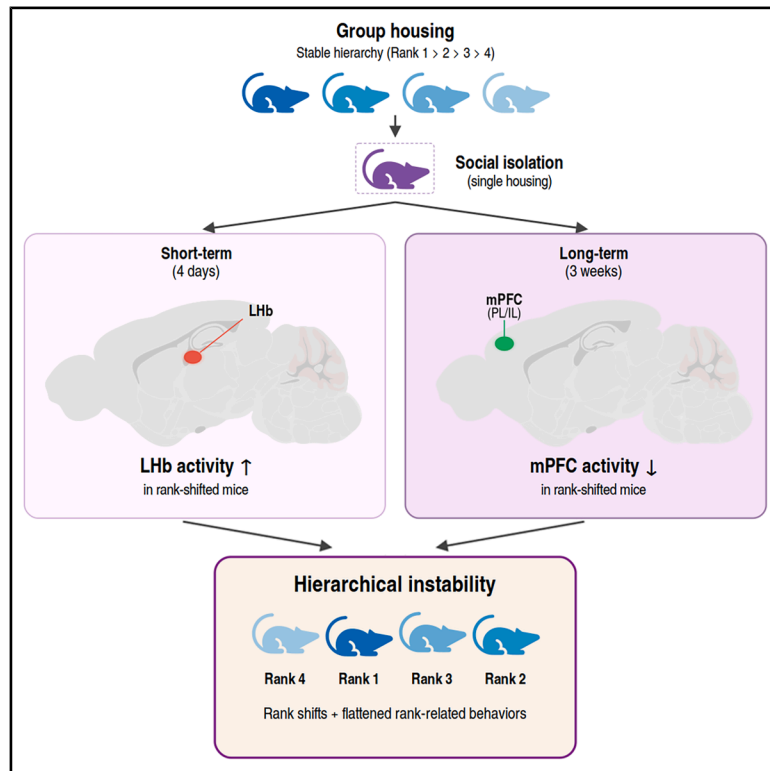


# Region-specific neural activity changes and hierarchical instability following social isolation

## Graphical abstract



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## In brief

Neuroscience; Behavioral neuroscience;  
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## Highlights

- Social isolation disrupts hierarchical stability in male mice
- Isolation flattens rank-related push and retreat behaviors in mice
- Region-specific c-Fos changes accompany isolation-driven rank shifts
- Short- and long-term isolation engage distinct regions during rank shifts



## Article

# Region-specific neural activity changes and hierarchical instability following social isolation

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## SUMMARY

Social hierarchy contributes to maintaining group stability and coordination; yet, how social isolation affects hierarchical dynamics remains poorly understood. Although social isolation is a potent psychosocial stressor, its influence on social rank, stability, and the underlying neural mechanisms has not been clearly delineated. We established two complementary tube-test-based behavioral paradigms to investigate how isolation modulates rank status, social stability, and competitive behaviors: a rank change model assessing pre- and post-isolation rank shifts and a daily rank-trace model capturing gradual changes over time. Behavioral analyses revealed attenuated distinctions in push and retreat behaviors following isolation. c-Fos mapping demonstrated region-specific neural responses: medial prefrontal cortex activity decreased in mice that advanced in rank after long-term isolation, whereas lateral habenula activity increased in the same group following short-term isolation. Collectively, social isolation is associated with disrupted hierarchical stability and distinct, region-specific patterns of neural activity in brain areas linked to social behavior.

## INTRODUCTION

Social hierarchy represents a structured system of relationships that organizes individuals within a group, promoting social stability, optimizing resource allocation, and facilitating coordinated behavior.<sup>1,2</sup> Once established, social hierarchies tend to remain stable but can, nonetheless, be altered by external factors such as the formation of new groups, exposure to physical or psychological stress, and changes in group dynamics.<sup>3–5</sup> Among various approaches to study organization, animal models of social stress have proven particularly valuable for experimentally dissecting fluctuations in social rank.<sup>6</sup>

Among different forms of social stress, social isolation, defined as the deprivation of social interactions, has received increasing attention due to its ecological relevance and profound psychological consequences.<sup>7,8</sup> Social isolation is observed under naturalistic conditions, such as during pandemics, or in pathological human states including depression.<sup>9</sup> However, its reported effects on social hierarchy are inconsistent. Some studies associate isolation with reduced cortisol levels and subsequent subordinate behavior,<sup>7,10</sup> whereas others reported increased aggression and enhanced dominance behavior.<sup>11</sup> These discrepancies suggest that baseline social rank, the duration of isolation, and the resulting neurobiological changes are jointly associated with the direction and magnitude of behavioral outcomes. In this study, we operationalized social isolation as single

housing—in which mice were individually housed without visual or tactile contact with conspecifics while auditory and olfactory cues were maintained—and systematically evaluated how isolation conditions modulate social hierarchy.

Social isolation is generally associated with broad changes in social behavior, which may subsequently lead to reorganization of hierarchical relationships.<sup>12,13</sup> Behavioral alterations such as increased anxiety, social withdrawal, and reduced social interaction are often interpreted as indicators of diminished social competitiveness, potentially leading to rank shifts.<sup>11,14</sup> However, the association between behavioral changes and hierarchy reorganization appears to depend critically on the duration of isolation. Extensive studies have shown that long-term isolation can markedly alter social rank when assessed using the rank change models,<sup>15</sup> whereas short-term isolation is commonly considered insufficient to induce such hierarchical modifications.<sup>16</sup> Although some investigations have reported rank alterations following short-term isolation under specific experimental conditions,<sup>13,17</sup> these findings have not been consistently replicated. This variability suggests that the influence of social isolation on hierarchical structure is not a simple function of isolation itself but rather modulated by its temporal characteristics. Recognizing isolation duration as a critical regulatory factor offers a refined conceptual framework for understanding the stability and plasticity of social hierarchies under environmental perturbations.<sup>17</sup>



Baseline social rank serves as a key determinant of social stress,<sup>18</sup> influencing stress reactivity,<sup>19</sup> neurobiological sensitivity, behavioral flexibility, and adaptability capacity.<sup>20</sup> However, the effects of social isolation must be interpreted within the broader context of social stability. Stable hierarchies buffer stress and promote predictable social interactions, whereas unstable or frequently reorganizing structures amplify uncertainty, vigilance, and vulnerability to stress-related outcomes.<sup>21</sup> Consequently, the impact of isolation may depend on an individual's initial rank, the stability of the preceding hierarchy, and whether rank shifts occur during or after isolation. Comparing conditions with and without rank change, while accounting for baseline rank and group stability, is, therefore, essential for dissecting the heterogeneous effects of isolation.

Several brain regions have been implicated in the regulation of social hierarchy. The medial prefrontal cortex (mPFC) plays a central role in the formation and maintenance of hierarchical relationships,<sup>22</sup> while the lateral habenula (LHb) regulates dominant and submissive behaviors and exhibits high sensitivity to social stress.<sup>23,24</sup> The mPFC contributes to self-referential processing and social decision-making, enabling individuals to evaluate their relative social position and modulate behavior accordingly.<sup>25</sup> Conversely, the LHb is particularly responsive to social defeat and isolation stress, potentially becoming hyperactive and leading to evaluated subordinate responses. Both regions dynamically adjust their activity in response to social competition and status transitions.<sup>24,26</sup> Additional brain areas, including the amygdala, nucleus accumbens (NAc), and hippocampus, contribute to the broader network supporting social behavior and hierarchy modulation.<sup>27</sup> The basolateral amygdala supports social recognition and emotional processing; under stress, its hyperactivity may increase threat perception and reduce dominance-related behaviors.<sup>28</sup> The NAc integrates motivational and reward-related signals that drive social approach behaviors, while reduced dopaminergic activity following isolation may diminish social motivation, fostering subordinate outcomes.<sup>29</sup> The ventral hippocampus contributes to contextual and social memory processing.<sup>30</sup> Together, these regions constitute an interconnected social-emotional network integrating cognitive, motivational, and affective components of social behavior. Altered activity across this network, particularly in the mPFC, LHb, amygdala, NAc, and hippocampus, has been observed following social isolation,<sup>31,32</sup> suggesting that functional reorganization within this circuitry may be associated with isolation-related modification in social hierarchy.

In this context, the present study aimed to elucidate how different durations of social isolation influence social hierarchy and the associated neural mechanisms. Specifically, we (1) implemented short-term and long-term isolation to examine temporal effects, (2) quantified baseline rank and rank transitions using the dominance tube test and David's score during and after isolation, and (3) assessed neural activation patterns in the mPFC and LHb using c-Fos expression as an indicator of neural activity. This multi-level approach was designed to clarify the interactions among isolation duration, baseline rank characteristics, and neural activation, thereby providing an integrative understanding of how social stress shapes hierarchical dynamics.

