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Female dominance hierarchies influence responses to psychosocial stressors

Highlights

- Behavior and endocrinology can predict female rank in future social pairings
- Female rank attributes are context dependent and influenced by circadian phase
- Social status influences phenotypes arising from chronic psychosocial stressors
- Rank informs neuronal activation patterns after chronic stress and social encounters

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In brief

Smith-Osborne et al. demonstrate that in female mouse dyads, there exists a motivation difference by rank that is stress-sensitive and engages various behavioral, endocrinological, and neurobiological responses to maintain homeostasis. The findings support the consideration of female social identity in rodent models of neurologic disease.

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Female dominance hierarchies influence responses to psychosocial stressors

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SUMMARY

Social species form dominance hierarchies to ensure survival and promote reproductive success. Traditionally studied in males, rodent hierarchies are considered despotic, and dominant social rank results from a history of winning agonistic encounters. By contrast, female hierarchies are thought to be less despotic, and rank is conferred by intrinsic traits. Both social buffering and elevated social status confer resilience to depression, anxiety, and other consequences of chronic stress. Here, we investigate whether female social hierarchies and individual traits related to social rank likewise influence stress resilience. We observe the formation of dyadic female hierarchies under varying conditions of ambient light and circadian phase and subject mice to two forms of chronic psychosocial stress: social isolation or social instability. We find that stable female hierarchies emerge rapidly in dyads. Individual behavioral and endocrinological traits are characteristic of rank, some of which are circadian phase dependent. Further, female social rank is predicted by behavior and stress status prior to social introduction. Other behavioral characteristics suggest that rank is motivation-based, indicating that female rank identity serves an evolutionarily relevant purpose. Rank is associated with alterations in behavior in response to social instability stress and prolonged social isolation, but the different forms of stress produce disparate rank responses in endocrine status. Histological examination of c-Fos protein expression identified brain regions that respond to social novelty or social reunion following chronic isolation in a rank-specific manner. Collectively, female rank is linked to neurobiology, and hierarchies exert context-specific influence upon stress outcomes.

INTRODUCTION

Social species form hierarchies for protection, for division of labor, to reduce aggression, and to raise their young.[1–3] Social networks have a buffering effect on the progression of numerous psychiatric conditions, including anxiety and major depression,[4–7] which disproportionately affect women.[8,9] Stress-resilient effects of social buffering have also been characterized in rodents.[10] Individual social status is not equitably rewarding, however; subordinate rank in male animals is typically associated with higher stress, secondary access to resources, and lower reproductive success.[1,3,11] Similar trends are seen in humans, wherein social rank is often approximated from socioeconomic status (SES) and social capital.[1,10] In humans, lower SES contributes to the prevalence of stress-related mental illness through the production of chronic, low-grade psychosocial stress.[4,10] Despite this connection, there is a lack of consensus on how social status influences—or is influenced by—stress status. Furthermore, female hierarchies are understudied. In rodents, it has been shown that female social hierarchies are formed based on intrinsic individual traits, while male hierarchies are formed based on a history of winning agonistic encounters.[12] However, the way in which social identity mediates neurobiological responses to psychosocial stressors is not well understood.

It is difficult to recapitulate a complex societal framework within a laboratory environment in a way that is also ethologically relevant. To research the neurobiological underpinnings of stress-related disorders, animal studies have employed various paradigms to produce a stressed behavioral phenotype. These include chronic unpredictable mild stress, social defeat stress, and chronic corticosterone administration. Researchers frequently employ somatic rather than psychological stressors—paradigms incomplete in their ability to reproduce the conditions predisposing an individual to depressive or anxious symptomology.[8] Most animal studies have been performed using male-only subjects despite abundant evidence that anxiety and depression are nearly twice as common in women.[8,9] It is possible though to improve experimental validity by recreating some of the psychosocial stressors experienced in modern life, such as social inequality and loneliness, by

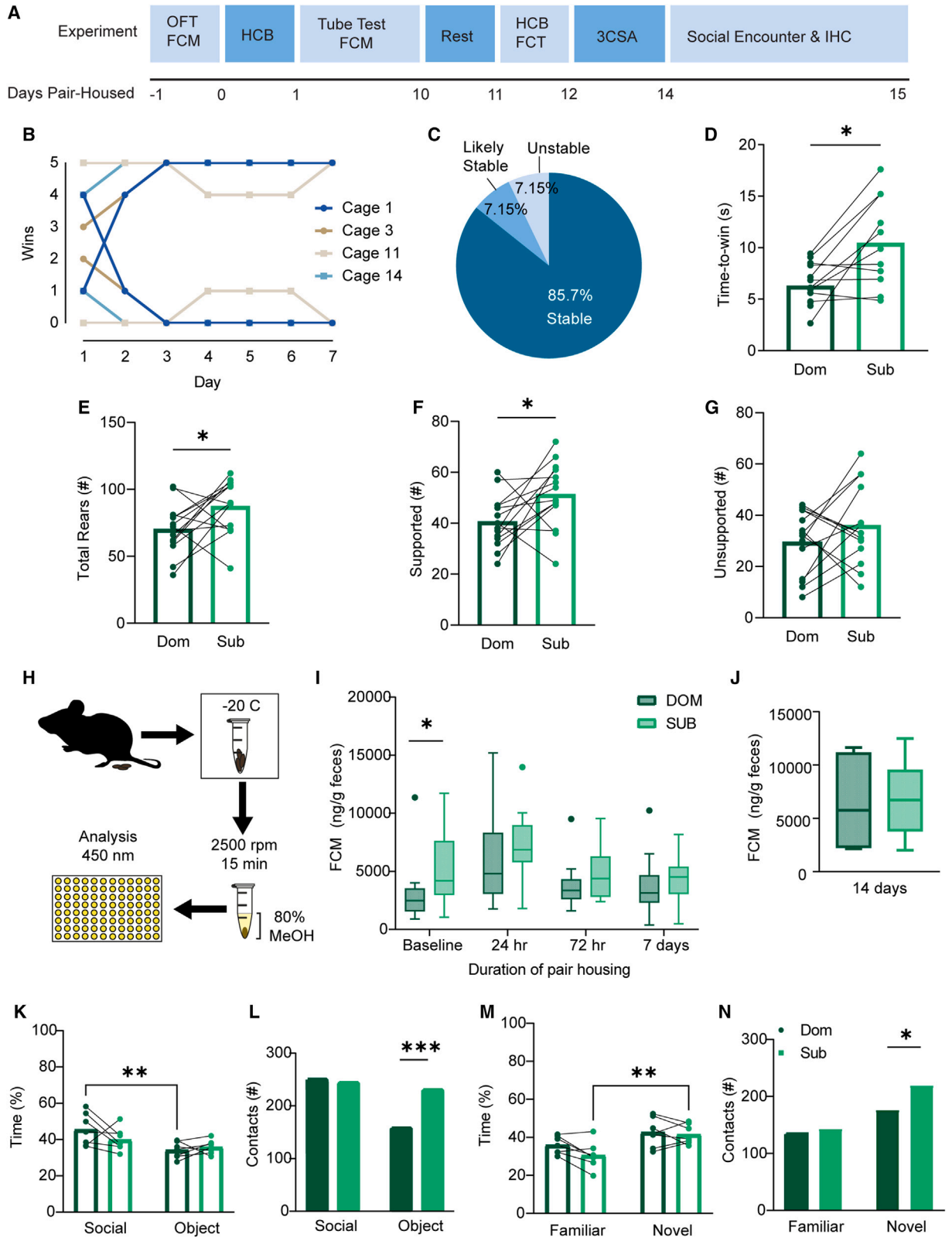

Figure 1. Determinants of future rank and variables associated with current rank

(A) Experimental timeline. OFT, open field test; FCM, fecal corticosterone metabolites; HCB, home cage behavior; FCT, food competition task; 3CSA, 3-chamber social approach; IHC, immunohistochemistry.

(legend continued on next page)

harnessing an animal's natural tendencies to form ranked social groups.

We hypothesized that, in female mice, social hierarchies directly influence stress status, thus conveying relative resilience or vulnerability to behavioral, endocrine, and neurobiological alterations arising from psychosocial stress. Here, we pair-housed age- and strain-matched female mice and analyze behavior and fecal corticosterone metabolites[13] before, during, and after hierarchy formation. We discover that divergent motivation, in addition to baseline endocrine status, defines female social rank. We also find that social rank affects the behavioral and endocrinological response to social isolation and social instability. Also, we report rank differences in brain c-Fos expression in two scenarios—after exposure to a novel social partner or after reunion with a familiar social partner, following extended social isolation. We find that subordinate animals show the greatest differences in regions related to cognitive processing and coordination of behavioral responses, whereas dominant animals had a greater difference in key regions associated with modulating homeostasis.

RESULTS

Subordinate social status is predicted by behavior and fecal corticosterone metabolites

We first investigated female hierarchy formation, rank characteristics, and social behaviors during the dark phase of the murine circadian cycle (Figure 1A). Social hierarchies were tested using competitive exclusion (tube test; STAR Methods)[14] and validated with a food competition task (FCT).[15] Before the tube test, mice are habituated to a short acrylic tube in the home cage; this serves both as a source of enrichment and a source of safety (hiding). During a match, two mice meet in an agonistic face-to-face challenge in a longer tube. One animal is forced out of the opposite end, either by active advancing or by passive resistance to removal and opponent retreat. The animal evicted from the tube is considered the loser.[14,15]

Dyads rapidly formed stable hierarchies (Figures 1B and 1C); most rank shifts occurred within the first 2 days of testing (Figure 1B). Time to run the length of the tube did not differ by rank, indicating that rank did not influence the ability to learn or perform the task (Figure S1A). As expected, dominants won most matches (Figure S1B), and when subordinates won, it took them significantly longer (Figure 1D). There were no rank differences in win style; most wins were active, meaning the conspecific was forcibly removed (Figure S1C). Results of the FCT strongly supported the ranks assigned by the tube test (Figure S1D).

Home cage behaviors, monitored before and after hierarchical establishment, did not reveal predictors of rank in social affiliative or non-social interactions (Figures S1E–S1G; Data S3). However, forepaw touch (placement of the forepaws on the conspecific accompanied by non-social sniffing) was overexpressed by subordinates during initial pairing (Figure S1F; Data S3B); this pattern disappeared after 11 days of continuous pair housing (Figure S1G; Data S3E).

In the open field test (OFT), future rank was predicted by total and exploratory supported rearing (Figures 1E and 1F) but not by stress-sensitive unsupported rearing,[16] locomotor behavior, or anxiety-like behavior[17–19] (Figures 1G and S1H–S1J).

Fecal corticosterone metabolite (FCM) excretion is representative of the animal's stress status several hours before passing the sample due to diurnal variations in gut transit time and thus metabolism of corticosterone in mice.[13] Rank was predicted by FCM before pair housing but not during hierarchy formation or stabilization (Figures 1H–1J). Most animals formed stable hierarchies within 4 days[14] (Figures 1B and 1C), indicating that an individual animal's rank in the first 24 h of testing matched its final rank. Therefore, in agreement with home cage behavior, the rank difference in FCM was present only during the initial period of hierarchical stabilization. At the end of testing, ranks did not differ in body or adrenal weight, suggesting there were no chronic stress effects from maintaining dominance status (Figures S1K and S1L).

