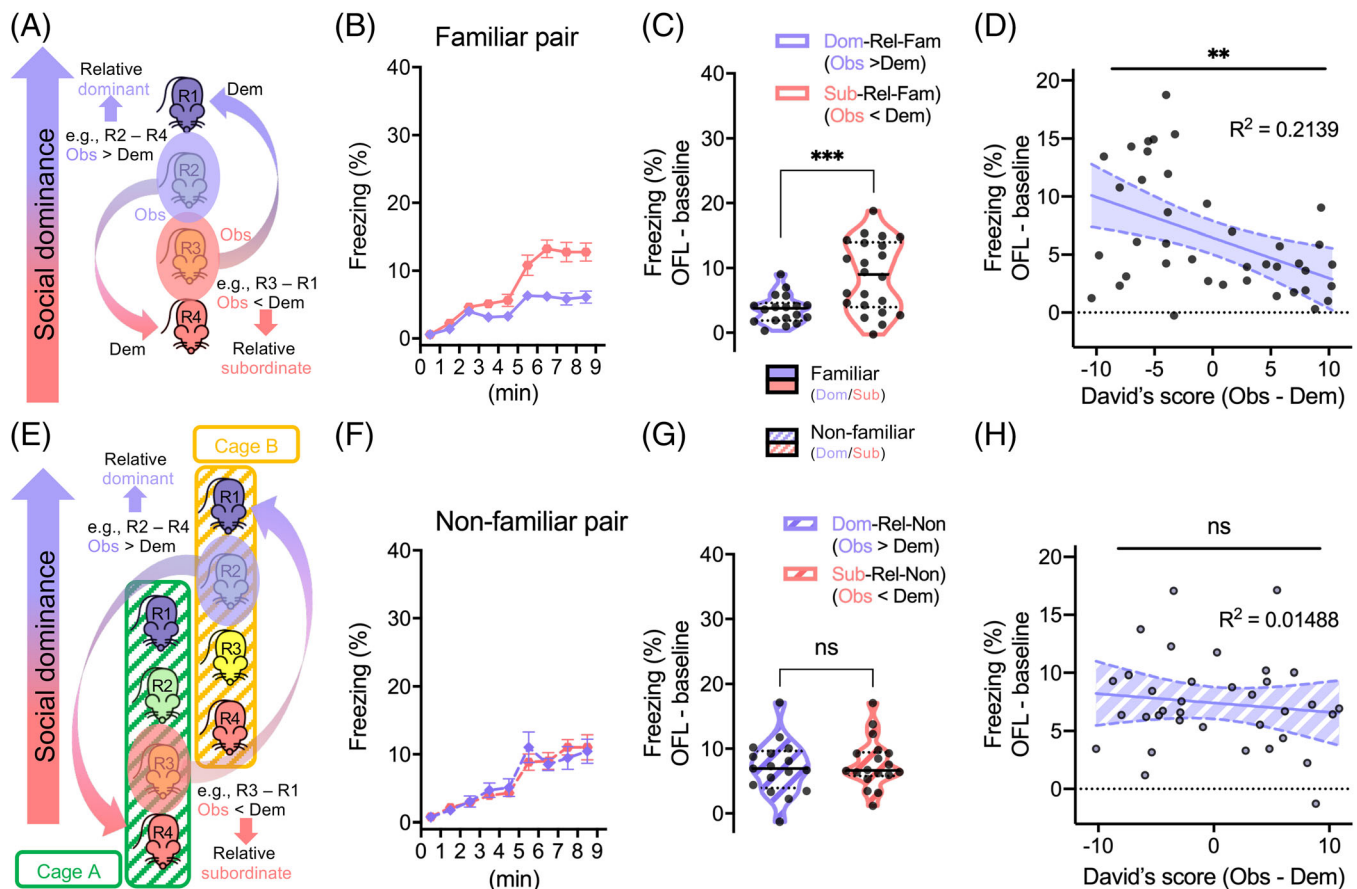
**FIGURE 5** Subordinate observers show elevated empathic fear response toward familiar demonstrators but not nonfamiliar demonstrators. (A, D) Illustration of the two behavioral analysis. Similar to Figure 3A and 4A, except that the rank-pair setting is reversed. Based on the results of the tube test, two observer groups were established: dominant (Dom-Obs-Fam) and subordinate (Sub-Obs-Fam). (A) Familiar cagemate demonstrators are paired with observers. (B, C) Compared with dominant observers (Dom-Obs-Fam;  $n = 8$ ), subordinate observers (Sub-Obs-Fam;  $n = 6$ ) showed higher freezing toward familiar demonstrators. (D) Nonfamiliar conspecific demonstrators from different cages are paired with dominant (Dom-Obs-Non;  $n = 10$ ) or subordinate (Dom-Obs-Non;  $n = 12$ ) observers. (E, F) There was no significant difference in the observer's freezing toward a nonfamiliar demonstrator

interrelationship experience between the observer and demonstrator from the effect of the observer's social rank on OFL. There was no difference in OFL response between the two different social rank groups of observers when they had not been paired with the demonstrator before and therefore were not familiar with the demonstrator (Figure 5F;  $T_{[20,00]} = 0.9940$ ,  $p = 0.3321$ , Welch's  $t$ -test, two-tailed). This indicates that previous social relationship or familiarity between observer and demonstrator is necessary for the observer to show different levels of OFL response depending on the social dominance rank.