## RESULTS

### Long-term social isolation disrupts the pre-established social hierarchy

Social isolation can disrupt the social interactions of mice, which are inherently social animals, and influence their pre-established social status by housing them alone instead of under group housing conditions. Four mice of same sex with similar age and weight were grouped together and housed for 2 weeks before conducting the tube test (tube test I). Most groups formed a stable social hierarchy within the group, which was determined using the total number of wins and David's score. After a 3-week social isolation or group housing period, the tube test (tube test II) was performed again to assess changes in the previously established hierarchy, followed by brain activation measurement through perfusion 1 h later (Figure 1A). The tube test was conducted 5 times per session, alternating sides to ensure fairness, using the round-robin rule so that all mice competed at least once. Mice ranked 1<sup>st</sup> and 2<sup>nd</sup> were defined as dominant, while those ranked 3<sup>rd</sup> and 4<sup>th</sup> were defined as subordinate (Figure 1B). When assessing rank change proportions under group housing conditions, 75% of mice showed no rank change (Figure 1C). A detailed rank-by-rank breakdown of these transitions is provided in Table S1, showing the destination rank for each pre-isolation rank group. A representative graph tracking trial-by-trial changes between tube tests I and II revealed that most mice maintained their rank (Figure 1D). Complete trial-by-trial rank trajectories of all four mice from every individual cage in the group-housed condition are provided in Figure S1 (left). Within-animal paired analyses confirmed that both rank 1 and rank 4 mice maintained their positions between tube tests I and II (rank 1:  $W = 3$ ,  $p = 0.500$ ,  $n = 4$ ; rank 4:  $W = 0$ ,  $p = 1.000$ ,  $n = 4$ ), with between-rank comparisons revealing consistent separation at both time points (Figure 1E; test I: Mann-Whitney  $U = 0$ ,  $p = 0.029$ ,  $r_{rb} = 1.00$ ; test II:  $U = 0$ ,  $p = 0.029$ ,  $r_{rb} = 1.00$ ). Tracking individual David's scores showed that the hierarchical difference between rank 1 and rank 4 was robustly preserved between tube tests I and II, with a strong main effect of group ( $F(1, 6) = 74.06$ ,  $p < 0.001$ ,  $\eta^2_p = 0.925$ ), a modest main effect of time ( $F(1, 6) = 6.755$ ,  $p = 0.041$ ,  $\eta^2_p = 0.530$ ), and no significant group  $\times$  time interaction ( $F(1, 6) = 1.574$ ,  $p = 0.256$ ,  $\eta^2_p = 0.208$ ) (Figures 1F and 1G; test I: Mann-Whitney  $U = 0$ ,  $p = 0.029$ ,  $r_{rb} = 1.00$ ; test II:  $U = 0$ ,  $p = 0.029$ ,  $r_{rb} = 1.00$ ). Results based on the number of wins also supported this finding (Figures 1H and 1I; test I: Mann-Whitney  $U = 0$ ,  $p = 0.029$ ,  $r_{rb} = 1.00$ ; test II:  $U = 0$ ,  $p = 0.029$ ,  $r_{rb} = 1.00$ ). Within-subject Wilcoxon signed-rank tests confirmed stable win counts between tube tests I and II in both rank 1 ( $W = -4$ ,  $p = 0.500$ , median  $\Delta = -0.6$ ,  $n = 4$ ) and rank 4 mice ( $W = -10$ ,  $p = 0.125$ , median  $\Delta = -0.4$ ,  $n = 4$ ). In contrast, for mice subjected to social isolation (single housing), the rank change proportions were markedly different. Here, only 45% of the mice retained their rank, while 55% exhibited changes in rank (Figure 1J). The detailed rank-by-rank transition pattern under single housing is presented in Table S1. A representative graph revealed rank shifts between tube tests I and II (Figure 1K; full per-cage trajectories of all four ranks are shown in Figure S1, right). Within-animal paired analyses revealed marked rank changes between tube tests I and II, with rank 1 mice showing a consistent tendency to descend

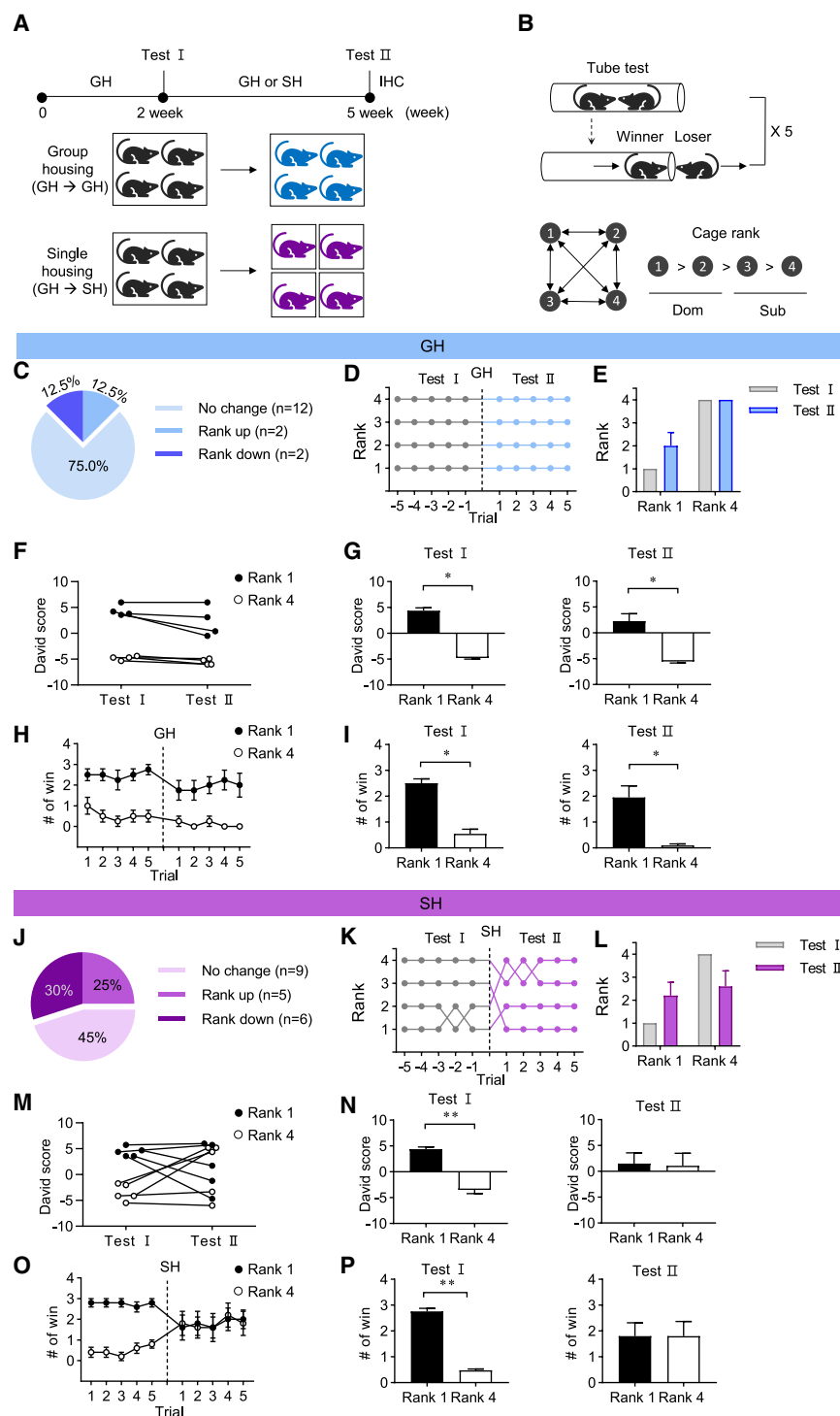


**Figure 1. Social isolation changes established rank**

(A) Schematic illustration of social isolation rank change model.

(B) Graphic scheme of tube test and rule of matching in the cage with classifying social hierarchy.

(C) Proportion of rank change spectrum in group housing.

(D) Representative rank trajectories of rank 1 and rank 4 mice before and after housing manipulation (group housing or single housing), illustrating the stability (group-housed) or reorganization (single-housed) of the hierarchical extremes across tube tests I and II.

(E and F) Rank positions of rank 1 and rank 4 mice between tube tests I and II under group housing, demonstrating the stability of hierarchical extremes (all bar graph  $n = 4$ ) (F) David's score between tests I and II in ranks 1 and 4.

(G) Difference of David's score between ranks 1 and 4 in tests I and II.

(H) Number of wins between ranks 1 and 4 in each trial (all bar graph  $n = 4$ , Mann-Whitney U test,  $*p < 0.05$ ).

(I) Difference of number of wins between ranks 1 and 4 in tests I and II (all bar graph  $n = 4$ , Mann-Whitney U test,  $*p < 0.05$ ).

(J) Proportion of rank change spectrum in single housing.

(K) Representative rank trajectories of rank 1 and rank 4 mice across 5 trials during tube test I (pre-single housing) and tube test II (post-single housing), illustrating the reorganization of hierarchical extremes under single housing conditions. (L) Rank positions of rank 1 and rank 4 mice between tube tests I and II under single housing (all bar graph  $n = 5$ ).

(M) David's score between tests I and II in ranks 1 and 4.

(N) Difference of David's score between ranks 1 and 4 in tube tests I and II (all bar graph  $n = 5$ , Mann-Whitney U test,  $**p < 0.01$ ).

(O) Number of wins between ranks 1 and 4 in each trial.

(P) Difference of number of wins between ranks 1 and 4 in tube tests I and II (all bar graph  $n = 5$ , Mann-Whitney U test,  $**p < 0.01$ ). Data are presented as mean  $\pm$  SEM.

(3/5 mice shifted from rank 1 to ranks 2–4;  $W = +6$ ,  $p = 0.250$ ,  $r_{rb} = +0.40$ ,  $n = 5$ ) and rank 4 mice showing the opposite pattern (2/5 mice ascended from rank 4 to ranks 1–3;  $W = -3$ ,  $p = 0.500$ ,  $r_{rb} = -0.20$ ,  $n = 5$ ), consistent with rank reversal following social isolation. Between-rank comparisons showed clear separation at baseline that was diminished at test II (Figure 1L; test I:

Mann-Whitney  $U = 0$ ,  $p = 0.008$ ,  $r_{rb} = 1.00$ ; test II:  $U = 7$ ,  $p = 0.222$ ,  $r_{rb} = +0.44$ ). Tracking David's scores revealed substantial changes between tube tests I and II in socially isolated mice, with the pre-established hierarchy between rank 1 and rank 4 disrupted at test II. A two-way repeated-measures ANOVA revealed a significant group  $\times$  time interaction ( $F(1, 8) = 8.403$ ,  $p = 0.020$ ,  $\eta^2_p = 0.512$ ), indicating that rank 1 and rank 4 mice exhibited divergent score trajectories across the two tests, while the main effects of time ( $F(1, 8) = 0.438$ ,  $p = 0.527$ ,  $\eta^2_p = 0.052$ ) and group ( $F(1, 8) = 4.773$ ,  $p = 0.060$ ,  $\eta^2_p = 0.374$ ) did not reach statistical significance (Figures 1M and 1N; test I: Mann-Whitney  $U = 0$ ,

$p = 0.008$ ,  $r_{rb} = 1.00$ ; test II:  $U = 10$ ,  $p = 0.643$ ,  $r_{rb} = 0.20$ ). Similarly, results based on the number of wins showed that previous differences observed in test I were no longer evident in test II (Figures 1O and 1P; test I: Mann-Whitney  $U = 0$ ,  $p = 0.008$ ,  $r_{rb} = 1.00$ ; test II:  $U = 12$ ,  $p = 0.984$ ,  $r_{rb} = 0.04$ ). Within-subject Wilcoxon signed-rank analyses revealed directional win count reversal under single housing: rank 1 mice showed a consistent decrease ( $W = -12$ ,  $p = 0.188$ , median  $\Delta = -0.8$ ,  $n = 5$ ; 4 of 5 mice decreased), while rank 4 mice showed a consistent increase ( $W = +13$ ,  $p = 0.125$ , median  $\Delta = +1.6$ ,  $n = 5$ ; 5 of 5 mice increased). Together, these results show that long-term single housing was associated with significant rank changes compared with group housing.