After 2 weeks of pair housing, a randomly selected cohort of 14 mice was tested in a three-chamber social approach (3CSA) test of sociability and social novelty preference. During the sociability test, only dominant animals exhibited a social preference (Figures 1K and 1L), whereas during the social novelty

(B) Sample match data for four pairs. Cages are identified by number (i.e., cages 1–14). Each cage is depicted by color-matched lines representing the two mice in that dyad; symbols indicate the number of individual wins per day, and rank shifts are represented by cage lines crossing.
(C) Percentage of pairs that was rank-stable by the end of dominance testing (12/14 total). Stability, 4 consecutive days of identical pair ranking. "Likely stable" describes one hierarchy that, on the final day of testing, was rank stable for the 3 previous days. "Unstable" describes one hierarchy in which mice switched ranks every day.
(D) Average time to win a match. N = 25 (14 dom/11 sub); *p = 0.0145 (Welch's t test). Three subordinates never won and are excluded.
(E) Pre-pairing OFT total rears. *p = 0.0445 (paired t test).
(F) Pre-pairing OFT supported rears. *p = 0.0388 (paired t test).
(G) Pre-pairing OFT unsupported rears. p = 0.2390 (paired t test).
(H) FCM collection and processing.
(I) Longitudinal FCM. Baseline, *p = 0.0168; 24 h, p = 0.2020; 72 h, p = 0.3164; 7 days, p = 0.1852 (Mann-Whitney).
(J) FCM after 14-day pairing (during 3CSA). p = 0.8850 (unpaired t test).
(K) Percentage of total exploration in social or novel object chamber. No interaction, chamber × rank ($F_{(1,24)} = 2.438$, p = 0.1315); effect of chamber ($F_{(1,24)} = 10.81$, **p = 0.0031, mixed-effects; Šidák's; dom, **p = 0.0044; sub, p = 0.4133).
(L) Direct contacts with social or novel object cup. Significant relationship, rank × object ($\chi^2_{(1,386)} = 13.03$, ***p = 0.0003); no relationship, rank × social ($\chi^2_{(1,489)} = 0.03260$, p = 0.8567, Yates-corrected χ^2).
(M) Percentage of total exploration in familiar or novel social chamber. No interaction, chamber × rank ($F_{(1,12)} = 1.989$, p = 0.1839); effect of chamber ($F_{(1,12)} = 8.866$, *p = 0.0115, mixed-effects; Šidák's; dom, p = 0.1140; sub, **p = 0.0061).
(N) Direct contacts with familiar or novel social cup. Significant relationship, rank × novel ($\chi^2_{(1,395)} = 4.457$, *p = 0.0348); no relationship, rank × familiar ($\chi^2_{(1,276)} = 0.2930$, p = 0.5883, Yates-corrected χ^2).
Pre-3CSA, N = 28 (14 dom/14 sub). 3CSA, N = 14 (7 dom/7 sub). Data in (D)–(G), (K), and (M) displayed as means with lines indicating matched dyads. (I) and (J) displayed using Tukey method.
See also Figure S1 and Data S1 and S3.

preference test, only subordinate animals displayed a preference for the novel social stimulus (Figures 1M and 1N).

In summary, stable hierarchies rapidly emerged during pair housing. Future subordinate status was predicted by exploratory rearing, home cage behavior and FCM. Dominants demonstrated greater sociability but no preference for social novelty in the 3CSA, and the reverse pattern of behavior was seen in subordinates.

Circadian phase influences rank characteristics

Since circadian rhythms and lighting conditions can affect behavior,[20–22] we compared data from baseline OFT behavior and FCM across the circadian cycle to evaluate their influence on predictive rank characteristics.

In the OFT, total (Figure 2A) and supported rearing (Figure 2B) were significantly higher in subordinates during the dark phase. Unsupported rearing was also lower in subordinates during the light phase (Figure 2C), suggesting a stress effect from light phase testing. During the dark circadian phase, subordinates also crossed the inner zone (IZ) more frequently (Figure 2D) but did not dwell there (Figure 2E), suggesting increased exploration but not anxiolysis.[17–19] Interestingly, there was no effect of circadian phase on total distance traveled (TDT; Figure 2F), which could be explained by increased thigmotaxis during the light phase, compared with increased IZ entries in the dark. Finally, subordinates excreted significantly higher FCM during the dark phase, indicating an influence on predictive rank associations (Figure 2G).

Social instability produces an avoidant phenotype in subordinate females

We next wanted to explore the intersectionality of rank, environmental context, and stress. Therefore, we subjected mice to a form of psychosocial stressor—social instability stress (SIS)[23–25]—to determine how rank influences behavior and FCM in the face of hierarchical uncertainty (Figure 3A). Since new pairings occurred during each session, match data were used to assign David's score (DS; STAR Methods)[26] to every subject in order to investigate the degree to which dominance correlates with rank characteristics.

Pre-SIS, there were no group differences in OFT behavior (Figures S2A–S2D), and SIS did not alter rearing (Figures S2E and S2F). However, SIS animals exhibited increased TDT and IZ crossings (Figures 3B and 3C). Since SIS did not affect IZ time (Figure 3D), this behavioral effect was attributed to elevated locomotion rather than an anxiolytic phenotype.[17–19]

We also evaluated behavior in the elevated plus maze (EPM). Pre-SIS, there were no group differences in arm activity (Figures S2G–S2J). Interestingly, supported rearing differed by rank in a pattern identical to that observed in the dark phase OFT (Figure 3E). In the post-test, control animals exhibited a rank difference in closed arm entries that was absent in SIS animals (Figure 3F), and stressed subordinates made fewer open arm entries than controls (Figure 3G). Also, SIS altered rank-characteristic rearing behavior in the EPM (Figure 3H). Since the EPM evokes an unconditioned approach-avoidance conflict,[27] this suggests an additive effect of environmental context and stress on behavior.

A novel object recognition test (NORT) was performed to identify rank differences in recognition memory. SIS reduced entries into the novel object zone (Figure 3I) without affecting overall object exploration (Figure S2M). Notably, SIS reduced subordinate novelty preference (Figure 3J) and dominants post-SIS possessed a significant negative correlation between DS and novelty preference (Figure 3K).

Coat state score (CSS) was also evaluated as a pharmacologically validated measure of well-being and depression-like behavior.[28] All animals' CSS worsened during the experiment (Figure 3L), which was likely caused by the repeated use of the tube test; after a 14-day rest, control CSS improved while SIS CSS remained elevated (Figure 3L). These data suggest a lasting, rank-independent stress effect (Figure S2N).

Overall, FCM did not differ significantly between groups, although there was a trend for greater FCM in stressed animals during several SIS sessions (Figure S2P). Prior to the final session, there were also no clear rank differences (Figure S2Q); at the experimental endpoint, however, SIS had disproportionately increased subordinate FCM (Figure 3N).

In summary, SIS produced a hyperlocomotive phenotype in the OFT, increased subordinate FCM, reduced subordinate novelty preference in the NORT, and caused subordinate avoidance in the EPM. Collectively, these findings indicate that SIS produced an avoidant phenotype in subordinate animals.

Social isolation produces contrasting rank effects on behavior and FCM

Different forms of stress elicit distinct behavioral and neurobiological responses.[29,30] To explore how social rank moderates the response to different stressors, we next evaluated the effects of chronic social isolation (Figure 4A). Pre-isolation, there were no underlying differences in OFT behavior (Figures S3A–S3H). Isolation affected subordinate rearing and reduced unsupported rears (Figures 4B–4D) without affecting locomotion or anxiety-like behavior (Figures S3I–S3K). Controls displayed a rank difference in sucrose preference that was absent in isolated animals (Figure 4E).

There were no differences in FCM after 48 h of isolation (Figure 4F). Interestingly, FCM increased significantly after 30 days, suggesting a period of chronicity was necessary to produce endocrine changes (Figure 4G). As observed during the dark circadian phase (Figure 2G), there was a trend for higher pre-isolation FCM in subordinates, which disappeared after 48 h of isolation (Figure 4H). Post-isolation, there was a significant effect of stress on FCM and adrenal weight in dominants only (Figures 4I and 4J). Finally, after 3 weeks of isolation, CSS was significantly worse in isolated animals (Figure 4K), independent of rank (Figure S3L).

In summary, social isolation produced a behavioral phenotype in subordinates in the OFT, affected CSS and sucrose preference independent of rank, and disproportionately elevated FCM in dominants. Collectively, these indicate rank differences in the behavioral and endocrine coping strategies to social isolation.

Rank differences in c-Fos expression following social novelty or social reunion

We next wanted to determine the extent to which rank influences neural activity in response to social stimuli and psychosocial stress. Therefore, we examined the expression of the immediate early gene product c-Fos after exposure to a novel social encounter (Figure S4) or after social reunion with a familiar conspecific following chronic social isolation (Figure S5). We