### 3.6 | Relative social dominance of an observer with respect to a demonstrator had a negative linear correlation with the empathic fear response

According to the behavioral results so far, the influence of the social rank on OFL was confirmed independently in each of the cases for

the observer and demonstrator. The context of prior social experience between the observer and demonstrator was necessary. More specifically, higher OFL responses were shown in two experimental conditions: when the observer and demonstrator had the social ranks of (1) intermediate and dominant or (2) subordinate and intermediate, respectively. Lower OFL responses were shown in the other two experimental conditions: when the observer and demonstrator had the social ranks of (1) intermediate and subordinate or (2) dominant and intermediate, respectively. These results are both consistent with the tendency of getting a higher OFL response when the social rank of an observer is relatively lower than that of the demonstrator (observer < demonstrator; e.g., subordinate < intermediate and intermediate < dominant). This implies that the relative social dominance interrelationship within a pair could be a critical factor affecting OFL in addition to the independent considerations of the absolute social dominance rank of each observer and demonstrator.



**FIGURE 6** Information on relative social hierarchical interrelationship toward each other without respective absolute dominance rank is enough to quantitatively predict the level of empathic fear response. (A) Classification of two groups based on the relative (Rel) dominance between observer and demonstrator. Regardless of the absolute social rank, subjects (Obs–Dem pairs) are grouped only depending on the sign of the value obtained by subtracting the demonstrator's David's score from the observer's David's score (Obs – Dem); that is, Obs > Dem: + (Dom-Rel-Fam), Obs < Dem: – (Sub-Rel-Fam). (B, C) A relatively subordinate observer (Obs < Dem;  $n = 20$ ) showed a higher OFL response than a relatively dominant observer (Obs > Dem;  $n = 18$ ). (D) Linear regression analysis. Relative dominance of the observer with respect to the demonstrator (David's score of Obs – David's score of Dem) was used as an independent variable, whereas empathic fear response of observer (OFL – baseline) was considered as the dependent variable. A significant negative linear correlation between the relative dominance score and the empathic response was observed. (E) Similar to (A) except that the pairs are nonfamiliar. Two groups are classified based on the relative dominance between the observer and demonstrator. Regardless of the absolute social rank, subjects (Obs–Dem pairs) are grouped only depending on the sign of the value obtained by subtracting the demonstrator's (from different cage) David's score from the observer's David's score; that is, Obs > Dem: + (Dom-Rel-Non;  $n = 14$ ), Obs < Dem: – (Sub-Rel-Non;  $n = 19$ ). (G) No statistical difference in nonfamiliar pair. (H) No significance in nonfamiliar pair. (D, H) 95% confidence interval around the linear regression line

Two additional approaches were applied to validate this hypothesis further. (1) The relative social dominance relationship between an observer and a demonstrator was considered a single independent variable, unlike previous analysis, which considered the social rank of the observer and demonstrator separately. (2) David's score was calculated to investigate the relationship between social interrelationship and OFL responses in a more quantitative way. <sup>14,25,38</sup>

First, we assessed if the OFL response depends on whether the observer has a relatively higher social dominance over the demonstrator. To this end, two groups were classified according to the relative social dominance of the observer over the demonstrator. The relative (“Rel”) dominance was set based on the sign of the value obtained by subtracting the demonstrator's social dominance score (David's score) from the observer's social dominance score regardless of the absolute

social rank within a cage; that is, Obs > Dem: + (Dom-Rel-Fam;  $n = 18$ ), Obs < Dem: – (Sub-Rel-Fam;  $n = 22$ ). The two groups of different relative social dominance conditions showed statistically significant differences in the level of OFL responses (Figure 6C;  $T_{[28.85]} = 3.985$ ,  $p = 0.0004$ , Welch's  $t$ -test, two-tailed). Consistent with the previous results, which independently considered the absolute social rank of each observer and demonstrator, the observer with relatively lower social dominance over the demonstrator showed a higher level of OFL response, and vice versa. In a nonfamiliar pair, there was no statistical difference between Obs > Dem: + (Dom-Rel-Non;  $n = 17$ ) and Obs < Dem: – (Sub-Rel-Non;  $n = 18$ ) groups (Figure 6G;  $T_{[32.36]} = 0.4662$ ,  $p = 0.6442$ , Welch's  $t$ -test, two-tailed).