### Social isolation induces coordinated, rank-specific changes in competitive behaviors

Competitive behavior can serve as one of the indicators for determining rank beyond simply deciding wins and losses in tube tests.<sup>28</sup> Competitive behavior consists of push, resist, and retreat, and in cases of closely matched ranks, resist may be more frequent, alongside other behaviors. In contrast, when ranks are clearly defined, each hierarchy exhibits representative behaviors more prominently.<sup>21,33</sup> In the tube test, two mice enter from opposite ends of the tube and meet at the center. To determine their rank, they engage in a standoff where neither move, defined as resist, followed by push (pushing the opponent), or retreat (being pushed out) (Figure 2A). Competitive behaviors were measured twice, in tube tests I and II, and stillness (time without behavioral judgment) was recorded separately. The duration of each behavior during the experimental period was measured (Figure 2B). Under group housing conditions, during tube test I, which initially established the hierarchy, rank 1 showed higher levels of push and lower levels of retreat compared to rank 4 ( $t(6) = 3.609$ ,  $p = 0.011$ ,  $d = 2.55$ ;  $t(6) = 0.931$ ,  $p = 0.388$ ,  $d = 0.66$ ;  $t(6) = 3.476$ ,  $p = 0.013$ ,  $d = 2.46$ ) (Figures 2C–2E). In tube test II, which tracked ranks over time, resist also showed significant differences, while push and retreat maintained the previously observed differences ( $t(6) = 3.090$ ,  $p = 0.021$ ,  $d = 2.18$ ;  $t(6) = 2.145$ ,  $p = 0.076$ ,  $d = 1.52$ ;  $t(6) = 4.760$ ,  $p = 0.003$ ,  $d = 3.37$ ) (Figures 2F–2H). Under group housing conditions, the differences in push and retreat between rank 1 and rank 4 observed in tube test I were similarly present in tube test II, with no marked differences in any of the three behaviors between the two time points at the group level. Under single housing conditions, tube test I was conducted before social isolation to measure competitive behavior and rank 1 and rank 4 showed differences in push and retreat ( $t(8) = 4.042$ ,  $p = 0.004$ ,  $d = 2.55$ ;  $t(8) = 0.166$ ,  $p = 0.872$ ,  $d = 0.11$ ;  $t(8) = 3.264$ ,  $p = 0.012$ ,  $d = 2.06$ ) (Figures 2I–2K). However, after 3 weeks of social isolation, within-animal paired analyses (Wilcoxon matched-pairs signed-rank test;  $n = 5$  per rank) revealed systematic behavioral changes between tube tests I and II (Figures 2L–2N). Rank 1 mice consistently decreased in both push duration ( $W = -15$ ,  $p = 0.063$ ,  $r_{rb} = -1.00$ , median  $\Delta = -0.68$  s; 5/5 mice decreased) and resist duration ( $W = -15$ ,  $p = 0.063$ ,  $r_{rb} = -1.00$ , median  $\Delta = -0.55$  s; 5/5 mice decreased), with modest, non-significant increases in retreat duration ( $W = +4$ ,  $p = 0.688$ ,  $r_{rb} = +0.27$ , median  $\Delta = +0.28$  s). In contrast, rank

4 mice showed opposite-direction changes in push ( $W = +7$ ,  $p = 0.438$ ,  $r_{rb} = +0.47$ , median  $\Delta = +0.45$  s; 3/5 mice increased) and retreat durations ( $W = -15$ ,  $p = 0.063$ ,  $r_{rb} = -1.00$ , median  $\Delta = -0.85$  s; 5/5 mice decreased), while resist duration decreased consistently ( $W = -15$ ,  $p = 0.063$ ,  $r_{rb} = -1.00$ , median  $\Delta = -0.37$  s; 5/5 mice decreased), paralleling the rank 1 pattern for this behavior. Note that with  $n = 5$  paired observations, the minimum achievable two-tailed exact Wilcoxon signed-rank  $p$  value is 0.063; the complete directional consistency (5/5 mice) and maximum effect sizes ( $|r_{rb}| = 1.00$ ) observed in the affected comparisons, therefore, represent robust evidence of systematic behavioral change despite structural limits on conventional statistical significance. Together, these within-animal analyses indicate that long-term social isolation is associated with coordinated, rank-specific changes in competitive behaviors, consistent with the hierarchical reorganization observed in the rank change model (Figure 1). The original between-rank comparisons at test II are retained in Figure S2 for completeness.

### Rank changes after social isolation are associated with reduced prelimbic c-Fos expression

To determine the relationship between brain activity and rank changes observed following social isolation, we investigated changes in related brain regions by examining c-Fos expression in targeted brain areas through immunohistochemistry. To compare the activation of these brain regions, we utilized the Quantification and Spatial Analysis of Features in Histological Images (QUINT) workflow to establish thresholds for c-Fos counting and to distinguish between ATLAS-defined brain regions (Figure 3A). For the comparison of brain region activation, we categorized individuals into no-change, rank-up, and rank-down groups on the basis of rank changes observed previously. We then compared c-Fos-positive cell counts between group housing and single housing conditions across these groups. Figure 3B presents a representative image of c-Fos immunohistochemistry in LHb and mPFC regions. The image highlights an increase in c-Fos-positive neurons in the LHb of single housing mice compared with group housing controls. We analyzed the c-Fos expression in mice under group housing and single housing conditions for the no-change, rank-up, and rank-down groups, comparing the differences between groups. When comparing c-Fos expression between the no-change and rank change groups in the LHb, PL (prelimbic cortex), and IL (infralimbic cortex) regions under group housing conditions, no significant differences were observed (region:  $F(2, 23) = 10.67$ ,  $p = 0.001$ ,  $\eta^2_p = 0.481$ ; condition:  $F(1, 23) = 5.245$ ,  $p = 0.032$ ,  $\eta^2_p = 0.186$ ; interaction:  $F(2, 23) = 1.026$ ,  $p = 0.374$ ,  $\eta^2_p = 0.082$ ) (Figure 3C). However, when the same comparison was performed under single housing conditions, only the PL region showed a significant decrease in c-Fos expression in the rank change group compared with the no-change group (region:  $F(2, 42) = 39.37$ ,  $p < 0.001$ ,  $\eta^2_p = 0.652$ ; condition:  $F(1, 42) = 9.957$ ,  $p = 0.003$ ,  $\eta^2_p = 0.192$ ; interaction:  $F(2, 42) = 3.312$ ,  $p = 0.046$ ,  $\eta^2_p = 0.136$ ). In the PL region, c-Fos expression was significantly lower in the rank change group compared with the no-change group ( $p = 0.012$ ) (Figure 3D). Next, we subdivided the rank change group into rank-up and rank-down groups

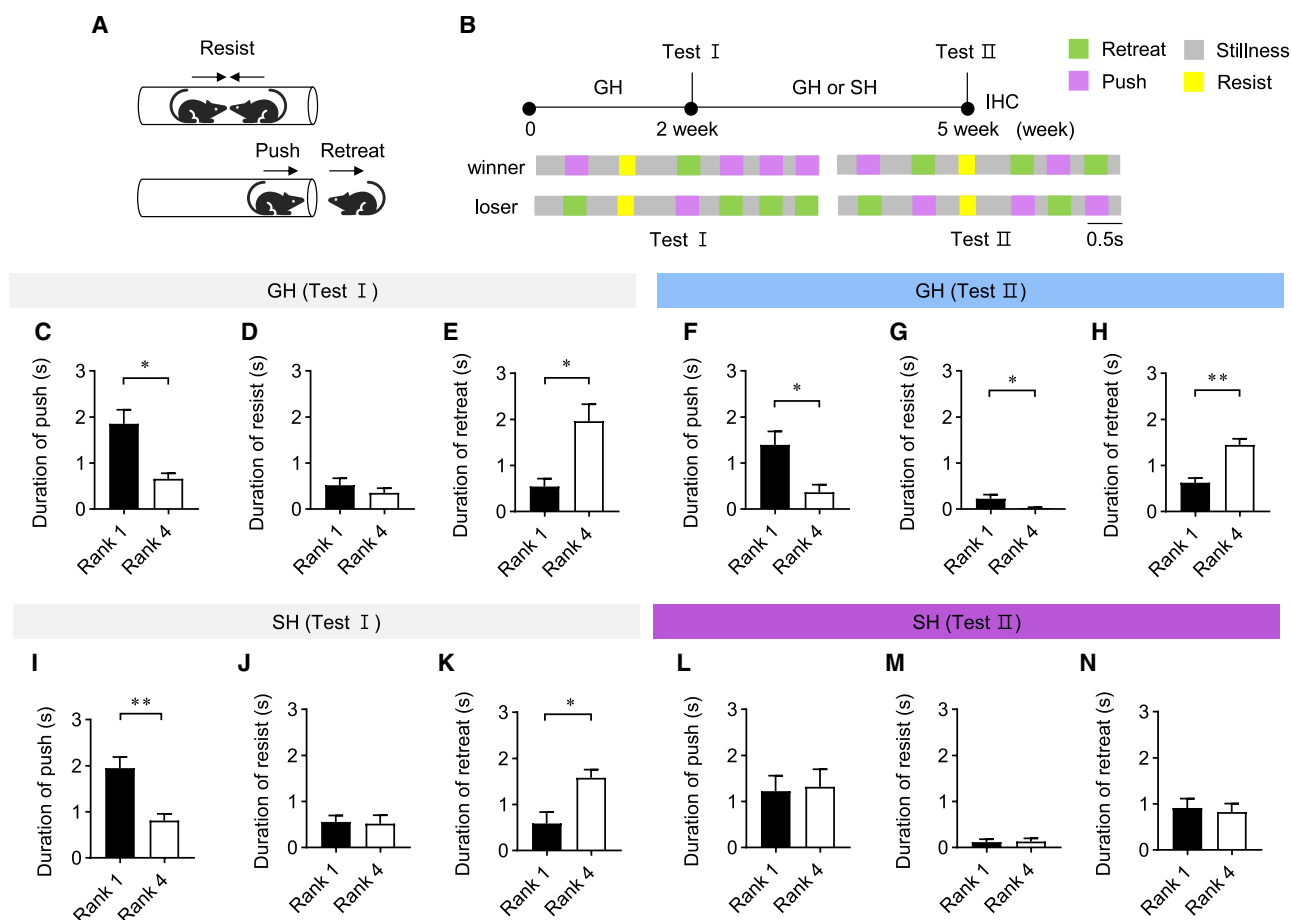


**Figure 2. Social isolation changes competitive behavior**

(A) Graphic image of competitive behavior in dominance tube test.