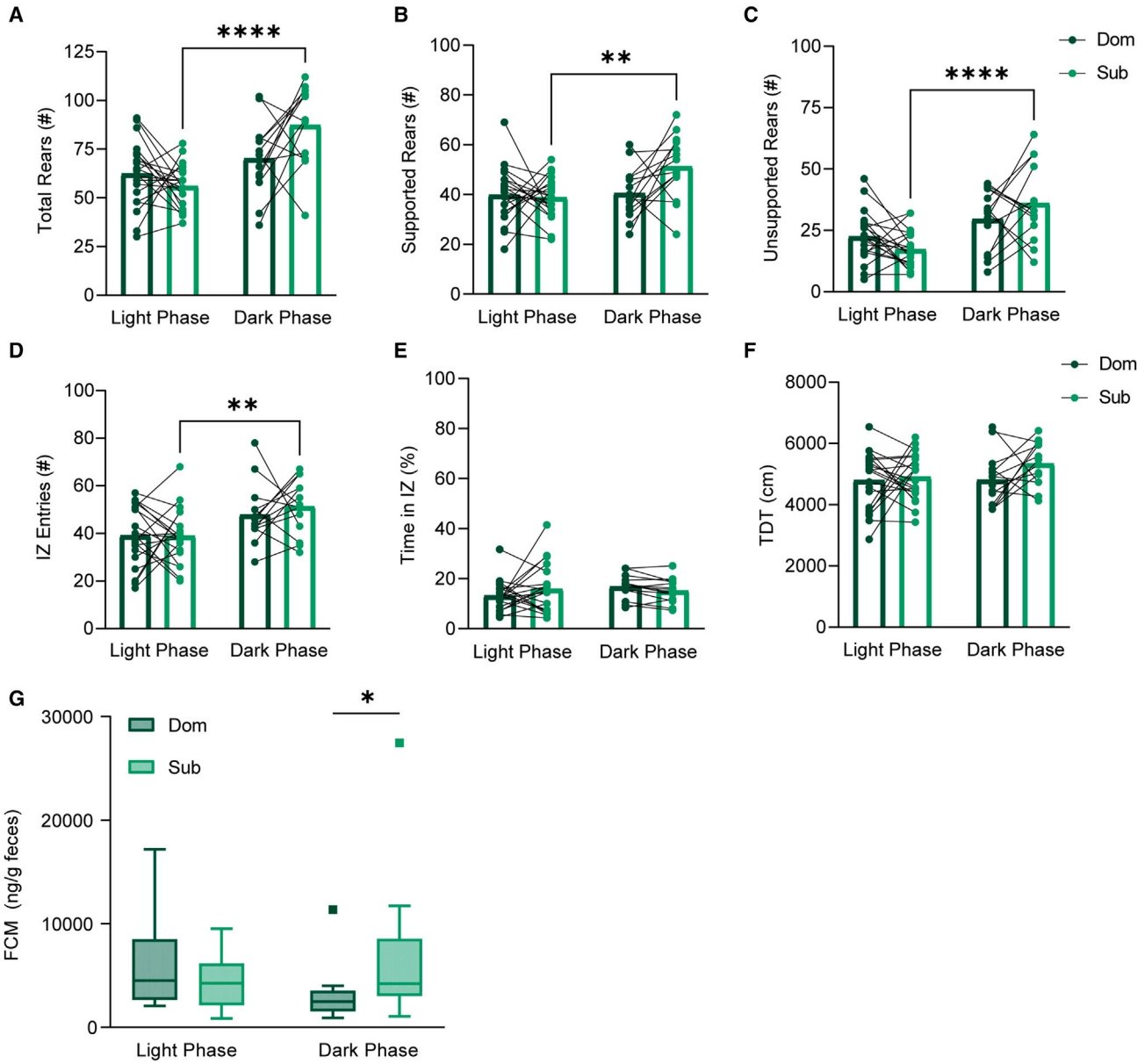

Figure 2. Circadian influence on behavioral and endocrinological rank predictors

(A) OFT total rears. Significant interaction, phase × rank ($F_{(1,64)} = 8.217$, **p = 0.0056); effect of phase ($F_{(1,64)} = 22.74$, ****p < 0.0001, mixed-effects; Šidák's; dom, p = 0.3333; sub, ****p < 0.0001).

(B) OFT supported rears. Significant interaction, phase × rank ($F_{(1,64)} = 5.057$, *p = 0.0280); effect of phase ($F_{(1,64)} = 6.272$, *p = 0.0148, mixed-effects; Šidák's; dom, p = 0.9796; sub, **p = 0.0026).

(C) OFT unsupported rears. Significant interaction, phase × rank ($F_{(1,32)} = 4.695$, *p = 0.0378); effect of phase ($F_{(1,32)} = 20.02$, ****p < 0.0001, mixed-effects; pair SD = 3.048; Šidák's; dom, p = 0.1473; sub, ****p < 0.0001).

(D) OFT IZ entries. No interaction, phase × rank ($F_{(1,32)} = 0.3954$, p = 0.5339); effect of phase ($F_{(1,32)} = 11.97$, **p = 0.0016, mixed-effects; pair SD = 2.754; Šidák's; dom, p = 0.0818; sub, **p = 0.0090).

(E) Time in IZ. No interaction, phase × rank ($F_{(1,32)} = 1.944$, p = 0.1729, mixed-effects; pair SD = 3.385).

(F) OFT distance traveled. No interaction, phase × rank ($F_{(1,64)} = 1.032$, p = 0.3136, mixed-effects).

(G) Baseline FCM. Significant interaction, phase × rank ($F_{(1,32)} = 6.468$, *p = 0.0162, mixed-effects; pair SD = 1141; Šidák's; light, p = 0.4630; dark, *p = 0.0471). Light, N = 40 (20 dom/20 sub); dark, N = 28 (14 dom/14 sub). All data, except for (G), expressed as means with lines indicating matched dyads. (G) displayed using Tukey method. Light phase ranks are assigned from initial pairings rather than final, DS-assigned ranks.

OFT, open field test; TDT, total distance traveled; IZ, inner zone; FCMs, fecal corticosterone metabolites.

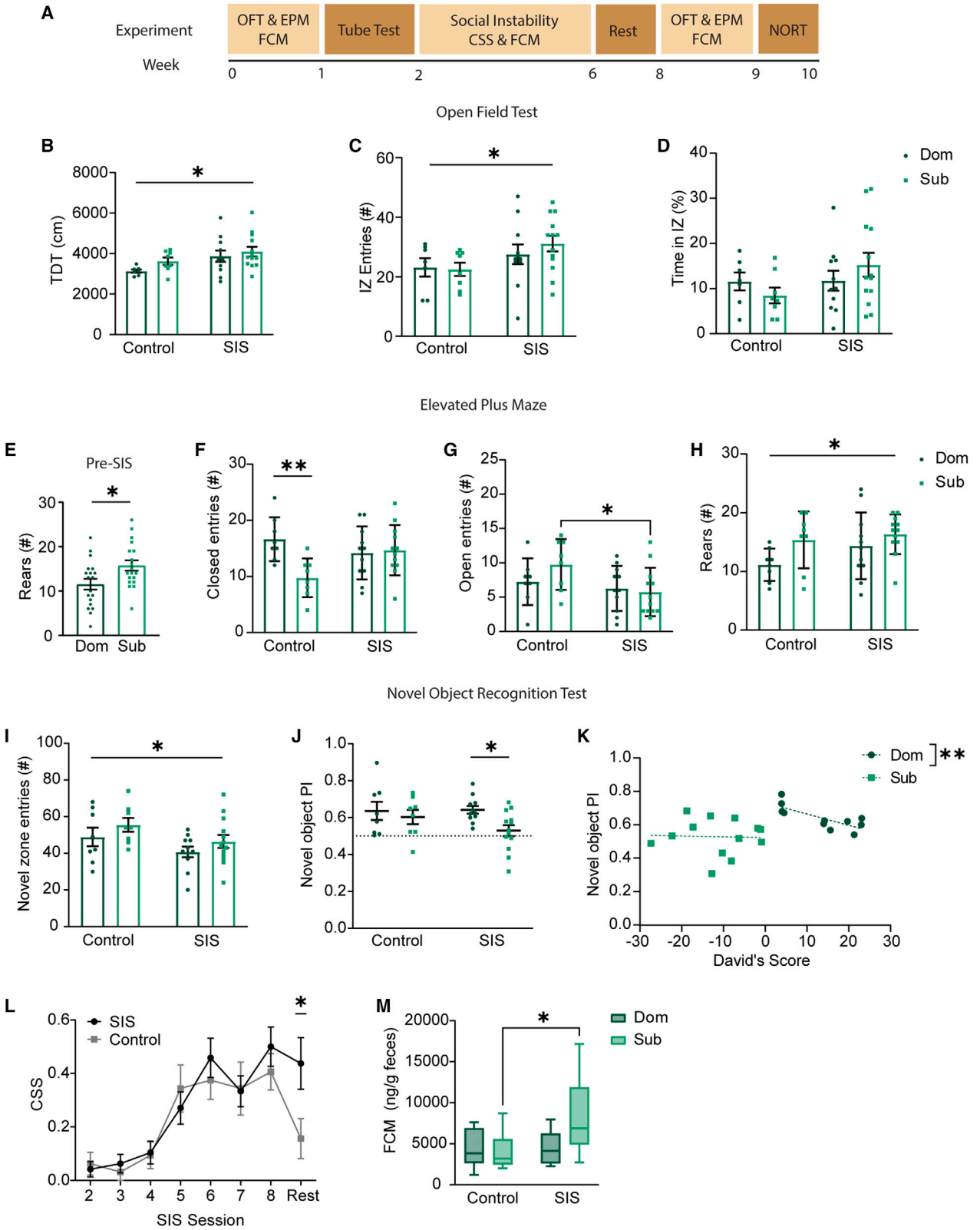

Figure 3. Rank differences in the effects of chronic social instability on behavior and stress status

(A) Experimental timeline. OFT, open field test; EPM, elevated plus maze; FCM, fecal corticosterone metabolites; CSS, coat state score; NORT, novel object recognition test.

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compared the magnitude and directionality of expression between the two social contexts by calculating the percent difference from the corresponding within-rank control mean (Figure 5). Seven regions were selected for analysis based upon their relevance to sociability and social identity, stress, and regulation of emotional valence.[1,31–52]

Social novelty increased claustral (CLA) c-Fos expression across ranks (Figure S4E),[31,32] whereas social reunion after isolation selectively increased CLA expression in subordinates (Figure S5C). In dominants, CLA expression increased after social reunion, compared with persistent isolation (Figure S5C), while in subordinates CLA c-Fos increased to a greater extent in both contexts (Figure 5B). Interestingly, control subordinates demonstrated reduced CLA c-Fos at "social baseline" conditions, such as during continuous pair housing or while alone (Figures S4E and S5C).

Social novelty significantly increased subordinate c-Fos expression in the prelimbic (PL) medial prefrontal cortex (mPFC) (Figure S4F), the lateral septum (LS) (Figure S4G), and the core of the nucleus accumbens (nAcc) (Figures S4H, S6A, and S6B). These regions are all associated with social recognition, social behavior, and regulating the emotional valence of social encounters.[1,33–42] Social reunion produced a rank-independent increase in PL expression (Figure S5D), but it significantly increased LS c-Fos in dominants only (Figure S5E). Isolation and reunion both increased expression in the nAcc core of subordinates (Figure S5F). Collectively, these data suggest that social reunion or novelty produces opposing rank effects on c-Fos expression in the LS but similar effects in the PL and nAcc core.

Compared with control, the greatest change in both the PL and the LS occurred in subordinates exposed to social novelty (Figures 5C and 5D), whereas in the nAcc core the subordinate effect was independent of context (Figure 5E). This relationship suggests that c-Fos expression in the PL and LS may be more sensitive to contextual differences arising from social novelty, while the changes in nAcc expression are more related to subordinate social identity.

The paraventricular nucleus (PVN) of the hypothalamus is implicated in regulating emotional state perception and social transmission of stress.[43–46] In dominants, both stimuli increased c-Fos in the PVN (Figures S4I and S5G), and there was a significant elevation from control after social reunion only in dominants (Figure 5G).

The paraventricular thalamus (PVT) is a homeostatic regulator involved in modulating arousal, valence, and motivation,[47–49] and it is essential for the execution of context-dependent approach or avoidance.[50–52] In dominants, social novelty reduced PVT c-Fos (Figure S4J), whereas in subordinates social isolation and reunion suppressed expression (Figure S5H). This rank effect was reproduced in the difference from control means, with the largest effect sizes in social novelty for dominants and reunion for subordinates (Figures 5H and 5I).