Next, the level of relative social hierarchy was considered on a continuous scale. The binary classifications such as Dom versus Sub

and Obs > Dem versus Obs < Dem were enough to show significant differences in the OFL responses. However, it has been reported that the social hierarchy within a particular group is not always composed simply of equal intervals or units.<sup>4,14</sup> This suggests that the hierarchical effect might not be binary or all-or-none but rather be on a continuous scale, thereby explaining the phenotypic variation in empathic responses. To reflect these details, David's score was used to calculate the dominance score of the observer for the demonstrator within a cage. The level of the relative social hierarchy of the observer over the demonstrator was determined by subtracting David's score of the demonstrator from that of the observer. Finally, regression analysis was performed with the level of the relative social dominance (David's score of observer – David's score of demonstrator) as an independent variable representing the difference of social dominance between the observer and demonstrator and the level of OFL response as a dependent variable. The results of regression analysis revealed a significant negative linear correlation between the relative social hierarchy level and the OFL response level (Figure 6D; linear regression,  $F_{(1,38)} = 10.34$ ,  $p = 0.0027$ ,  $R^2 = 0.2139$ ,  $Y = -0.3487 \cdot X + 6.462$ ,  $n = 40$ ); the lower the observer's social hierarchy relative to the demonstrator's, the higher the observer's level of OFL response. This finding is consistent with the results of the behavioral experiments described above and further provides a general negative correlation between the relative social hierarchy and the OFL response on a continuous scale. No statistical significance was observed in nonfamiliar pairs (Figure 6H; linear regression,  $F_{(1,33)} = 0.4983$ ,  $p = 0.4852$ ,  $R^2 = 0.01488$ ,  $Y = -0.07977 \cdot X + 7.422$ ,  $n = 35$ ).

## 4 | DISCUSSION

This study demonstrated a relationship between a mouse's rank in a social hierarchy and affective empathic behavior in the context of familiarity, that is, prior social interrelationship. Observers of intermediate social ranks showed elevated OFL responses with demonstrators of relatively higher social dominance, which corroborates the findings of a previous study demonstrating a relationship between dominance and social transmission of fear.<sup>17</sup> The OFL responses of subordinate observers were also higher than those of dominant observers in the presence of intermediate demonstrators.

However, intermediate observers did not distinguish between dominant and subordinate stranger demonstrators from different cages, and a similar trend was observed in a reverse setting; no difference was noted in dominant and subordinate observers with stranger intermediate demonstrators. This result corroborates the findings of a previous study showing that the subordinate deer mice observer displayed better vicarious social learning of defensive responses to biting flies than the dominant observer only in the presence of a familiar demonstrator.<sup>39</sup> Thus, although familiarity bias was not significant in the nociceptive response (i.e., more closely related to affective empathy paradigm) from the reference, these independent experimental approaches across various species with different behavioral paradigms seem to result in a similar conclusion; familiarity bias is critical for the

hierarchical effect on the behaviors under the umbrella of empathy. These findings strengthen the hypothesis that not intrinsic attributes but the prior social interrelationship is a significant factor influencing the appropriate processing of social information.

This better coincides with the view of utility maximization through role distribution during hierarchy formation within a social group<sup>19,40</sup> than the aforementioned simple interpretation based on intrinsic attributes. Under a stable social hierarchy, the subordinate may avoid the fight and minimize their energy consumption in exchange for conceding priority of resources.<sup>19,41</sup> In exchange for gaining priority of resources, the dominant may be obligated to inform and respond to external potential threats on behalf of the group members.<sup>17</sup> Hence, only with tacit mutual consent based on the prior social interrelationship between them, the subordinate observer might show a higher OFL response based on elevated social sensitivity to the demonstrator's aversive social alert signal that could imply putative external threat.

This study is the first to demonstrate that an extrinsic factor (familiarity; i.e., acquired social interrelationships) between two individuals (observer and demonstrator) rather than an intrinsic factor of each individual is crucial to the hierarchical effects on emotional empathic responses. Although most previous studies on social hierarchy focused on intrinsic factors, such as neuroendocrine regulation or neural circuit, a notable review presented an intrinsic–extrinsic factor framework in the realm of dominance hierarchy research.<sup>1</sup> In addition, the literature mentioned the “winner effect” as a rare but noteworthy behavioral example representing an extrinsic factor in dominance hierarchy.<sup>1,42</sup> Additionally, our study contributes to resolving the imbalance between the tests of intrinsic/extrinsic factors (relative lack of examples of extrinsic factors versus intrinsic factors) by providing additional evidence that highlights familiarity, or prior social interrelationship, as one of the extrinsic factors in social hierarchy studies.