(B–E) Representative data of competitive behavior in tube tests 1 and 2. Comparisons in (C)–(N) are restricted to rank 1 and rank 4 mice, with each *n* representing one mouse from an independent cohort of four co-housed mice. Duration of push (C), resist (D), and retreat (E) between ranks 1 and 4 in group housing test I (all bar graph *n* = 4, unpaired *t* test, \**p* < 0.05).

(F–H) Group housing tube test II (all bar graph *n* = 4, unpaired *t* test, \**p* < 0.05, \*\**p* < 0.01).

(I–K) Single housing tube test I (all bar graph *n* = 5, unpaired *t* test, \**p* < 0.05, \*\**p* < 0.01).

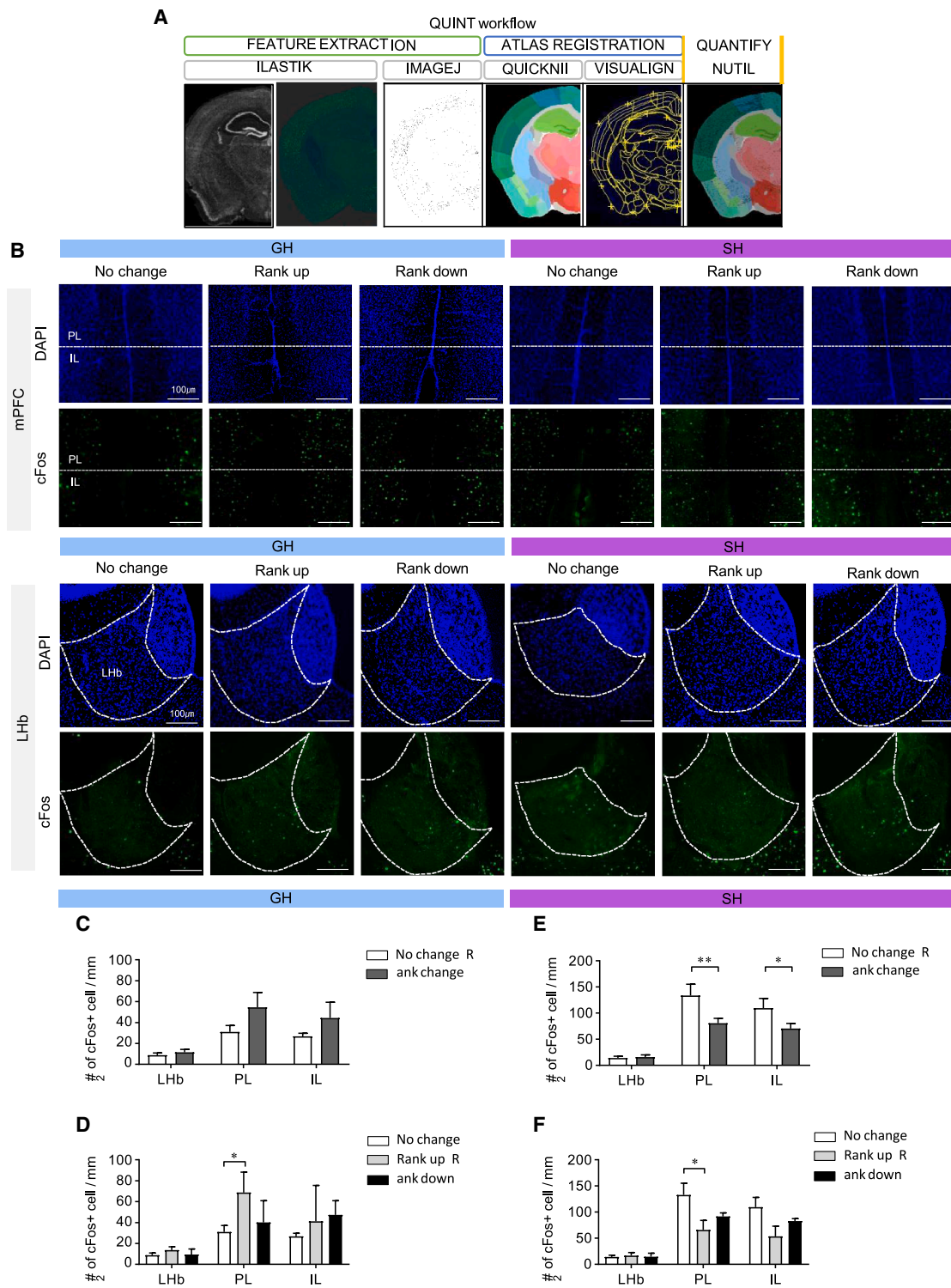
(L–N). Within-animal paired comparisons of competitive behaviors (push, resist, and retreat) between tube test I (pre-isolation) and tube test II (post-3-week single housing) in the same animals (*n* = 5 per rank; Wilcoxon matched-pairs signed-rank test, two-tailed exact). Rank-biserial correlation (*r*<sub>rb</sub>) is reported as effect size. Data are presented as mean ± SEM.

under group housing conditions and compared them with the no-change group (region:  $F(2, 20) = 10.48$ ,  $p < 0.001$ ,  $\eta^2_p = 0.512$ ; condition:  $F(2, 20) = 3.101$ ,  $p = 0.067$ ,  $\eta^2_p = 0.237$ ; interaction:  $F(4, 20) = 1.124$ ,  $p = 0.373$ ,  $\eta^2_p = 0.184$ ). In the PL region, rank-up individuals showed increased c-Fos expression compared with the no-change group ( $p = 0.037$ ) (Figure 3E). However, under the same conditions in single housing, both the PL and IL regions showed a decrease in c-Fos expression in the rank-up group compared with the no-change group (region:  $F(2, 39) = 27.68$ ,  $p < 0.001$ ,  $\eta^2_p = 0.586$ ; condition:  $F(2, 39) = 5.742$ ,  $p = 0.007$ ,  $\eta^2_p = 0.227$ ; interaction:  $F(4, 39) = 1.977$ ,  $p = 0.117$ ,  $\eta^2_p = 0.169$ ). In the PL and IL region, rank-up individuals showed decreased c-Fos expression compared with the no-change group (PL:  $p = 0.015$ ; IL:  $p = 0.047$ ) (Figure 3F). It is important to identify changes in hierarchy formed

by single housing and determine when such changes begin to manifest during the period of isolation.

### Daily rank tracking reveals hierarchical destabilization within the first four days of single housing

Previous studies have shown that the effects of social isolation can vary depending on its duration.<sup>6</sup> While our earlier experiment confirmed the changes in hierarchy due to social isolation, the exact timing of these changes remained unclear. Therefore, we aimed to pinpoint when hierarchical changes occur by conducting daily experiments using a tracking model. Initially, group housing was maintained for two weeks, during which a daily tube test was conducted to track ranks. To independently validate the temporal dynamics of rank stability, we conducted a separate follow-up experiment using an independent cohort, in which



(legend on next page)

the tube test was performed daily for 6 days during group housing and 14 consecutive days after single housing (Figure S3). This extended testing regimen confirmed progressive rank stabilization during the group-housing phase (day -6 to day -1), followed by marked destabilization within the first 3–4 days of single housing, consistent with the short-term rank dynamics observed in the main experiment. After establishing stable ranks, social isolation was implemented, and rank changes were tracked daily. The results showed that rank changes peaked on the fourth day of single housing. At this time point, perfusion was performed an hour after the tube test (Figure 4A). The tube test was conducted daily following a round-robin rule, and cage ranks were evaluated using the same method as before (Figure 4B). When tracking the rank of rank 1, it was observed that under group housing conditions, the rank remained close to rank 1. However, following isolation, rank changes occurred, and a significant increase in  $\Delta$ rank (rank change compared with day before) was observed (Figures 4C and 4D;  $t(11) = 2.865$ ,  $p = 0.015$ ,  $d = 1.65$ ). Similarly, rank 4 exhibited the same pattern of rank and  $\Delta$ rank changes (Figures 4E and 4F;  $t(11) = 2.582$ ,  $p = 0.026$ ,  $d = 1.49$ ). To further examine rank changes, we applied a stability index, which measures the difference between the previous day's rank and the following day's rank. Comparisons of rank 1 and rank 4 under group housing and social isolation conditions revealed significant differences after social isolation in both ranks (Figures 4G and 4H; rank 1:  $t(11) = 2.760$ ,  $p = 0.019$ ,  $d = 1.59$ ; rank 4:  $t(11) = 2.278$ ,  $p = 0.044$ ,  $d = 1.31$ ). Lastly, to assess how these rank changes impacted rank stability within the cage, we measured instability, a metric representing the variance of rank changes within a rank. Under group housing conditions, instability remained low but increased significantly after social isolation (Figures 4I and 4J;  $t(11) = 2.893$ ,  $p = 0.015$ ,  $d = 1.67$ ). Significant changes in  $\Delta$ rank, stability index, and instability were observed within the first 4 days of single housing in both rank 1 and rank 4 mice.

### Competitive-behavior differences between ranks disappear during the early single-housing rank-change period

To investigate the effect of rank changes observed during a specific period of social isolation on competitive behaviors, push, resist, and retreat behaviors were measured, employing the same methodology as in the previous experiment. Measurements were taken at two distinct time points: during the 3–4 days period in group housing, where rank stability was most pronounced, and during the 3–4 days period following social isolation, when rank changes were evident (Figure 5A). In the group housing conditions, where stable ranks were established, significant differences in push and retreat behaviors were observed between rank 1 and rank 4, aligning with the findings

of previous studies ( $t(22) = 7.268$ ,  $p < 0.001$ ,  $d = 2.97$ ;  $t(22) = 1.186$ ,  $p = 0.248$ ,  $d = 0.48$ ;  $t(22) = 4.919$ ,  $p < 0.001$ ,  $d = 2.01$ ) (Figures 5B–5D). In contrast, during the period of rank changes associated with social isolation, these differences in push and retreat behaviors between rank 1 and rank 4 were no longer observed ( $t(22) = 1.230$ ,  $p = 0.232$ ,  $d = 0.50$ ;  $t(22) = 0.307$ ,  $p = 0.762$ ,  $d = 0.13$ ;  $t(22) = 0.833$ ,  $p = 0.414$ ,  $d = 0.34$ ) (Figures 5E–5G). The differences in push and retreat between rank 1 and rank 4 observed during the stable group housing period were no longer present during the 3–4 days period of single housing, coincident with the rank changes described earlier.