The PVT has spatially and genetically distinct neuronal subpopulations with divergent roles in processing rewarding and aversive stimuli.[48,49] The anterior PVT conveys arousal information to the cortex, whereas the posterior PVT responds to somatic aversive stimuli such as footshock.[48] Within the anterior PVT, further differences in functional connectivity exist between the most anterior (aPVT) and middle (mPVT) portions.[49] The mPVT has more connectivity to the brainstem and may be more involved in conveying arousal information in response to aversive stimuli.[49] Therefore, we examined the distribution of c-Fos expression in the aPVT and mPVT. Social novelty reduced expression in the mPVT of dominant animals (Figures S4K and S4L), whereas social reunion and persistent isolation reduced expression only in the aPVT of subordinates. Interestingly, social reunion increased c-Fos expression in the mPVT of dominants, compared with persistent isolation, but not to the control state (Figures S5I and S5J), suggesting pair housing elicits a degree of mPVT activity.

(B) Post-SIS OFT distance traveled. No interaction, stress × rank ($F_{(1,35)}$ = 0.284, p = 0.5974); effect of stress ($F_{(1,35)}$ = 6.074, *p = 0.0188, two-way ANOVA; Šidák's; dom, p = 0.0943; sub, p = 0.3025).
(C) Post-SIS IZ entries. No interaction, stress × rank ($F_{(1,35)}$ = 0.4906, p = 0.4883); effect of stress ($F_{(1,35)}$ = 4.628, *p = 0.0384, two-way ANOVA; Šidák's; dom, p = 0.5495; sub, p = 0.0852).
(D) Post-SIS time in IZ. No interaction, stress × rank ($F_{(1,35)}$ = 1.793, p = 0.1891, two-way ANOVA).
(E) Pre-SIS rears in the EPM. N = 38 (18 dom/20 sub); *p = 0.0424 (unpaired t test).
(F) Post-SIS EPM closed arm entries. Significant interaction, rank × stress ($F_{(1,35)}$ = 7.030, *p = 0.0120); effect of stress ($F_{(1,35)}$ = 5.300, *p = 0.0274, two-way ANOVA; Šidák's; SIS, p = 0.9545; control, **p = 0.0054).
(G) Post-SIS EPM open arm entries. No interaction, stress × rank ($F_{(1,35)}$ = 1.789, p = 0.1897); effect of stress ($F_{(1,35)}$ = 4.850, *p = 0.0343, two-way ANOVA; Šidák's; dom, p = 0.7960; sub, *p = 0.0322).
(H) Post-SIS EPM rears. No interaction, stress × rank ($F_{(1,35)}$ = 0.6461, p = 0.4269); effect of rank ($F_{(1,35)}$ = 4.807 *p = 0.0351, two-way ANOVA; Šidák's; SIS, p = 0.4903; control, p = 0.1146).
(I) Novel object zone entries. No interaction, rank × stress ($F_{(1,36)}$ = 0.0135, p = 0.9082); effect of stress ($F_{(1,36)}$ = 5.017, *p = 0.0314, two-way ANOVA; Šidák's; dom, p = 0.2746; sub, p = 0.1878).
(J) Novel object PI. No interaction, rank × stress ($F_{(1,36)}$ = 1.351, p = 0.2527); effect of rank ($F_{(1,36)}$ = 4.482, *p = 0.0412, two-way ANOVA; Šidák's; SIS, *p = 0.0275; control, p = 0.7894).
(K) Correlation between DS and novel object PI in SIS subjects. Dom, R = −0.7728, **p = 0.0053, $F_{(1,9)}$ = 13.34, R^2 = 0.5972; sub, R = −0.03603, p = 0.9070, Pearson's, $F_{(1,11)}$ = 0.01430, R^2 = 0.001298 (SLR).
(L) Stress versus control CSS during SIS and post-rest. Rest, *p = 0.0267 (Mann-Whitney).
(M) FCM after final SIS session. Significant interaction, rank × stress ($F_{(1,36)}$ = 4.163, *p = 0.0487); effect of stress ($F_{(1,36)}$ = 4.354, *p = 0.0441, two-way ANOVA; Šidák's; dom, p = 0.9993; sub, *p = 0.0106).
Stress, N = 24 (11 dom/13 sub); control, N = 16 (8 dom/8 sub). Data expressed as mean ± SEM except for (M). (M) displayed using Tukey method. One outlier was excluded for post-SIS OFT data (Grubb's, α = 0.05). Post-SIS OFT: stress, N = 24 (11 dom/13 sub); control, N = 15 (7 dom/8 sub). Two animals fell during pre-SIS EPM, and one during post-SIS EPM; these were excluded. Post-SIS EPM: stress, N = 23 (11 dom/12 sub); control, N = 16 (8 dom/8 sub).
SIS, social instability stress; PI, preference index.
See also Figure S2.

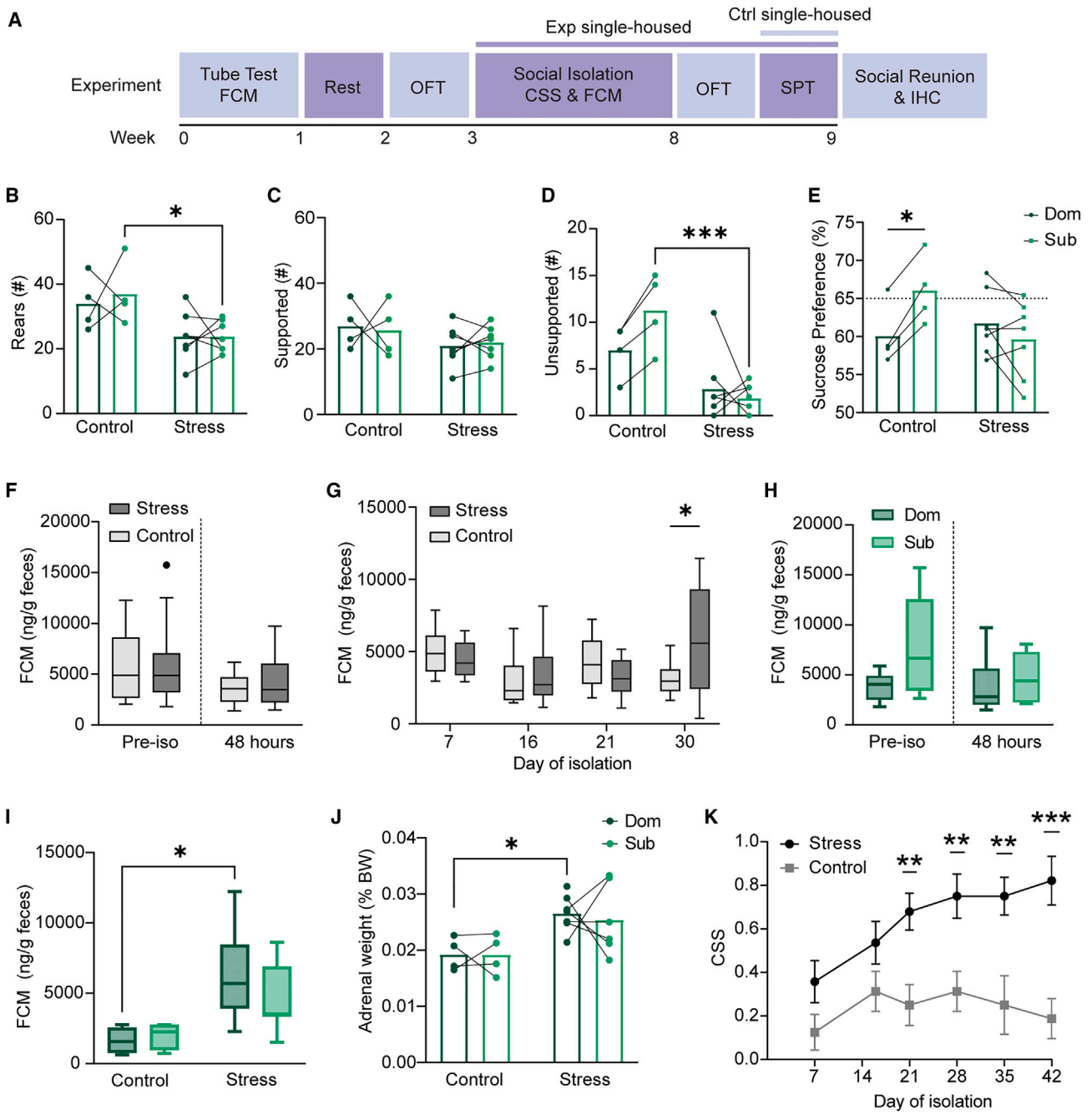

Figure 4. Social isolation induces contrasting rank effects in stress status and stress-sensitive behaviors

(A) Experimental timeline. FCM, fecal corticosterone metabolites; OFT, open field test; CSS, coat state score; SPT, sucrose preference test; IHC, immunohistochemistry.

(B) Post-isolation OFT total rears. No interaction, stress × rank ($F_{(1,18)} = 0.2079$, $p = 0.6539$); effect of stress ($F_{(1,18)} = 12.53$, **$p = 0.0023$, mixed-effects; Šidák's; dom, $p = 0.0837$; sub, *$p = 0.0223$).

(C) Post-isolation OFT supported rears. No interaction, stress × rank ($F_{(1,18)} = 0.1583$, $p = 0.6954$, mixed-effects).

(D) Post-isolation OFT unsupported rears. Significant interaction, stress × rank ($F_{(1,9)} = 5.408$, *$p = 0.0451$); effect of stress ($F_{(1,9)} = 17.48$, **$p = 0.0024$, mixed-effects; pair SD = 1.852; Šidák's; dom, $p = 0.0978$; sub, ***$p = 0.0003$).

(E) Sucrose preference as percentage of volume consumed over 3 days. Significant interaction, stress × rank ($F_{(1,9)} = 15.75$, **$p = 0.0033$, mixed-effects; pair SD = 3.909; Šidák's; control, *$p = 0.0101$; stress, $p = 0.2267$).

(F) FCM pre-isolation and after 48 h. Pre-iso, $p = 0.9734$ (Mann-Whitney); 48 h, $p = 0.5322$ (unpaired t test).

(G) FCM over 30 days of isolation. Day 30, *$p = 0.0213$ (Welch's t test).

(H) Ranked FCM in stress group pre-isolation and after 48 h. Pre-iso, $p = 0.1065$ (paired t test); 48 h, $p = 0.4688$ (Wilcoxon).

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DISCUSSION

Dyadic hierarchy characterization and predictive traits associated with rank

We studied the formation and maintenance of dyadic female social hierarchies and discovered key behavioral and neurobiological rank differences in baseline characteristics and stress responsivity (Figures 6A and 6B). Dyads rapidly formed rank-stable hierarchies exhibiting several attributes traditionally associated with rank in male mice. Notably, subordinate females had higher FCM (Figure 1I) and exhibited more exploratory behavior in the OFT (Figure 1F), whereas dominant females had increased access to a food reward (Figure S1D), greater resistance to losing competitive encounters (Figure 1D), and greater sociability, which have variably been reported as characterizing subordinate and dominant rank in male mice, respectively.[1,15,53]

The literature describing male rank characteristics is extensive, but findings are frequently contradictory.[54] Therefore, we replicated our analyses under differing contexts to further elucidate how context influences manifestations of rank. Indeed, in this study, rank associations were found to be context dependent; future subordinates engaged in more exploratory rearing and had higher baseline FCM during the dark phase (Figures 1F and 1I), and they overexpressed "forepaw touch" behavior only upon initial social introduction (Figure S1F). Interestingly, the EPM reproduced rank differences during the light phase (Figure 3E), indicating that environmental context, as well as circadian phase, can influence rank-associated phenotypes.