Furthermore, the recently published intensive meta-analysis on emotional empathy reported several controversial results, particularly concerning familiarity.<sup>43</sup> Concerning this issue, our study emphasizes the necessity to consider social hierarchy in addition to familiarity and therefore inspires an appropriate experimental design for further studies to establish a unified framework for the investigation of affective empathy.

In such context, regardless of the absolute dominance rank of the observer and demonstrator, observers were classified into two groups only depending on the relative social hierarchy of the observer over the familiar demonstrator. This revealed a consistent tendency of the observer groups of relatively lower social hierarchy to show higher OFL responses. We further showed that the difference in relative social hierarchy level between the observer and demonstrator quantified by David's score has a significant negative linear relationship with the level of OFL response of the observer to the demonstrator. The results of regression analysis suggest that the hierarchical effect is not simply all-or-none—responsive or not—but is proportional to the relative social hierarchy within a pair reflecting the prior social interrelationship between the individuals.

Here, we investigated almost all possible combinations of social rank pairs of observer-demonstrator, except the extreme social rank pairs (rank 1–rank 4, or vice versa). If the negative linear correlation between relative dominance and affective empathy shown here is a more ubiquitous phenomenon, extreme social rank pairs will also be aligned with the linear regression line, distributed at both ends of the line in the result (from Figure 6D). However, several studies (including results Section 3.2 of this article) have reported that social rank formation within a cage does not always correspond to the linear characteristic rather nonlinear and could vary depending on the cage environment.<sup>4,14,42</sup> Further study with extreme social rank pairs in various social environmental conditions could be a challenging but promising and valuable approach to elucidate this issue more clearly.

In the present study, we refrained from thoroughly scrutinizing the concept of development of social interrelationship itself, for example, what happens during cohousing in home-cage or tube test and how it affects the OFL response. Herein, several questions could be raised; to what extent could mice be affected by prior social experiences? Could a 3-day tube test sufficiently and reliably result in the formation of familiarity and social hierarchy relationships with unfamiliar stranger mice and reflect them as a difference in affective empathy in behavioral responses? According to most of the previous literature, cohousing for a long-term period of >3–10 weeks is necessary to induce familiarity-based enhancement of emotional empathic responses.<sup>10,11</sup> However, contrary to the previous consensus, a recent report showed that a short period of 3 days of excessive social experiences accompanying physical contact with an unfamiliar stranger was enough to result in an increased level of emotional contagions, reflecting the formation of familiarity.<sup>44</sup>

In line with this, it has not yet been examined how rodents process and acquire dynamic social information and how much complexity they can handle. Because studying the dominance hierarchy in a social environment of >5 mice per cage is usually tricky without congested housing issues, these are intriguing but difficult questions to answer. Several phenomenal studies suggested an alternative breakthrough for investigating complex social hierarchies. Up to 12 mice were reported to show linear and stable dominance rank under the environment of large vivaria.<sup>45</sup> Moreover, under more laboratory-friendly conditions, a stable social rank of up to eight mice could be established when the tube test is repeatedly applied with mice from different cages with tetrad home-cage conditions.<sup>46</sup> This recent progress in social behavioral studies may enable more advanced approaches to unveil the underlying mechanisms of complex social dynamics, which were previously difficult to explore in detail.

## 5 | CONCLUSION

In this study, we verified the effect of hierarchy on emotional empathic behavior in mice using the tube test and OFL behavioral paradigm and clarified that it is not the intrinsic attributes of an individual but the prior social interrelationship between the individuals that determine the effect of social hierarchy on emotional empathic

responses. We also quantitatively presented the negative linear correlation between the relative social hierarchy of the observer to the demonstrator and the OFL response of the observer. By considering the combinations of several social contexts, for example, familiarity, social hierarchy, and prior social interrelationship, more carefully as putative crucial factors for various social behaviors, we will better understand the complex social behavior and underlying mechanisms. In the social behavior research field, it would be possible to identify consistent influential factors explaining phenotypic variations that were previously unrecognized or uncontrollable. Consequently, we could further uncover subtle but definite meanings from what was previously considered as mere meaningless experimental noise to be excluded.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## DATA AVAILABILITY STATEMENT

Data reported in this study are available from the corresponding author upon request.

## GLOSSARY

OFL	Observational fear learning
ANOVA	one-way analysis of variance
Obs	observer
Dem	demonstrator
Fam	familiar
Non	non-familiar
Sub	subordinate
Dom	dominant
Rel	relative

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