### Rank elevation during early social isolation is associated with increased LHb c-Fos expression

To determine whether rank changes observed after a specific period of social isolation were associated with changes in the brain, we performed immunohistochemistry on brain samples following the rank change, using the same methodology as in the previously established rank change model. Figure 6A presents a representative image of c-Fos immunohistochemistry in the mPFC and LHb region. We analyzed the c-Fos expression in single housing conditions for the no-change, rank-up, and rank-down groups. When comparing c-Fos expression in the LHb, PL, and IL regions between the no-change and rank change groups under single housing conditions, no significant differences were observed (region:  $F(2, 24) = 5.95$ ,  $p = 0.008$ ,  $\eta^2_p = 0.331$ ; condition:  $F(1, 12) = 0.942$ ,  $p = 0.351$ ,  $\eta^2_p = 0.073$ ; interaction:  $F(2, 10) = 2.351$ ,  $p = 0.146$ ,  $\eta^2_p = 0.320$ ) (Figure 6B). Next, we subdivided the rank change group into rank-up and rank down groups under single housing conditions and compared them with the no-change group (region:  $F(1.44, 14.44) = 4.182$ ,  $p = 0.048$ ,  $\eta^2_p = 0.295$ ; condition:  $F(1.56, 15.60) = 0.530$ ,  $p = 0.556$ ,  $\eta^2_p = 0.052$ ; interaction:  $F(2.08, 2.61) = 1.228$ ,  $p = 0.424$ ,  $\eta^2_p = 0.495$ ). In the LHb region, rank-up individuals showed increased c-Fos expression compared to the no-change group ( $p = 0.020$ ) (Figure 6C). Rank-up individuals under single housing showed significantly higher LHb c-Fos expression than no-change individuals ( $p = 0.020$ ), whereas no significant differences were observed in the mPFC subregions.

## DISCUSSION

In this experiment, we tracked shifts in social status across two social isolation models and examined corresponding brain region alterations. First, the “rank change model” measured pre-existing ranks and evaluated changes after three weeks of single housing. Second, the “daily rank trace model” monitored ranks on a daily basis to identify the specific time points of status changes throughout the isolation period.

**Figure 3. Social isolation does not change brain activity in LHb but in mPFC in rank change model**

(A) Schematic illustration of QUINT workflow.

(B–F) (B) Representative image of c-Fos in mPFC and in LHb between group housing and single housing. Comparing the difference in the expression of c-Fos in the LHb, PL, and IL between no-change and rank-change groups under group housing conditions (C) and single housing conditions (E). Comparing the difference in the expression of c-Fos in the LHb, PL, and IL between no-change, rank-up, and rank-down groups under group housing conditions (D) and single housing conditions (F). Two-way ANOVA was used to assess the effects of brain region and rank change, with both factors included as between-subject variables.

\* $p < 0.05$ . Data are presented as mean  $\pm$  SEM. Scale bars, 100  $\mu$ m.

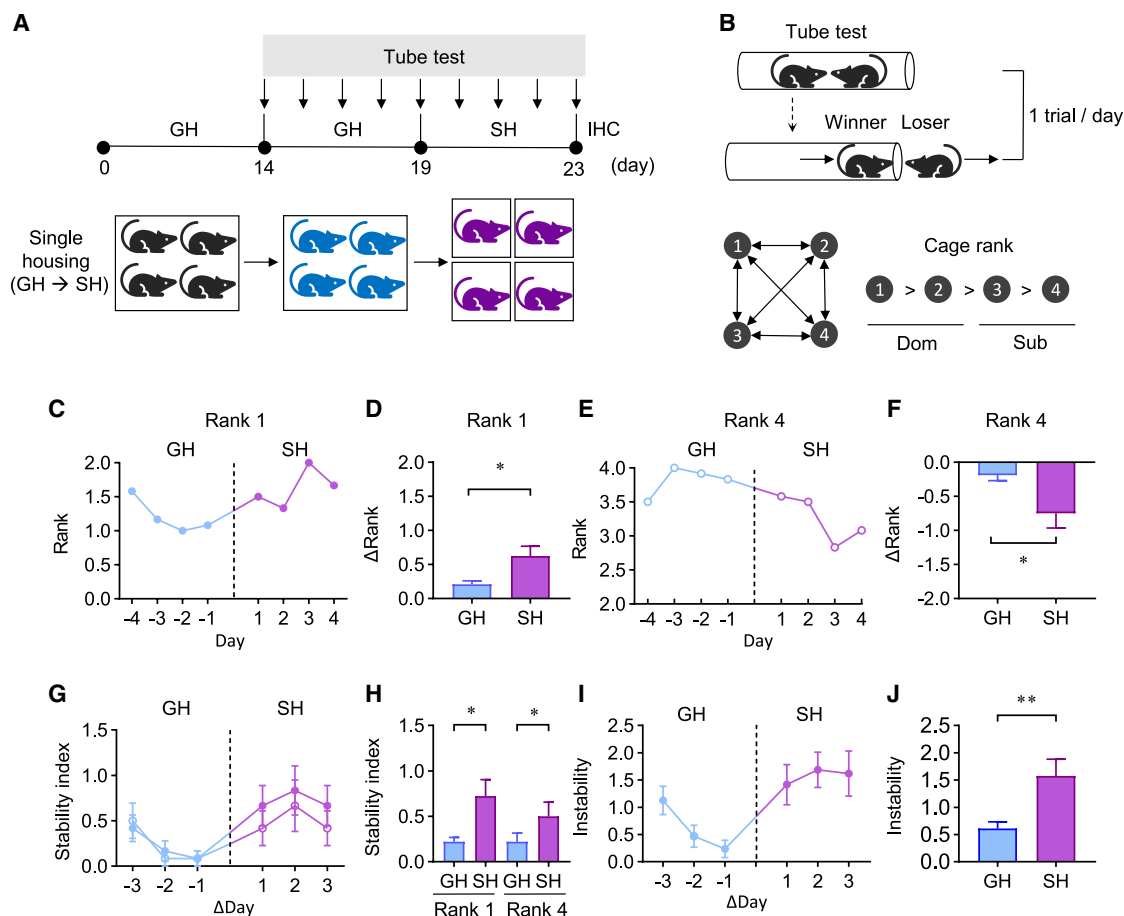


**Figure 4. Social isolation induces changes in rank after 3–4 days**

(A) Schematic illustration of social isolation daily rank trace model.

(B–D) Graphic scheme of tube test and rule of matching in the cage with classifying social hierarchy. Rank change (C) and Δrank (D) between group housing and single housing in rank 1 (all bar graph  $n = 12$ , unpaired  $t$  test,  $*p < 0.05$ ).

(E and J) Rank 4 (all bar graph  $n = 12$ , unpaired  $t$  test,  $*p < 0.05$ ). Stability index (G and H), instability (I), and instability average (J) between group housing and single housing.  $n = 12$ , unpaired  $t$  test,  $*p < 0.05$ ,  $**p < 0.01$ ; one-way ANOVA,  $*p < 0.05$ . Data are presented as mean  $\pm$  SEM.

In the rank change model, we selected groups with stable hierarchies, quantified by David's score, to ensure a robust baseline for subsequent tracking. Following prolonged social isolation, we observed statistically significant rank shifts compared with group housing conditions. Social isolation alters competitive behavior, resulting in rank shifts. Previous studies suggest that isolation duration significantly influences outcomes.<sup>34</sup> To address this, we utilized two models targeting both short- and long-term effects. While prolonged isolation was associated with clear behavioral changes, neural responses varied across time. Therefore, the rank change model was deemed suitable for studying long-term isolation, while the daily rank trace model was more appropriate for monitoring short-term changes and identifying when rank shifts occurred.

The comparison revealed that rank changes typically occurred within 3–4 days of social isolation. During this early stage, alterations in brain activity were concentrated in the LHb, with the rank-up group showing significantly higher activity than the no-change group. The rank-up group consisted of originally subor-

dinate individuals that increased their status following isolation. This finding contrasts with earlier studies suggesting that LHb activation is mainly linked to negative stimuli and submissive behavior.

Because social isolation is commonly associated with stress-related neural responses, a certain degree of LHb activation might be expected across all groups.<sup>35</sup> However, in the present study, elevated LHb c-Fos expression was observed specifically in rank-up individuals relative to no-change individuals, rather than uniformly across isolated mice. One speculative interpretation of this regional association can be framed within the broader literature on social rank and stress. LeClair et al. (2021) reported that, under stable group housing conditions, rising to the top rank through a dynamic history of winning confers stress resilience in mice, whereas losing an established dominant position is associated with reduced resilience.<sup>33</sup> Their findings emphasize that the affective and physiological meaning of a rank transition depends on the individual animal's history of winning and on the stability of the surrounding social landscape—an idea