After hierarchies stabilized, dominants exhibited greater sociability, whereas subordinates demonstrated a greater preference for social novelty (Figures 1K–1N). Notably, after stabilization, the rank difference in home cage forepaw touch disappeared (Figure S1G). Given the evidence for social novelty and exploratory preferences in subordinates, forepaw touch may be novelty-sensitive and ethologically both a social and exploratory behavior. Collectively, these findings suggest greater exploratory drive in subordinates.

Behavioral and biological characteristics may underlie a broader context-sensitive difference in motivation that influences, or is influenced by, an animal's social status. For example, subordinate male mice assume exploratory behaviors upon first exposure to the OFT.[53,55] Despite this, a recent meta-analysis[54] found that study heterogeneity has resulted in lack of a clear relationship between exploratory behavior and male social rank. It should be noted that all behavioral tests in that analysis were outcome measures—none were used in a predictive capacity prior to dominance testing. The characteristics identified in this study may therefore represent some of the unknown intrinsic traits defining female social status,[12] since they were independent of a history of winning agonistic encounters. Further studies are necessary to determine the importance of context novelty in reproducing these traits across circadian phases.

Circadian phase and testing context exert influence over rank associations

Mice are primarily nocturnal, and they engage in spontaneous social behaviors more frequently during the dark phase of their circadian cycle.[20–23,56] To optimize the conditions for social interactions during home cage behavior analysis, in the first experiment, mice were housed on a reverse light cycle. In subsequent experiments mice were tested during the light phase to facilitate behavioral tests that are performed under lighting conditions that would disturb the circadian rhythm of dark-housed animals.[22]

Circadian phase affected exploration; changes in rearing and IZ entries suggest that there is a stress component associated with light phase testing and that subordinate characteristics are disproportionately affected (Figures 2A–2D). Lighting conditions and circadian phase have both been shown to affect rodent locomotor behavior in laboratory settings; this has been proposed as one cause of inter-study variability in behavioral research.[57] Interestingly, and in contrast to other studies, circadian differences were not observed for TDT (Figure 2F), indicating rearing is ethologically distinct from locomotion. This change may be attributed to the lighting conditions employed throughout testing; regardless of circadian phase of testing, the OFT was performed under dim lighting that may have reduced the aversiveness of the open field and promoted exploration.

Absolute FCM recovery was not influenced by circadian phase as expected,[13] but baseline rank differences were only significant during the dark phase (Figure 2G). It is possible that prerequisite conditions were not met during the light phase due to differences in diurnal excretion patterns.[13] Therefore, the relationship between FCM and rank should be interpreted with careful attention to context. Indeed, the literature remains conflicted regarding the relationship between social identity, sex, and stress status.[53,54,58] It is therefore prudent to consider variables influencing steroid recovery, including species- and sex-specific excretion patterns, sample source, and the temporal proximity to any stressors.

Ranks respond differently to alternate forms of psychosocial stress

Stress-naive subordinates demonstrate a pro-exploratory motivation in the EPM that is abolished by SIS (Figures 3E–3G). Since this motivation was established as characterizing subordinate rank at baseline, these findings are evidence for a loss of trait behavior. Further, loss of novelty preference and changes in the pattern of EPM arm entry were interpreted as the development of an avoidance phenotype in SIS subordinates (Figures 3G and 3J). Interestingly, SIS did not increase FCM in

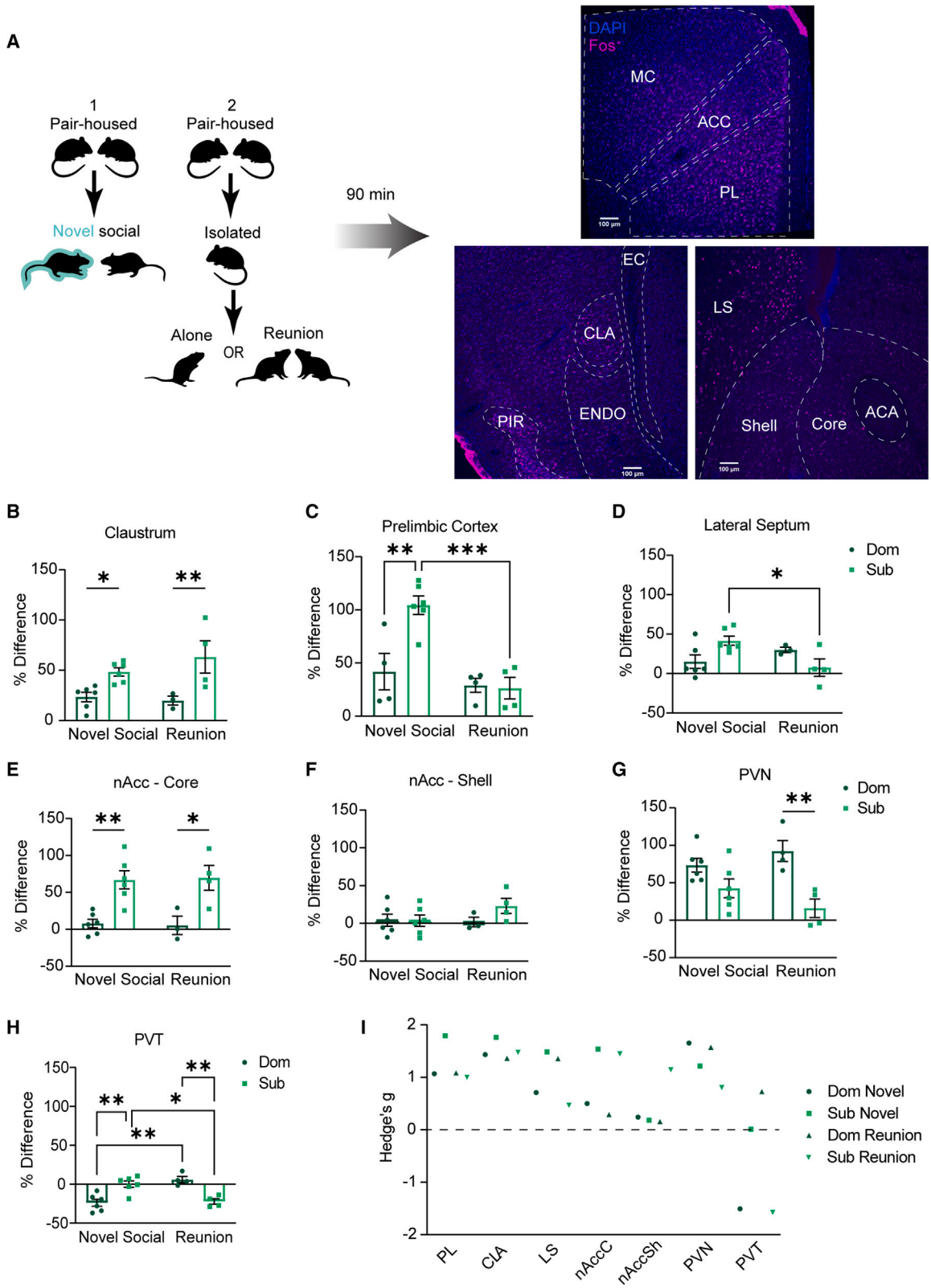
(I) FCM at the end of isolation (42 days). No interaction, stress × rank ($F_{(1,18)}$ = 1.077, p = 0.3132); effect of stress ($F_{(1,18)}$ = 11.57, **p = 0.0032, mixed-effects; Šidák's; dom, *p = 0.0113; sub, p = 0.2113).

(J) Paired adrenal weight (as percentage of final body weight [BW]). No interaction, stress × rank ($F_{(1,17)}$ = 0.0817, p = 0.7785); effect of stress ($F_{(1,17)}$ = 11.84, **p = 0.0031, mixed-effects; Šidák's; dom, *p = 0.0317; sub, p = 0.0823). One adrenal was lost during processing; data were excluded.

(K) Weekly CSS by group. 21 days, **p = 0.0091; 28 days, **p = 0.0089; 35 days, **p = 0.0055; 42 days, ***p = 0.0004 (Mann-Whitney).

Stress, N = 14 (7 dom/7 sub); control, N = 8 (4 dom/4 sub). Data in (B)–(E) and (J) displayed as means with lines indicating matched dyads. (F)–(I) displayed using Tukey method. (K) displayed as mean ± SEM. Final FCM samples were taken pre-SPT, during which control animals were isolated.

See also Figure S3.



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dominant animals, which contributed to the lack of a clear stress effect at the experimental endpoint (Figure 3M).

In contrast to SIS, social isolation stress produced overt effects on behavior, increased FCM, and reduced sucrose preference (Figures 4B–4E and 4G). Unsupported rearing is considered stress-sensitive because acute stress and aversive environments reduce its occurrence in the OFT.[16] Isolation reduced unsupported rearing only in subordinate animals (Figure 4D). Interestingly, post-isolation FCM and adrenal weight were disproportionately elevated in dominant animals (Figures 4I and 4J).

In summary, there appear to be contrasting behavioral and endocrinological effects of social instability and social isolation on ranks. Subordinate females are resilient to the endocrinological effects of social isolation but susceptible to behavioral changes, with the reverse being true for dominants. After SIS, subordinates develop an avoidance phenotype that is uncharacteristic of the rank in stress-naive conditions. By contrast, dominants are more resilient to SIS than to social isolation and do not manifest stress changes behaviorally in the OFT. These findings suggest a difference in coping strategy that is predicated on social rank, wherein subordinate animal behavior is sensitive to changes in social environment while dominants experience endocrinological consequences to the absence of social reinforcement.

Effects of social novelty on subordinate neuronal activity

The PL is involved in social decision-making and responses to novel vs. familiar social encounters.[1,33] Increased excitability in the dorsal mPFC, including the PL, is associated with dominance in male mice.[1,34] Interestingly, social novelty disproportionately increased PL c-Fos in subordinates (Figure 5C), supporting its role in novelty salience while also suggesting a sex-difference in the PL manifestation of dominance.

The CLA likewise coordinates salience detection, vigilance, and attention, but is primarily associated with the integration and relay of cortical and limbic information.[31,32] Whereas the CLA is an integratory hub, the LS has well-established roles in mediating social behavioral responses including social aggression, recognition, and preference.[35–38] Similarly, the nAcc core regulates motivated behavior involving social reward and decision-making and importantly, reward approach and acquisition.[39–42] In agreement with behavior, the subordinate pattern of enhanced c-Fos expression in these three regions (Figures 5B–5E) is consistent with a pro-exploratory motivation engaging salience evaluation, attention, and social approach in the context of social novelty. This is further supported by lower PL engagement during social reunion (Figure 5C), suggesting a role in regulating novel social experiences differentially in subordinate females. In contrast to the nAcc core, the shell regulates the assignment of hedonic value to stimuli.[42] In this study, neither intervention produced a significant change in the nAcc shell (Figure 5F), suggesting rank does not influence the hedonic value of social interaction in females. Collectively, this suggests a disproportionate effect of social novelty on decision-making, information relay, and potentially social reward in subordinates, which may reflect the rank difference in social novelty preference identified in the 3CSA (Figures 1K–1N).

Effects of social novelty on dominant neuronal activity

The ubiquitous increase in CLA c-Fos expression, following social novelty, likely reflects its role in relaying attentional state information.[31,32] In either context, dominant controls exhibited trending greater baseline CLA expression (Figures S4E and S5C), which reduced the significance of the c-Fos response to social reunion. Together, these data suggest that the CLA of subordinates may be more sensitive to high-arousal social interactions (Figure 5B).

Both contexts increased PVN c-Fos expression in dominants, supporting its known role in regulating social encounters[43] (Figures S4I and S5G). Interestingly, while social novelty produced a non-significant increase in expression in the PVN of subordinates, reunion had no effect (Figure 5G). One interpretation is that PVN activity is associated with social investigation or challenge in dominants and is more relevant for novelty recognition in subordinates. This is supported by the fact that increased expression was seen in the LS and PL of novelty-exposed subordinates—regions linked to social novelty recognition[35,59,60] (Figures 5C and 5D). Higher

Figure 5. Differential c-Fos expression in stress-naive animals exposed to a novel social partner and socially isolated animals following social reunion

(A) Schematic depiction of the c-Fos experiments (STAR Methods).

(B) CLA difference from control. Novel, N = 12 (6 dom/6 sub); reunion, N = 7 (3 dom/4 sub); no interaction, stimulus × rank ($F_{(1,15)} = 1.275$, $p = 0.2767$); effect of rank ($F_{(1,15)} = 17.42$, ***$p = 0.0008$, two-way ANOVA; Šidák's; novel, *$p = 0.0461$; reunion, **$p = 0.0092$).

(C) PL difference from control. Novel, N = 10 (4 dom/6 sub); reunion, N = 8 (4 dom/4 sub); significant interaction, stimulus × rank ($F_{(1,14)} = 8.652$, *$p = 0.0107$); effects of stimulus ($F_{(1,14)} = 16.80$, **$p = 0.0011$) and rank ($F_{(1,14)} = 7.294$, *$p = 0.0172$, two-way ANOVA; Šidák's; novel:dom × novel:sub, **$p = 0.0055$; novel:sub × reunion:sub, ***$p = 0.0008$).

(D) LS difference from control. Novel, N = 12 (6 dom/6 sub); reunion, N = 7 (3 dom/4 sub); significant interaction, stimulus × rank ($F_{(1,15)} = 8.372$, *$p = 0.0111$, two-way ANOVA; Šidák's; dom, $p = 0.4403$; sub, *$p = 0.0184$).

(E) nAcc core difference from control. Novel, N = 12 (6 dom/6 sub); reunion, N = 7 (3 dom/4 sub); no interaction, stimulus × rank ($F_{(1,15)} = 0.04516$, $p = 0.8346$); effect of rank ($F_{(1,15)} = 25.23$, ***$p = 0.0002$, two-way ANOVA; Šidák's; novel, **$p = 0.0024$; reunion, *$p = 0.0101$).

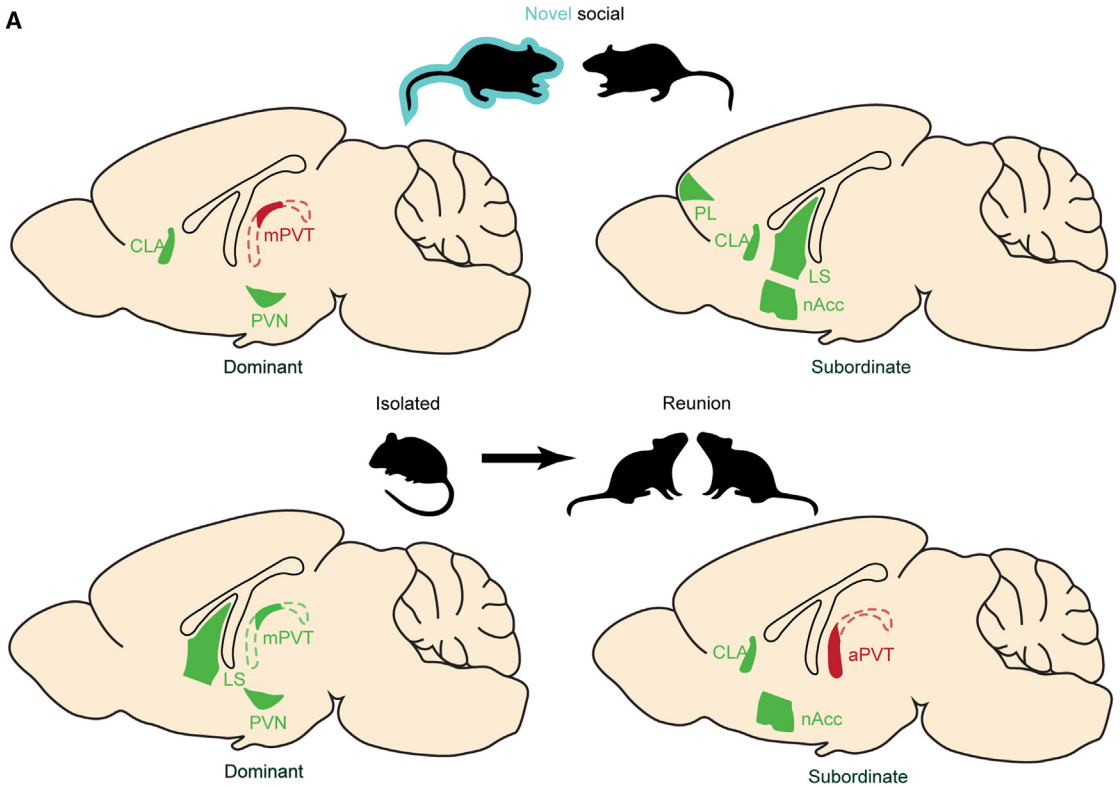
(F) nAcc shell difference from control. Novel, N = 12 (6 dom/6 sub); reunion, N = 7 (3 dom/4 sub); no interaction, stimulus × rank ($F_{(1,15)} = 1.556$, $p = 0.2313$).

(G) PVN difference from control. Novel, N = 12 (6 dom/6 sub); reunion, N = 8 (4 dom/4 sub); no interaction, stimulus × rank ($F_{(1,16)} = 3.464$, $p = 0.0812$); effect of rank ($F_{(1,16)} = 19.17$, ***$p = 0.0005$, two-way ANOVA; Šidák's; novel, $p = 0.1238$; reunion, **$p = 0.0019$).

(H) PVT difference from control. Novel, N = 12 (6 dom/6 sub); reunion, N = 8 (4 dom/4 sub); significant interaction, stimulus × rank ($F_{(1,16)} = 35.18$, ****$p < 0.0001$, two-way ANOVA; Šidák's; novel:dom × novel:sub, **$p = 0.0032$; novel:dom × reunion:dom, **$p = 0.0012$; novel:sub × reunion:sub, *$p = 0.0148$; reunion:dom × reunion:sub, **$p = 0.0047$).

(I) Hedge's g statistic by region. Refer to Data S2B for a complete list of values.

Data expressed as mean ± SEM. mPFC, medial prefrontal cortex; PL, prelimbic cortex; ACC, anterior cingulate cortex; MC, motor cortex; CLA, claustrum; PIR, piriformis; ENDO, endopiriformis; LS, lateral septum; nAcc, nucleus accumbens; PVN, paraventricular hypothalamic nucleus; PVT, paraventricular thalamic nucleus. See also Figures S4 and S5, Data S2, and Tables S1 and S2.



B

Condition	SR		UR		TDT		FCM		Sociability		Novety	
	Dom	Sub	Dom	Sub	Dom	Sub	Dom	Sub	Dom	Sub	Dom	Sub
Stress naïve		↑						↑	↑			↑
Social instability					↑	↑		↑	NT	NT		↓
Social isolation				↓			↑		NT	NT	NT	NT

Figure 6. Key differences between female ranks and stress responsivity

(A) Schematic of rank-specific changes in c-Fos expression. Green indicates regions with increased expression and red indicates regions with decreased expression.

(B) Summarized rank effects on behavior in stress paradigms. Arrows indicate directionality of change: ↑ increased after stress or higher for that rank (stress-naive); ↓ decreased after stress or lower for that rank (stress-naive); – no difference after stress, or between ranks (stress-naive); NT, not tested.

SR, supported rears; UR, unsupported rears; TDT, total distance traveled; FCM, fecal corticosterone metabolites; PL, prelimbic cortex; CLA, claustrum; LS, lateral septum; nAcc, nucleus accumbens; PVN, paraventricular hypothalamic nucleus; a/mPVT, anterior/middle paraventricular thalamic nucleus.

baseline FCM status could represent biological coping or priming to lower social status in novel social encounters, which, in agreement with changes in PVN c-Fos expression, becomes attenuated in the context of social or hierarchical familiarity. Further studies are necessary to determine the relative contribution of PVN cell types in signaling social context by rank.

Both the PVN and PVT influence pro-social behavior.[43,47,52] The aPVT primarily conveys information about arousal to the cortex and fear state information to the brainstem.[48,49] The mPVT can be distinguished from the aPVT by enhanced connectivity to the parabrachial nucleus, indicating the mPVT may be more sensitive to aversion than the aPVT.[49] The decreased c-Fos expression in mPVT (Figure S4L) therefore suggests that the PVT of dominant animals exposed to social novelty suppresses aversive signaling pathways and facilitates social investigation through PVN and CLA activation.

Effects of social reunion after chronic social isolation on subordinate neural activity

Increased nAcc core expression in both social contexts (Figure 5E) suggests that social investigation may dictate an approach or avoidance response of greater magnitude in subordinates. This supports a generalized pro-exploratory motivation, and elevated expression during persistent isolation could represent a form of biological coping (Figure S5F). Further experiments investigating the types of neurons activated during social

exposure, and their involvement in mediating motor output, are necessary to explore the salience of social encounters by rank.

Social reunion and isolation produced similar expression patterns, with the exception that only reunion increased expression in the CLA (Figure S5C), supporting a role for the CLA in mediating social investigation in subordinates.

Social isolation may induce a pro-homeostatic state in the aPVT of subordinate animals by suppressing neuronal subpopulations associated with arousal when alone and during social reunion (Figure S5I). This could represent another biological coping mechanism employed because of the intrinsic state of subordinates under the conditions of social isolation. Whether this could be recreated in a familiar social encounter without prolonged social isolation requires further experiments.