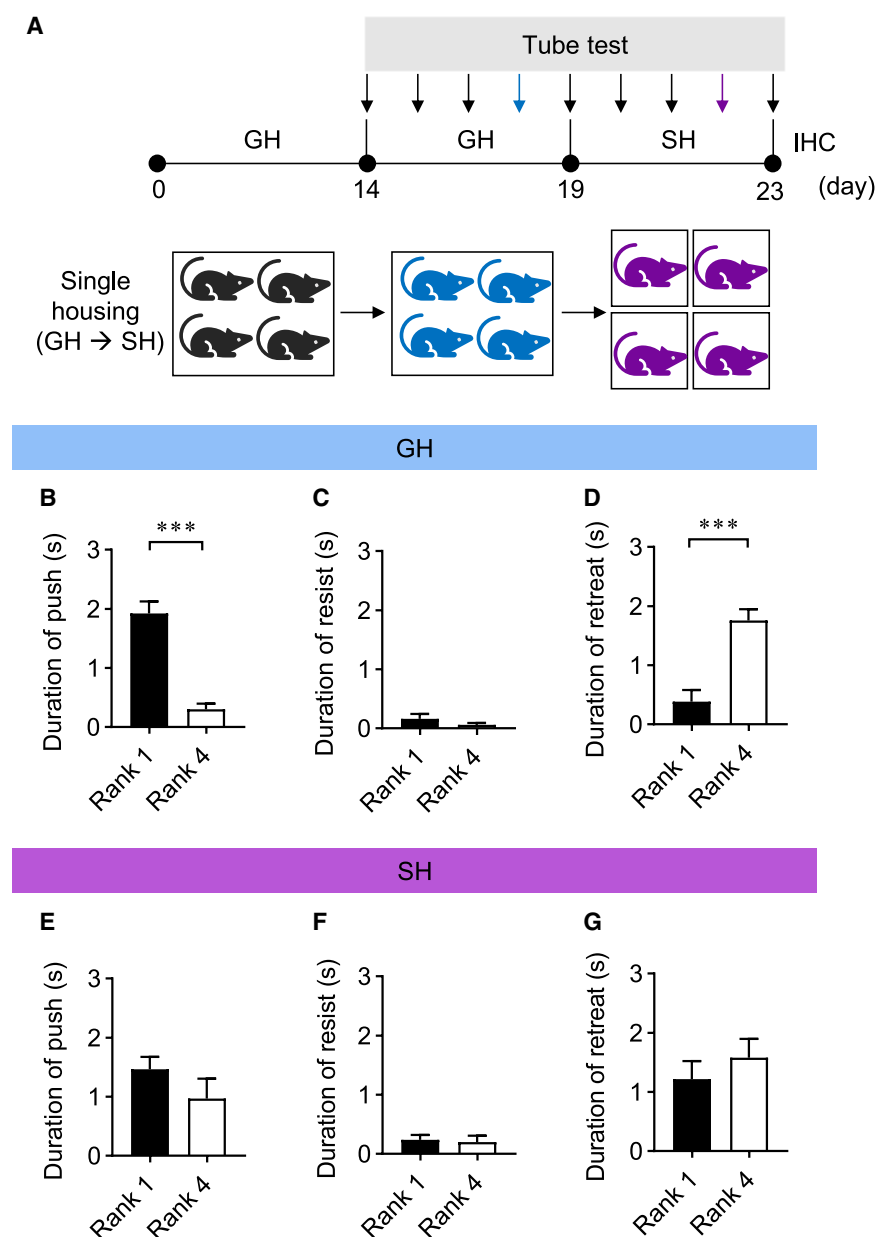


**Figure 5. Social isolation induces changes in competitive behavior after 3–4 days**

(A–D) Schematic illustration of point about competitive behavior in daily rank trace model. Duration of push (B), resist (C), and retreat (D) between ranks 1 and 4 in group housing (all bar graph  $n = 12$ , unpaired  $t$  test,  $***p < 0.001$ ). (E–G) Single housing (all bar graph  $n = 12$ , unpaired  $t$  test). Data are presented as mean  $\pm$  SEM.

pretation that Lhb engagement in rank-up individuals reflects affective or motivational components associated with rank ascension under a disrupted social landscape, rather than moment-to-moment competitive behavior per se. We note, however, that the modest per-group sample sizes in the behavioral correlation analyses ( $n = 7$  rank-up,  $n = 6$  rank-down, and  $n = 11$  no-change) limit the sensitivity of within-group correlation tests, and that c-Fos captures only regional activation at a single time point. Causal, cell-type-specific approaches—for example, circuit-specific manipulations or *in vivo* recordings from defined Lhb populations during rank transitions under different social landscapes—will be required to firmly link Lhb activity to the affective component of rank transition.

In the present study, rank-up individuals ascended in rank not under stable group housing conditions but against the backdrop of social isolation—that is, during a period in which the pre-existing hierarchical landscape had been substantially disrupted. It is, therefore, plausible, though speculative, that rank ascension in this disrupted context may carry a different affective load than rank ascension observed in stable hierarchies, and that the selectively elevated Lhb activity in rank-up individuals may reflect

sometimes discussed in the dominance literature under the informal label of a “winning cost,” referring to the notion that the process of status change itself carries affective and motivational demands that are distinct from the steady-state occupation of a given rank. Prior reports have further suggested that the Lhb responds not only to negative valence and aversive outcomes but also to high-demand motivational states and social prediction errors,<sup>31</sup> which would be consistent with the selective Lhb activation observed in rank-up individuals here. Although Lhb c-Fos density was selectively elevated in rank-up individuals at the group level, this activation did not track push, retreat, or their ratio either between groups or within groups, indicating that the Lhb signal is dissociable from the kinematic execution of tube test competition. This pattern is consistent with the inter-

this context-dependent component rather than rank ascension per se.

We further note that this association between elevated Lhb activity and the rank-up phenotype is observed at the group level—that is, between animals classified as rank-up or no-change on the basis of behaviorally defined rank transitions—rather than as a within-animal correlation between trial-level competitive behavior, such as push duration and Lhb c-Fos counts. Because push duration is strongly dependent on baseline rank and is distributed in opposing directions across rank-up (predominantly originally rank 4) and rank-down (predominantly originally rank 1) individuals, a direct cross-animal correlation between push behavior and Lhb activity would be dominated by pre-existing rank rather than by the rank-transition process itself.

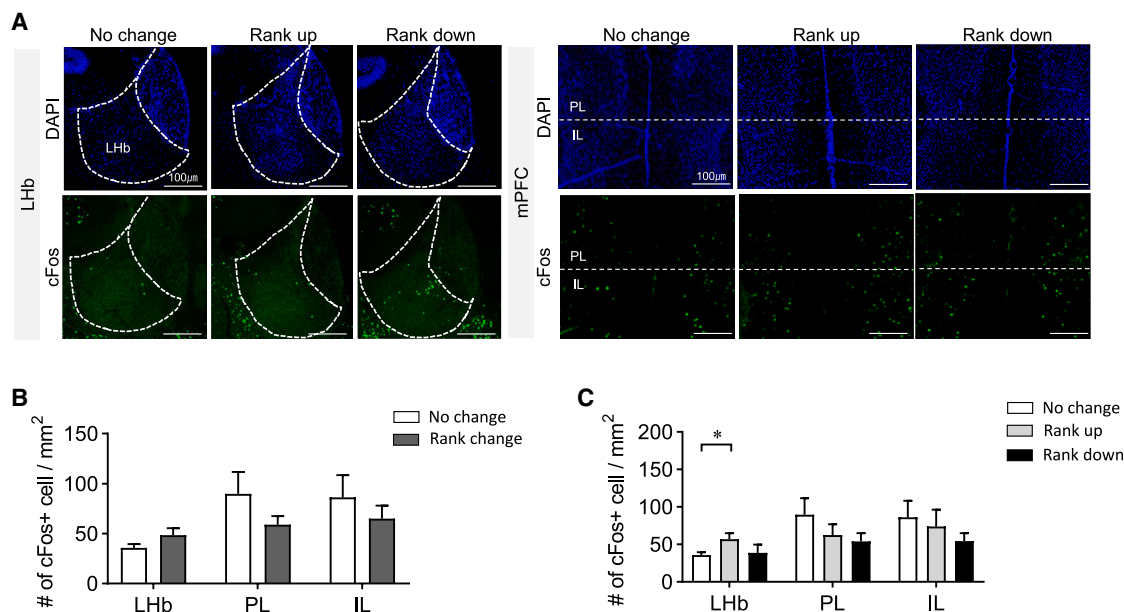


**Figure 6. Social isolation increases brain activity in only rank-up group LHb in daily rank trace model**

(A) Representative image of c-Fos in mPFC and LHb after social isolation. Comparing the difference in the expression of c-Fos in the LHb, PL, and IL between no-change and rank-change groups under single housing conditions (B). Comparing the difference in the expression of c-Fos in the LHb, PL, and IL between no-change, rank-up, and rank-down groups under single housing conditions (C). Two-way ANOVA was used to assess the effects of brain region and rank change, with both factors included as between-subject variables  $*p < 0.05$ . Data are presented as mean  $\pm$  SEM. Scale bars, 100  $\mu$ m.

We emphasize, more broadly, that all of the above interpretations remain speculative. Our c-Fos data capture regional activation at a single time point and do not provide direct evidence of affective state, motivational valence, resilience, or any specific mechanistic role of LHb neurons in rank ascension. Nor do our data directly compare rank transitions across stable and disrupted social conditions within the same experiment. Further causal and cell-type-specific investigations—for example, using circuit-specific manipulations or *in vivo* recordings from defined LHb populations during rank transitions under different social landscapes—will be required before a context-dependent “winning cost” framework can be firmly linked to LHb activity, and before, the group-level association reported here can be extended to a within-animal account of LHb engagement during competitive interactions.

In contrast, the rank change model revealed that long-term social isolation was associated with significantly reduced the density of c-Fos-positive cells in the PL, specifically in individuals undergoing rank shifts. While group-housed counterparts exhibited a trend toward increased PL activity associated with rank changes,<sup>35</sup> this pattern was inverted following prolonged isolation, underscoring that isolation alters PL engagement in a context-dependent manner.<sup>36</sup> These observations align with prior reports of attenuated PL activity in response to social isolation. Given the pivotal role of the PL in emotional regulation and executive planning, such hypoactivity may underpin symptoms of social withdrawal or emotional blunting.

A remaining question is why the PL, but not the IL, exhibited a significant isolation-associated reduction in c-Fos expression, given the close anatomical proximity of these two mPFC sub-

regions. The PL and IL are increasingly recognized as functionally dissociable subdivisions of the mPFC, with the PL preferentially engaged in the expression and maintenance of goal-directed, assertive behaviors, including social dominance, effortful competition, and sustained action initiation, whereas the IL is more strongly associated with the inhibition of acquired responses and flexible behavioral adaptation, such as extinction and passive coping.<sup>37,38</sup> This functional divergence is further reinforced by distinct projection patterns: the PL preferentially targets the basolateral amygdala, NAc core, and mediodorsal thalamus, a circuit supporting sustained dominance behaviors, whereas the IL projects more strongly to amygdala intercalated cells, NAc shell, and autonomic brainstem nuclei, a circuit supporting response suppression and emotional regulation.<sup>39,40</sup> Because the maintenance of dominance behaviors depends on sustained PL-dependent top-down drive, the selective PL hypoactivity observed in our data is consistent with a preferential vulnerability of PL-mediated functions under prolonged isolation, while IL-mediated inhibitory functions remain relatively preserved. Although we also observed a trend toward reduced c-Fos expression in the IL, this change did not reach statistical significance, consistent with the notion that PL and IL contribute complementary but dissociable roles in stress-related and social behavioral adaptations.<sup>41</sup>

Crucially, individuals experiencing rank changes under long-term isolation displayed a more pronounced reduction in PL activity.<sup>36,42</sup> While reduced cortical activity can sometimes reflect neural efficiency, the concurrent observation of social instability in these mice argues against this interpretation. Instead, the hypoactivity may reflect reduced in top-down regulatory control.<sup>43</sup>

This pattern is consistent with the possibility that sustained social isolation, combined with the cognitive load of unstable hierarchy, is associated with reduced engagement of the prefrontal executive systems involved in maintaining consistent social behavior. Synthesizing these findings with short-term LHB data, we propose a temporal shift in neural activity: early isolation is associated with elevated activity in subcortical stress- and salience-circuits (LHB), whereas chronic isolation is associated with reduced activity in prefrontal regulatory regions (PL).