Effects of social reunion after chronic social isolation on dominant neural activity

There is evidence for PVN-LS co-regulation of social interactions; a PVN-LS circuit has been identified as essential for social recognition in mice,[38] and both regions are sensitive to social defeat.[46] Further, both the LS and the PVN have a potential role in the valence modulation of social stimuli.[38,44] Since social reunion, but not social novelty, increased expression in both regions (Figures S5E and S5G), this suggests that dominants may experience enhanced recruitment of social recognition pathways after social isolation. However, enhanced LS activation could represent pro-social behavior, or it could belong to a larger pro-stress, pro-dominance behavioral circuit, especially given the well-described role of the LS in mediating aggression.[38] This latter interpretation is supported by the isolation-induced reversal of mPVT suppression (Figure S5J), possibly engaging aversive state signaling while the PVN and LS facilitate social recognition. Since neither persistently isolated nor pair-housed dominants demonstrated an increase in mPVT c-Fos (Figure S5J), this further suggests that social isolation is necessary to make familiar social interactions aversive. Given the divergent nature of the LS in salience and behavioral output regulation, future studies should determine which cell types are involved and whether there are identifiable traits that could be used to designate dominance assertion in female mice, since in our study, we did not observe agonistic behaviors. Further studies are also indicated to determine what effect a novel social encounter after prolonged isolation would have on PVN and mPVT c-Fos expression in dominant females, as well as to determine whether isolation alters social reward salience.

Concluding remarks

Collectively, these findings suggest a motivation difference by rank that is stress-sensitive and engages various behavioral, endocrinological, and neurobiological responses to maintain homeostasis. We propose a scenario wherein subordinate status is associated with baseline endocrinological priming, a pro-exploratory motivation that is sensitive to psychosocial stress, and a neuromodulatory strategy that responds with pro-exploratory and approach signaling to social encounters, and which engages homeostatic coping pathways to reduce arousal following chronic social isolation. By contrast, dominant status is associated with a pro-social motivation reinforced by a lower endocrine stress status in baseline social situations. However, dominants do not engage the endocrinological coping mechanisms of subordinates and are therefore susceptible to social isolation, which is associated with an increase in aversive state processing upon familiar social encounters. This makes subordinates more susceptible to the consequences of social uncertainty and dominants to those of social isolation.

STAR★METHODS

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

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cub.2023.03.020.

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AUTHOR CONTRIBUTIONS

Conceptualization, L.S.-O.; methodology, L.S.-O., R.P., and J.P.F.; validation, L.S.-O.; formal analysis, L.S.-O. and A.D.; investigation, L.S.-O., A.D., and A.R.; resources, L.S.-O., A.D., R.P., and J.P.F.; writing – original draft, L.S.-O.; writing – review & editing, A.D., R.P., and J.P.F.; visualization, L.S.-O.; supervision, J.P.F.; project administration, L.S.-O.; funding acquisition, L.S.-O. and J.P.F.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti-cFos	Synaptic Systems	Cat# 226 003; RRID: AB_2231974
Rabbit anti-cFos	Synaptic Systems	Cat#226 004; RRID: AB_2619946
Goat anti-Rabbit AlexaFluor 647	Thermo Fisher Scientific	Cat# A-21206; RRID: AB_2535792
Experimental models: Organisms/strains		
C57BL6/J Mouse	Jackson Laboratory	RRID: IMSR_JAX:000664
Software and algorithms		
CinePlex Studio	Plexon	v3.9.0
Prism	GraphPad Software	v.9.4.1
FIJI ImageJ	National Institutes of Health	v.2.3.1

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jonathan P. Fadok (jfadok@tulane.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- Raw data have been deposited at Harvard Dataverse and are publicly available as of the date of publication. https://doi.org/10.7910/DVN/C1QSQ2.
- 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.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals

Healthy adult 3–4-month-old female C57BL6/J mice weighing 19-25g (Jackson Laboratory, Bar Harbor, Maine) were housed in pairs for the duration of these studies, except for the social isolation experiment. In the initial dark phase experiment, animals were housed on a 12:12 reverse light cycle. During subsequent experiments, animals were housed on a 12:12 traditional light schedule. Food (PicoLab Rodent Diet 5053, LabDiet) and water were provided *ad libitum*. After arrival, mice were acclimated to the facility for a minimum of 7 days and then randomly assigned to experimental groups. All animal procedures were in accordance with relevant institutional and national guidelines and regulations and were approved by the Institutional Animal Care and Use Committee at Tulane University.

METHOD DETAILS

Behavioral tests

Mice were brought into the testing room in their home cage and allowed to acclimate for at least 30 minutes before all behavioral tests. Activity in the open field test and elevated plus maze was recorded via an overhead camera and tracking was recorded using Cineplex (Plexon, Dallas, TX) software.

Open field test

The open field test took place in a 45 x 45 x 45 cm square, white PVC arena under either dim red light (< 15 lux) or white light (< 15 lux) conditions during the dark or light circadian phase of testing, respectively. Mice were placed in the arena and allowed to explore for 10 minutes. After each trial the arena was cleaned with 70% ethanol.

Homecage behavior

Animals were evaluated for social behaviors in the home cage at two time points: initial meeting (pre-CE) and after 11 days of pair housing (after CE). All behavioral evaluation was performed under dim red-light conditions (< 10 lux) during the dark (active) circadian phase. Animals were placed in a clean cage with bedding and without enrichment (nesting material) and allowed to interact freely. Upon the first social encounter, a 20-minute timer was started, and interactions were recorded by overhead camera and manually scored by two unbiased observers and averaged.

Behaviors with relevance for sociability, dominance, anxiety, and all direct contacts were recorded. Behaviors were treated as counted variables (i.e., +1 added for every occurrence) and categorized as follows:

I. "Social affiliative" – nose-to-nose sniffing, head sniffing, flank sniffing, anogenital sniffing, allogrooming, and following.
II. "Forepaw touch" – one animal places its forepaws on the head, shoulders, or body of another animal followed by vigorous nonsocial environmental sniffing in a sweeping motion above the conspecific. This behavior most closely resembled supported rearing[16] with the conspecific being used in place of a wall or similar support. Since the behavior was performed frequently but the sniffing was not directed towards the conspecific, this behavior was scored as its own category.
III. "Nonsocial" – scent marking, scent mark sniffing, autogrooming, crawling over/under a conspecific towards a nonsocial goal arising from space constraints in the cage.
IV. "Agonistic" – chasing, biting, attack and retreat, boxing, pinning. Agonistic behaviors were not observed during home cage behavior analysis.

Competitive exclusion (tube test)

CE took place in 2 phases: training and testing. Prior to training, group-housed animals were habituated to the presence of a 6 x 2.5 cm diameter clear acrylic tube in the home cage.

i. Training – The testing apparatus consisted of a clear acrylic 30 x 2.5 cm diameter acrylic tube, wide enough for the passage of one mouse at a time. During 10 trials per day, mice were individually trained to run the length of the tube from either direction.
ii. Testing – Dominance testing began after the last day of training. Each animal underwent two trial runs prior to every testing session. After the trial runs, mice were placed on opposite sides of the tube and allowed to meet in the middle. The test was considered complete when one mouse forced the other completely back to its starting side. This was recorded as a win for the mouse that successfully ran the length of the tube, and a loss for the mouse forced out of the tube. A total of 5 dominance trials were performed per day. The dominant status for each testing session was defined as 3 or more wins. Time to complete the test as well as win style was recorded. A win strategy was considered active if the winner employed agonistic behaviors to force the opponent out (e.g., pushing, push-back, advancing) or passive if non-agonistic methods were employed (i.e., winner refused to retreat but did not actively advance to force out the opponent).

The tube test is considered agonistic in that it creates a social challenge that involves a physical altercation, and competitive in that the animal expelled from the tube is forced into an open, exposed arena while the winner maintains access to the protective and familiar environment of the tube.[14,15] An alternate name for the tube test is competitive exclusion.

Food competition task

The food competition task was performed in unfasted animals to remove the variable of subjective satiety as a primary motivator. 48 hours prior to the experiment, animals were acclimated to receiving a palatable food (Honey Nut Cheerio; General Mills, Minneapolis, MN) in the home cage to reduce novelty-induced suppression of feeding. Immediately after behavior analysis, a single Cheerio was placed in the center of the home cage. Behavior was recorded by overhead camera for 5 minutes after initial interaction or until the food item was completely consumed. No other food, water, or enrichment was available during this task. Animals were scored for overall time in possession of the food item as a percentage of total time.

3-Chamber social approach

The test took place in a 50 x 30 x 30 cm apparatus constructed from white PVC sheets. The device was divided into 3 chambers by walls separated with removable guillotine-style dividers. Each iteration of this test consisted of three distinct groups of mice: experimental (or control) mouse, novel mouse #1 (will become Familiar mouse), and novel mouse #2. The test took place in three phases: habituation, sociability test, and social novelty test.

i. Habituation – On the first day, animals are allowed to explore the apparatus with all dividers removed for 10 minutes.
ii. Sociability – Sociability and social novelty testing occurred on the same day. Two identical wire mesh cups were placed in either chamber, one to contain the naïve (nonexperimental/control) mouse and the other to remain empty in the opposite chamber (novel object). The central chamber remained empty. The subject mouse was placed in the central chamber for a 5-minute habituation period with the dividers in place, preventing access to the outer chambers. After habituation, novel mouse #1 was

placed under the wire mesh cup in one side chamber. The dividers were removed, and the subject mouse was allowed to explore chambers freely for a total of 10 minutes.

iii. Social Novelty – The subject mouse was returned to the central chamber while novel mouse #2 was placed under a new mesh cup in the chamber previously containing the novel object. The dividers were removed, and the subject mouse allowed to explore freely for another 10 minutes. Tests were recorded to video via an overhead camera for later analysis by individuals blinded to the experimental parameters. After each trial chambers were cleaned with 70% ethanol.

Outcome measures include percentage of total time exploring each chamber during the two iterations, and direct contacts with the mesh cup containing a social partner (or remaining empty as a novel object). Direct contacts include sniffing, rearing-to-sniffing, and touches. Climbing was prevented by covering the top of the cup with a weighted barrier.

Elevated plus maze

Mice were placed on an elevated (60 cm), cross-shaped opaque PVC maze consisting of two 5 x 28 cm open arms and two 5 x 28 cm arms enclosed by 38 cm walls; testing occurred under bright lighting conditions (white light > 100 lux). Animals were allowed to freely explore the apparatus for 5 minutes. After each trial the maze was cleaned with 70% ethanol.

Social instability stress

Adapted from the protocol described by Yohn et al.,[25] every 3rd day pair-housed (n=24) mice were introduced to a novel age- and strain-matched conspecific and these pairs lived continuously together until the next social group rearrangement. Pairs that had co-habitated for the previous 3 days were subjected to competitive exclusion prior to introduction to the next social partner to determine the social rank achieved in the previous pairing. Control animals (n=16) were continuously pair housed but tested with CE during each session along with experimental animals. Coat state score, body weight, and fecal samples were taken at this time. This was repeated for a total of 4 weeks. This model requires mice to re-form social groups and disables social support structure, a model of social stress that is thought to be more ethologically relevant to the development of major depression in humans.[23–25]

As with the social isolation experiment, behavioral testing was performed prior to initiating SIS and then repeated after the completion of stress paradigm. Given the stress-associated differences seen in the social isolation experiment were independent of anxiety behaviors in the OFT, in this experiment we further explored the behaviors expressed in the anxiogenic context of the elevated plus maze (EPM). Additionally, at the end of SIS a novel object recognition test (NORT) was performed to identify underlying and SIS-responsive rank differences in recognition which could be responsible for the differences observed in the 3CSA.

David's Score

David's score is a widely used measure of individual dominance that is calculated from a sociomatrix of the win/loss results of each tube test session.[26] DS ranged from -27.4 (most subordinate animal) to 23.1 (most dominant animal), such that the population was slightly skewed towards subordinate status (11 dominant animals, 13 subordinate animals at experimental endpoint). The mean DS (-0.01) was used to assign the experimental animals into ranked groups (i.e., Sub = DS < -0.01, Dom = DS > -0.01).

Social isolation

Pair-housed mice underwent competitive exclusion to determine rank over a period of 4 days; ranks were considered stable if consistent for all 4 days. Mice were then continuously pair housed for 10 days, after which they underwent baseline (pre-stress) OFT. Afterwards, experimental mice (n=14) were transitioned to isolation housing with standard enrichment for a total of 7 weeks. Control mice (n=8) remained in pair-housing for the same period. Fecal samples, coat state score, and weight were assessed periodically during the first 5 weeks of isolation, but animals were otherwise undisturbed. Post-stress behavioral testing began during week 6 and control mice were isolated for the sucrose preference test in week 7. At the end of the isolation period, experimental mice were re-introduced to their original social partner for 15 minutes, then euthanized via transcardial perfusion. Adrenal glands and brains were extracted, and brain slices were processed for immunohistochemical detection of c-Fos expression. Adrenal glands were weighed as pairs and expressed as a percentage of the final total body weight.

Sucrose preference test

This test was performed in the home cage and mice were individually housed during testing. Mice were acclimated to the presence of two sipper tubes filled with water in the cage for 72 hours prior to testing. Afterwards, the two tubes were replaced with one containing water and the other containing a 1% sucrose solution. Over the next four days, each tube was weighed once daily to determine the amount of water or sucrose solution consumed over the previous 24 hours. The tube position alternated each day to account for potential side bias. Sucrose preference was calculated as the percentage of the volume of sucrose consumed divided by the total fluid intake.

Novel object recognition test

The open field apparatus was used to conduct this test, which was performed over two trials. During trial 1, two identical cubes (Rubik's Ltd, Hungary) were placed in opposing corners of the open field, 5 cm away from the walls. The subject was placed in the apparatus and allowed to explore freely for 10 minutes. 24 hours later the second trial was performed, in which one of the identical objects was replaced with a novel object, a Rubik's pyramid (Rubik's Ltd, Hungary), and an identical cube used in the first trial. The subject was again allowed to explore for 10 minutes. Behavior was recorded via an overhead camera for later analysis.

Non-behavioral tests

Coat state score

Animals were assessed for changes in coat state associated with poor well-being. Eight body regions (head, neck, back, forepaws, hindpaws, tail, abdomen, and anogenital region) were assessed and assigned a score based on appearance as follows: 0 = well-

kempt, clean, and shiny, 0.5 = moderately unkempt or dull, 1.0 = poorly kept, very dull and dirty or patchy. The points for all regions were summed to assign a single coat state score for each animal. Higher scores therefore indicate a worse coat state.

Analysis of fecal corticosterone metabolites

Fecal samples were taken at the same time each day within experiments, and at least 2 hours into the light cycle of testing. Mice were individually removed from their cage and placed in a plastic beaker on a scale to take body weight measurements. This process typically resulted in the mouse passing fecal pellets which were immediately collected in microcentrifuge tubes on dry ice. Mice that did not pass during the weighing were gently handled for several minutes until fecal pellets were produced. Samples were stored at -20°C until analysis using an enzyme immunoassay specific for glucocorticoid metabolites sharing a 5α-3β, 11β-diol structure, as described by Touma et al.[13,61]

Samples and standards were run in duplicate. All presented values have intra-assay CV < 10%; values with intra-assay CV > 10% were excluded from analysis. The average inter-assay CV for each experiment is listed below:

Experiment 1 (stress-naïve; n = 6 assays): 5.79%
Experiment 2 (SIS; n = 10 assays): 7.38%
Experiment 3 (isolation; n = 11 assays): 5.84%

Contexts for immunohistochemistry experiments

Novel social encounter

c-Fos expression was examined in response to two different contexts to evaluate how social rank alters neural activity in response to social novelty and psychosocial stress. All animals were acclimated to the testing environment for 30 minutes prior to testing. Social contexts occurred under dim (< 10 lux) red light conditions.

Context 1: Following behavioral testing in the three-chamber social approach, mice from the original dark phase experiment (Figure 1) were divided into groups based on exposure to a brief social encounter (5 minutes after observation of the first social interaction) with a novel age-, strain-, and sex-matched conspecific in a clean cage. Control animals were placed alone into a clean cage for the same amount of time.

Afterwards, all mice were immediately dark housed (lights off, covered cages) for 90 minutes.

Social reunion

Context 2: After chronic social isolation, experimental animals were reunited with their former co-housing partner in a new cage and allowed to interact for 15 minutes (Figure S5). In order to explore the effect of social isolation alone (i.e. in the absence of social stimuli) and control for the effect of a novel environment, a subset of isolated animals underwent continued isolation in a clean empty cage for the same amount of time. Control animal pairs were placed together into a novel empty cage.

Afterwards, all mice were immediately dark housed (lights off, covered cages) for 90 minutes.

Histology

Brain tissue collection and adrenal dissection

c-Fos expression was assessed in mice that were perfused 90 min following a social context. Animals were deeply anesthetized with 0.5 - 0.7 mL Avertin (12.5 mg/mL 2,2,2-tribromoethanol, IP, Sigma). Mice were transcardially perfused with 40-100 mL cold phosphate-buffered saline (PBS) followed immediately by 40-100 mL cold 4% paraformaldehyde (PFA) in PBS. After whole-body fixation, the brain was extracted, and the adrenals were removed from the abdominal cavity and post-fixed in 4% PFA for 24 hours. Afterwards, left and right adrenals were cleansed of peri-adrenal adipose tissue and weighed together.

Immunohistochemistry

60 μm coronal slices of PFA-fixed brains were cut on a compresstome vibrating microtome (Precisionary, Greenville, NC). Antibody staining was performed on free-floating tissue slices. Slices were permeabilized with 3 x 15 min washes with 0.5% PBST then blocked in 5% goat serum in PBST for 2 hours at room temperature. Sections were incubated overnight in primary antibodies at 4°C on a shaker. Next the slices were washed in 0.5% PBST (3 X 15 min) and then incubated in secondary antibodies for 2 hours at room temperature. After final washes in PBS (3 x 10 min) slices were mounted onto slides with mounting medium with DAPI (Biotium, Fremont, CA) and cover slipped. The primary antibody was rabbit anti-cFos (1:2000; 226 003, Synaptic Systems, Germany or 1:1500; 226 004, Synaptic Systems, Germany), and the secondary antibody was goat anti-rabbit AlexaFluor 647 (1:500; A-21206, Thermo Fisher Scientific, Waltham, MA).

Imaging and analysis of c-Fos immunolabeling

Images were obtained using an AxioScan.Z1 slide-scanning microscope (Zeiss, Germany) and a Nikon A1 Confocal microscope (Nikon, Japan). C-Fos positive nuclei were quantified from 20x images using Fiji ImageJ software (NIH, Bethesda, USA) and reported as c-Fos positive cells per mm2, averaged between hemispheres. c-Fos expression was quantified in 2-5 slices per structure, per mouse. Final counts were averaged for each animal.

In addition to obtaining absolute values, we characterized the magnitude and directionality of c-Fos expression arising from stress-naïve animals exposed to social novelty and socially isolated animals reunited with their familiar social partner. To accomplish this, we compared the rank effects between paradigms by calculating the percent difference of every animal from the control mean of their corresponding rank in that experiment. These data are shown in Figure 5; the within-rank mean control state is represented as baseline and experimental state as percent derivation from baseline.

Coordinates for PVT slice analysis

The PVT c-Fos expression was further analyzed for the anteroposterior distribution of slices. The subregions were defined according to the coordinates designated by Gao et al.[48] and Zhu et al.[49] as anterior PVT (from Bregma: –0.22mm), middle PVT (from Bregma: –0.94mm) and posterior PVT (from Bregma: –1.82mm). Slices were grouped by subregion and analyzed across groups by rank and experimental condition.

Roughly 55% of the PVT images in this study were obtained from the aPVT (84/152 slices or 55.26%) and 45% from the mPVT (68/152 slices or 44.74%). No images were taken from the pPVT.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis was performed using Prism version 9.4.1 (GraphPad Software, San Diego, CA). Specific details for each experiment are listed in the associated figure legends. Significance is designated in figures as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Normality of groups was tested with the Shapiro-Wilk test. Two independent samples were compared using a two-tailed unpaired Student's t-test for unpaired data or a paired t-test for pair-matched data, unless data were non-Gaussian in distribution, in which case a Mann-Whitney U-test (or Wilcoxon test for pair-matched data) was used to compare ranks. All tests were two-tailed.

Fecal corticosterone metabolite data are frequently non-Gaussian. These data are reported in box-and-whisker plots, which are displayed according to the Tukey method; boxes extend from Q1 to Q3, inner fences extend +/- 1.5 IQR, dots convey outliers, and horizontal lines designate the median.

If the variances of normally distributed data differed significantly in the F test, unpaired samples were compared using Welch's test. When more than two independent samples were compared, two-way ANOVA on factors of Condition (or Stress) and Rank was used for normally distributed data and post-hoc analysis was performed using Sidak's test when significant main effects or interaction effects were found. Tukey's multiple comparisons test was used when more than two groups were compared.

In the case of consistent pairs (I.e., when animals were continuously pair housed with the same partner, or reunited with the same partner), a mixed-effects model was run instead using Pair ID as a random factor. The model uses a compound symmetry covariance matrix and is fitted using Restricted Maximum Likelihood (REML). Unless provided in the figure legend, random effect (Pair ID) SD = 0 and it is excluded from the model.

Non-Gaussian data with more than two independent samples were analyzed using Kruskal-Wallis test and post-hoc analysis was performed using Dunn's test when significant main effects were found.

Home cage behavioral interactions were scored manually and reported as tabulated events into 2x2 contingency tables where columns represent social rank and rows represent behavioral categories, then analyzed using the Yate's corrected Chi-square test. Counted variables (direct contacts in the 3CSA, homecage behaviors) were scored manually and reported as simple counts in 2x2 contingency tables where columns represent social rank and rows represent independent variables of interest, then analyzed using the Yate's corrected Chi-square test.

Normally distributed correlation data were analyzed using a Pearson correlation, otherwise a Spearman correlation was used.

c-Fos percent difference from control mean effect size was calculated with Hedge's g statistic to account for the sample size < 50. Differences were considered significant if their probability of occurring by chance was less than 5% (i.e., tests returned a p-value of less than 0.05).