Consistent with this interpretation, the daily rank trace model revealed that short-term social isolation selectively increased LHB activity in the rank-up group, while other brain regions remained largely unaffected at this stage. Notably, these LHB-specific changes appear to diminish over time; instead, neural alterations become more prominent in the mPFC under long-term conditions. This temporal progression is conceptually supported by evidence that sustained LHB hyperactivity can engage glutamatergic projections that indirectly reduce mesocortical monoaminergic drive, including to prefrontal regions.<sup>44</sup> This temporal dissociation is further supported by evidence that the mPFC-LHB circuit exhibits bidirectional functional dynamics, depending on stress context and behavioral state, allowing neural responses to shift across time and conditions.<sup>45</sup>

Social isolation is widely recognized as a social stressor that is associated with broad alterations in neural activity across multiple brain regions.<sup>43</sup> Prior studies have reported that exposure to social isolation is associated with altered activity in the social brain, including the prefrontal cortex, amygdala, and insula, which has been linked to diminished sociability and blunted responses to social novelty.<sup>44</sup>

However, because c-Fos immunohistochemistry labels active cells only at the level of regional activation and does not distinguish between excitatory and inhibitory populations or resolve cell-type-specific contributions, our findings should be interpreted strictly at the regional level. We, therefore, refrain from inferring specific circuit-level or downstream mechanisms from the present dataset. The selective elevation in LHB activity observed during short-term isolation and the reduction in PL activity observed during long-term isolation are best understood as regional associations with rank transitions rather than as evidence of engagement of specific downstream or network-level pathways. Validation of any such circuit-level interpretation will require future studies employing cell-type-specific, projection-defined, or intersectional genetic approaches.

In synthesis, these findings suggest that social isolation is associated with widespread yet temporally structured reorganization of neural circuits.<sup>46</sup> Short-term isolation was associated with elevated activity in LHB-centric pathways-implicated in stress and salience processing-during the initial stages of social status transitions,<sup>47</sup> whereas long-term isolation was associated with reduced activity in prefrontal regions involved in emotional and behavioral regulation. Ultimately, these neural shifts are best framed within a distributed network perspective, transcending localized regional deficits to encompass a systemic reconfiguration of the social brain.

In conclusion, this study elucidates the neurobiological substrate underlying social hierarchy reconfiguration through distinct, spatio-temporally defined mechanisms. We report that

the early phase of social isolation was associated with volatile rank transitions, a process marked by elevated LHB activity, identifying this region as a salient neural signature of early status instability. Conversely, chronic isolation was associated with reduced PL activity within the mPFC, particularly among individuals exhibiting rank instability, which may reflect altered prefrontal regulatory control. By synthesizing social rank dynamics with longitudinal neural adaptations, our findings establish a robust, network-level framework for conceptualizing social status plasticity and its inherent vulnerability to stress.

### Limitations of the study

Several limitations should be considered when interpreting the present findings. First, only male mice were examined. This choice was made because social dominance hierarchies in female mice have been reported to be less stable and more difficult to reliably quantify using the dominance tube test,<sup>43</sup> as also noted in the [STAR Methods](#). Nevertheless, social hierarchy and stress-related neural responses are known to be sexually dimorphic in rodents, and the present findings, therefore, cannot be directly generalized to female mice. Future studies employing female-appropriate behavioral paradigms will be needed to determine whether the associations between social isolation, rank transitions, and LHB/mPFC activity observed here extend across sexes.

Second, although the number of mice used in each experimental group was consistent with that of previous studies employing similar dominance tube test and c-Fos mapping paradigms, the relatively modest group sizes may limit statistical power. To allow readers to assess the magnitude and stability of the observed effects independently of *p*-values, we have additionally reported effect sizes (Cohen's *d* for *t* tests; partial  $\eta^2$  for ANOVA) throughout the [results](#) and figure legends. Replication in larger cohorts and across different laboratories will be important to confirm the robustness of the present findings.

Third, while the dominance tube test is widely regarded as a gold standard for assessing social hierarchy, exclusive reliance on this single paradigm may constrain the generalizability of our behavioral findings. Complementary assays, such as the urine marking or warm spot tests, and longer-term observations across multiple independent time points, would provide more multi-dimensional validation of social rank and help mitigate trial-specific variability.

Fourth, all neural measurements in this study were based on c-Fos immunohistochemistry, which labels active cells at the regional level but does not distinguish between excitatory and inhibitory neurons or resolve cell-type-specific or projection-defined contributions. Accordingly, all interpretations in the present study are restricted to the level of regional activation, and inferences regarding specific neural circuits, cell types, or downstream pathways, such as dopaminergic or limbic projection systems, cannot be drawn from the current dataset. Future studies employing cell-type-specific markers, intersectional genetic approaches, or *in vivo* recordings from defined neuronal populations will be required to dissect the circuit- and cell-type-level contributions to the rank-related neural patterns reported here. In addition, the behavioral and neural measurements in this study are not temporally aligned—push duration

is recorded during the tube test, whereas c-Fos expression is assessed approximately 1 h later as an integrated marker of neural activity—and push duration is further confounded by baseline rank, being distributed in opposing directions across rank-up (predominantly originally rank 4) and rank-down (predominantly originally rank 1) individuals. For these combined reasons, a direct within-animal correlation between LHb activity and competitive behavior is not methodologically appropriate in the present dataset, and future studies employing *in vivo* recordings from defined LHb populations during tube test interactions will be needed to directly evaluate this association with behavior-behavior alignment.

While the present study employed sample sizes consistent with those of established tube test paradigms and ethically constrained by the 3R principles, replication with larger cohorts in independent studies would further substantiate the generalizability of these findings. Finally, expanding the neural analysis beyond the mPFC and LHb to broader brain networks may offer deeper insights into the neurobiological underpinnings of isolation-related social hierarchy disruptions.

## RESOURCE AVAILABILITY

### Lead contact

Requests for further information and resources should be directed to and will be fulfilled by the lead contact, Jihyun Noh ([jihyun2@dankook.ac.kr](mailto:jihyun2@dankook.ac.kr)).

### Materials availability

This study did not generate new unique reagents.

### Data and code availability

- All data reported in this paper will be shared by the [lead contact](#) upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

## STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- [EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS](#)
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  - Social isolation model
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  - David's score
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## AUTHOR CONTRIBUTIONS

Conceptualization, T.J., J.N., and J.K.; methodology, T.J.; investigation, T.J.; formal analysis, T.J. and S.U.; data curation, T.J. and S.U.; validation, M.K.; visualization, T.J. and S.U.; writing – original draft, T.J.; writing – review and editing, J.N., J.K., M.K., and S.U.; funding acquisition, J.N. and J.K.; supervision, J.N. and J.K. All authors read and approved the final manuscript.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

## SUPPLEMENTAL INFORMATION

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## STAR★METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE                           | SOURCE                              | IDENTIFIER                                                                                      |
|-----------------------------------------------|-------------------------------------|-------------------------------------------------------------------------------------------------|
| <b>Antibodies</b>                             |                                     |                                                                                                 |
| c-Fos (9F6) Rabbit mAb                        | Cell Signaling Technology           | Cat# 2250S; RRID:AB_2247211                                                                     |
| Alexa Fluor™ 488 Goat anti-rabbit IgG (H + L) | Invitrogen/Thermo Fisher Scientific | Cat# A-11034; RRID:AB_2576217                                                                   |
| <b>Experimental models: Organisms/strains</b> |                                     |                                                                                                 |
| Mouse: C57BL/6N (male, 8–9 weeks, 20–30 g)    | Orient Bio Inc.                     | N/A                                                                                             |
| <b>Software and algorithms</b>                |                                     |                                                                                                 |
| Ilastik                                       | ilastik.org                         | <a href="https://www.ilastik.org">https://www.ilastik.org</a>                                   |
| QuickNII                                      | HBP/EBRAINS                         | <a href="https://www.nitrc.org/projects/quicknii">https://www.nitrc.org/projects/quicknii</a>   |
| VisuAlign                                     | HBP/EBRAINS                         | <a href="https://www.nitrc.org/projects/visualign">https://www.nitrc.org/projects/visualign</a> |
| Nutil                                         | HBP/EBRAINS                         | <a href="https://www.nitrc.org/projects/nutil">https://www.nitrc.org/projects/nutil</a>         |
| GraphPad Prism 8.0                            | GraphPad Software                   | <a href="https://www.graphpad.com">https://www.graphpad.com</a>                                 |
| ImageJ                                        | NIH                                 | <a href="https://imagej.nih.gov/ij/">https://imagej.nih.gov/ij/</a>                             |

## EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

## Animals

C57BL/6N mice (20–30g, male, Postnatal 8–9 weeks) were purchased from Orient Bio Inc (Seongnam, South Korea). A total of 84 male C57BL/6N mice were used in this study. In Experiment 1 (long-term social isolation, 3 weeks), 16 mice were assigned to the Group housing condition and 20 mice to the Single housing condition. In Experiment 2 (short-term social isolation, 4 days), 48 mice were used. To maintain scientific integrity, variables such as age and body weight were carefully aligned throughout the experiment. Mice were maintained on a 12-h light-dark cycle in a specific-pathogen-free facility with controlled temperature ( $21 \pm 1^\circ\text{C}$ ) and humidity (60%) and had *ad libitum* access to water and food. Before the dominance tube test, Group Housing (GH) mice were housed with 4 mice per cage for 2 weeks. Only male mice were tested, as dominance hierarchies in female mice are less stable.<sup>48</sup> All animal experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Korea Brain Research Institute (IACUC-24-00051-M1). Only male mice were studied; consequently, the influence or association of sex on the present findings could not be assessed and is discussed as a limitation to the generalizability of the findings (see Limitations of the Study).

## Social isolation model

Mice were housed in groups of 4 mice 2- weeks before social isolation. Before isolation, a dominance tube test was performed to measure established rank. Then mice were randomly assigned to group-housed (GH) and single housing (SH) conditions. Mice in the GH group were housed in groups of 4 mice per cage just like before, and those in the SH group were individually housed in a standard mouse cage alone. We established two social isolation paradigms; 1) SH groups exposed to 3 weeks of social isolation were housed individually. While visual and tactile contacts with other mice were completely blocked, auditory and olfactory cues maintained to stimulate naturalistic isolation conditions within a colony room. Meanwhile, GH groups were continually housed four per cage during this period. At the end of the isolation period, a tube test was performed once more to confirm the rank, and the experimental mice were euthanized via transcardial perfusion after 1 h. Both tube tests were conducted with five trials each, alternating the direction each time. The matchups were arranged following the round-robin rule, ensuring that each group faced each other at least once while minimizing repeated pairings as much as possible. 2) Social competition was conducted once per day following the round-robin rule, and social isolation began when the same rank was maintained consistently for 3–4 consecutive instances during the GH period. Perfusion was performed after 4 days of social isolation to examine brain activity during the period when rank showed the most significant changes.

## METHOD DETAILS

### Dominance tube test

In a laboratory setting, the tube test is a reliable and effective behavioral assay that is commonly used to assess the social hierarchy among rodents.<sup>49</sup> The apparatus is a transparent tube 30 cm in length and 3 cm inner diameter, a size just sufficient to permit one adult mouse to pass through without reversing the direction. The dominance tube test used a plastic stick for cleaning the tube and manual intervention in behavioral training. Prior to testing, all mice were habituated to the testing room for at least 1 h on the day of the experiment and were further acclimated to the apparatus by being allowed to traverse the empty tube 5 times alternately from both ends. During the test, the two mice were released simultaneously into the opposite ends of the tube and one mouse would attempt to show dominance and force the other back out of the tube. The dominance tube test was conducted with 5 dyadic trials per day, in which the mouse that first moved forward from the tube within 2 min was designated the 'winner' of each trial. Daily rank was determined based on a majority-consistent outcome across the 5 trials (typically 3–4 wins out of 5), reflecting the probabilistic nature of tube-test dominance interactions. Each trial was limited to a maximum duration of 2.5 min. If neither mouse had retreated from the tube within this period, the trial was classified as a 'draw' and excluded from rank determination; in such cases, both mice were gently removed from the tube before the next trial. In order to prevent learning of a directional win bias during the test, the test was carried out alternately in both directions. For the matches between mice, the round-robin rule was used so that all mice could compete once. In a group, four mice eventually formed six pairs in the tube test, and the final ranking was determined according to the total number of wins and David's score. For analyses of competitive behaviors (Figures 2C–2N), comparisons were restricted to the Rank 1 (most dominant) and Rank 4 (most subordinate) mice from each cohort, consistent with established tube test paradigms<sup>50,51</sup>; each Rank 1–Rank 4 pair was therefore drawn from one independent cohort of four co-housed mice. After each trial, the tube was cleaned with 70% ethanol to remove odor, urine, or feces. Between consecutive trials, an inter-trial interval of at least 1 min was maintained to allow tube cleaning and to minimize residual olfactory cues and carry-over effects. Competitive behavior was measured using the manual counting method of Ethovision 17 and three canonical tube test behaviors: push, resist, and retreat. The push and resistance actions represent the active and passive forms of dominant behaviors, whereas the voluntary retreat and forced retreat actions represent the active and passive forms of submissive behaviors. To minimize observer bias, all mice were identified solely by individual mouse identification numbers during behavioral testing and offline scoring; the experimenter performing trial outcome determination and behavioral quantification (push, resist, retreat) had no access to housing condition information at the time of scoring, providing a *de facto* blinded scoring procedure.

### David's score

The advantage of David's score is that the overall success of an individual is determined by weighing each dyadic success measure by the unweighted estimate of the interactant's overall success, so that relative strengths of the other individuals are taken into account.<sup>52</sup> David's score consists of four elements, the first two increasing the final score, while the last two decrease it, with higher scores representing better competitive ability and, thus higher dominance.<sup>53</sup>

$$\text{David score} = W + W_{\text{weight}} - L - L_{\text{weight}}$$

The David Score evaluates performance through four components: W (Wins), the total number of victories; W\_weighted (weighted Wins), victories adjusted for opponent strength or importance; L (Losses), the total number of defeats; and L\_weighted (weighted Losses), defeats adjusted for the opponent's strength or significance. This method ensures a balanced evaluation of both the quantity and quality of wins and losses.

After measuring David's score, the rank needs to be clearly distinguished to evaluate changes after 3 weeks. Therefore, at the time of measuring the established rank in Tube test 1, a stable rank within the group must be formed to confirm rank changes caused by social isolation.<sup>4</sup> A higher David's score for Rank 1 and a large difference compared to Rank 4 indicate the presence of a stable hierarchy. Consequently, experimental results where David's score of Rank 1 was less than 3 were excluded from the analysis, and only groups showing a clear difference between Rank 1 and Rank 4 were used for further analysis.

### Free-floating immunohistochemistry

After social isolation, mice were anesthetized under isoflurane and transcardially perfused with phosphate buffer saline (PBS). Brains were removed and fixed in 4% paraformaldehyde for 24 h. Brains were then stored in a 30% sucrose solution in PBS at 4°C. Brains were sectioned at 35µm coronal sections with a cryostat (CM1860, Leica). To confirm c-fos expression, the brain slices were washed two times with PBS for 10 min and incubated in a blocking solution (0.2% Triton X-100, 0.5% bovine serum albumin, and 4% normal goat serum in PBS) for 1.5 h at room temperature, followed by overnight incubation with primary antibody at 4°C. The primary antibodies were rabbit anti-cFos (Cell signaling technology, Cat. # 2250S, 1:1000). The secondary antibodies were Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen, 1:500). Coronal sections containing the regions of interest were selected according to the Allen Mouse Brain Atlas: PL and IL were sampled from approximately bregma +2.0 to +1.3 mm, and LHb was sampled from approximately bregma –2.0 to –1.3 mm. Six sections per mouse were analyzed for each region of interest. All images were subsequently overlaid with the corresponding atlas section to anatomically define the regions of interest.

### Imaging and quantification

Images were acquired using confocal microscopy (A1/Ni-E, Nikon; 10× objective lens magnification) and a slide scanner (Pannoramic scanner; 20× objective lens magnification). Z-stacks of brain areas were obtained (step size: 4 μm) using sequential scanning in confocal imaging. Images were acquired using the slide-scanner system with two channels, DAPI and FITC, under exposure times of 200 ms and 400 ms, respectively. The acquired images were processed using the CaseViewer program, where TIF file conversion was performed. Regions of Interest (ROIs) were defined, and individual raw data images were obtained by cropping the images slice by slice according to the designated ROI. All images were processed and analyzed on ImageJ and Fiji software (NIH). Prior to counting cFos-positive cells, all brain sections were overlaid with the corresponding Allen Mouse Brain Atlas maps. Images were acquired under identical exposure settings (DAPI: 200 ms; FITC: 400 ms) and microscope parameters that were held constant across all samples to ensure comparable signal intensity and were converted to 8-bit grayscale. cFos-positive nuclei were identified by applying Otsu's automatic thresholding method (ImageJ/Fiji) uniformly to all samples within a given region of interest, ensuring that the same algorithmic criterion was used across the entire dataset and eliminating operator-dependent variability in threshold selection. Each region of interest was individually outlined on the basis of the overlaid Allen Mouse Brain Atlas map, and the number of cFos-positive nuclei was recorded. For quantification, six sections per mouse were analyzed per region of interest. cFos-positive cells were counted unilaterally from a single hemisphere per section; counts from the six sections analyzed per region of interest were averaged to yield a single value per mouse.

### QUINT workflow

The workflow for serial brain section image analysis comprises several parts (Figures 3 and 6): namely, image pre-processing (ImageJ); registration of images to a reference atlas (QuickNII, Visualign); segmentation of labeled features (Ilastik); and quantification of features per atlas region (Nutil using the Quantifier feature). To prepare raw data, The QUINT workflow started with organizing individual image files, renaming them in a standardized format (\_sXXX) to ensure software compatibility, and matching unique identifiers to avoid errors. Raw data in designated folders was verified before proceeding to prevent interruptions. Using Ilastik, pixel classification distinguished foreground features from the background through supervised machine learning. Manually labeled examples trained the model, generating pixel probabilities that were exported. Batch processing applied the model to all data, creating consistent segmentation maps for analysis. Histological images were registered to a 3D brain atlas, like the Allen Mouse Brain Atlas, using QuickNII. Adjustments to scale, orientation, and transparency ensured precise alignment, with results saved as XML and JSON files for further use. VisuAlign refined QuickNII-based registration for datasets with distortions or needing higher precision. Manual marker placement enabled detailed atlas adjustments, and the refined data was saved for improved spatial mapping accuracy. Segmentation results and registration data were processed in Nutil to quantify and analyze feature distribution across brain regions. Input included segmented images, registration outputs, and the reference atlas, generating detailed reports on feature counts and localization for brain structure insights.

### QUANTIFICATION AND STATISTICAL ANALYSIS

Sample sizes (n) indicated in figure legends refer to the number of individual mice, unless otherwise specified as cages. To account for the interdependence of mice housed within the same cage, statistical comparisons for behavioral metrics involving group dynamics were verified by averaging values per cage or using mixed-effect models where appropriate. Degrees of freedom reported in the results reflect this adjustment to prevent pseudo replication. All data are presented as the mean ± SEM with statistical significance set at  $p < 0.05$ . Students unpaired *t* test and one or two-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons was applied to data that conformed to a normal distribution. Statistical analysis was performed using GraphPad Prism 8 software. For paired within-subject comparisons, Wilcoxon matched-pairs signed-rank tests were used. Effect sizes were reported alongside statistical test results. For Mann-Whitney U tests and Wilcoxon signed-rank tests, rank-biserial correlation ( $r_{rb}$ ) was calculated. For *t*-tests, Cohen's *d* was reported, and for ANOVAs, partial  $\eta^2$  ( $\eta^2_p$ ) was calculated. Effect size magnitudes were interpreted according to Cohen's conventional benchmarks. All statistical tests, the exact value of *n* (number of individual mice unless otherwise specified as cages), test statistics, degrees of freedom, exact *p* values, and effect sizes are reported in the Results and in the corresponding figure legends. Across all figures, asterisks denote  $*p < 0.05$ ,  $**p < 0.01$ , and  $***p < 0.001$ , and the specific statistical test used is stated in each figure legend.