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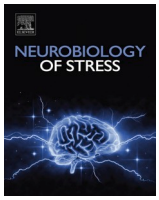

Effects of social isolation on locus coeruleus opioid receptor expression and affective behavior

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ABSTRACT

Social isolation is a stressor that impairs homeostatic neuroendocrine functions and is associated with the development of several mood disorders characterized by persistent negative affect. Persistent feelings of loneliness have been growing public health concerns for several years and were greatly exacerbated by the onset of the COVID-19 pandemic. The problem has grown so severe the U.S. Surgeon General recently declared loneliness to be an epidemic health concern that is associated with poor mental and somatic health outcomes. Therefore, identifying mechanisms of neuroadaptation that contribute to the development of persistent negative affect is a critical step in the identifying better treatments for mood disorders. One region of the brain that becomes dysregulated in neuropsychiatric disease is the locus coeruleus. It is innervated by multiple stress-related peptidergic afferents, including those that release endogenous opioids to affect behavior. It is a major contributor to the behavioral limb of the stress response, but its role in the neurobiology of social behavior is understudied. Here we show that in laboratory rats, six weeks of social isolation leads to increased neophobia, reduced sociality, and passive stress coping. These behavioral changes are also associated with downregulation of the δ-opioid receptor and upregulation of the κ-opioid receptor in locus coeruleus. These findings suggest that extended social isolation promotes dysregulation of several opioid receptor subtypes in a brain structure that has an important role in regulating affective behavior, implicating them as potential targets for the treatment of neuropsychiatric disease associated with social isolation and loneliness.

1. Introduction

Social isolation and persistent feelings of loneliness are growing public health concerns that have been exacerbated by the COVID-19 pandemic. In social species, loss of social contact is a stressor that disrupts normal neuroendocrine and physiological functions, and the resultant neuroadaptations are thought to contribute to the development of social isolation (SI) stress-associated mood disorders such as anxiety and depression (Meade, 2021; Wilkialis et al., 2021; Wickramaratne et al., 2022; Giacco, 2023). Importantly, loneliness is also associated with increased risk for neurodegenerative and cardiovascular disease, dementia, stroke, and premature death (Mann et al., 2022; Singh et al., 2023; Usama et al., 2024). Thus, clarifying the impact of SI on the brain and body is a critical step in alleviating a public health concern that has grown so severe that it was recently declared an epidemic of loneliness by the U.S. Surgeon General. Because SI is a stressor that disrupts brain homeostasis, we sought to investigate how it affects the locus coeruleus (LC), a small but broadly projecting structure whose activity influences virtually the entire central nervous system with a well-characterized role in mediating the behavioral response to stress (Van Bockstaele et al., 2010; Chaijale et al., 2015; McCall et al., 2015; Reyes et al., 2015; Bangasser et al., 2016; Borodovitsyna et al., 2018; Chandler et al., 2019; Reyes et al., 2019; Serova et al., 2019; Borodovitsyna et al., 2020). Specifically, during stress, various afferents to LC release the peptide transmitter corticotropin releasing factor (CRF) onto its dendrites and cell bodies, leading to neuronal depolarization, enhanced noradrenergic neurotransmission in the forebrain and a hypervigilant negatively-valenced affective behavioral state (Jedema and Grace, 2004; McCall et al., 2015, 2017). LC is also innervated by structures that release the endogenous opioids Leu-enkephalin and dynorphin which preferentially interact with δ-opioid receptors (DORs) and κ-opioid receptors (KORs), respectively.

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Although the role for these receptors in LC physiology and its dependent behaviors are not well studied, some evidence suggests that enkephalinergic neurotransmission at LC-expressed DORs promotes stress resilience: activation of enkephalinergic neurons that innervate LC is associated with a more resilient active coping-like behavioral phenotype during social defeat stress (Reyes et al., 2015, 2019). It is thought that enkephalin release onto LC cell bodies occurs at the end of a stressor and is inhibitory to counteract CRF actions, contributing to the terminatation of the stress response (Williams and North, 1984; Aghajanian and Wang, 1987; Valentino and Van Bockstaele, 2015). Although this is a normal physiological reaction that promotes brain function and behavioral compensation in the face of stressful situations, CRF signaling in LC influences multiple genetic, cellular, and neuroplastic changes (Mamalaki et al., 1992; Rusnak et al., 2001; Swinny and Valentino, 2006; Salim et al., 2007; Swinny et al., 2010; Fan et al., 2013; George et al., 2013) that may persist well beyond the stressful episode itself. For example, studies from our lab have previously shown that DOR expression is lower one week after a single episode of combined predator odor and physical restraint (Tkaczynski et al., 2022), potentially limiting the ability of LC to respond to enkephalin at the end of the stress response to facilitate a return to a normative behavioral state. Numerous other studies have also investigated the neurobiological bases of LC dysregulation following various preclinical stress models that rely on the application of aversive stimuli (i.e., restraint (George et al., 2013; Sabban et al., 2015; Serova et al., 2019; Arikawe et al., 2021), predator odor (Curtis et al., 2012; Borodovitsyna et al., 2018, 2020, 2022; Tkaczynski et al., 2022), social defeat (Chaijale et al., 2015; Reyes et al., 2015; Wood et al., 2015; Reyes et al., 2019), foot shock (Wang et al., 2017; Sabban et al., 2018; Giustino et al., 2020; Tillage et al., 2021; Kelberman et al., 2023). However, only a very small handful of studies (Angulo et al., 1991; Nakamoto et al., 2017; Atmore et al., 2020; Broadfoot et al., 2023) have specifically investigated how chronic SI stress affects the LC. Rodents, like humans, are social animals that experience neuroadaptation and behavioral change following SI (Meade, 2021, Wilkialis et al., 2021; Mann et al., 2022; Broadfoot, Lenell et al. 2023; Csikos et al., 2024; Pitcairn, Ortelli et al. 2024). For example, SI has been shown to reduce sociability (A, Hansson et al., 2024), impair spatial memory (Mohammadkhanizadeh et al., 2024, and increase anxiety-like and depressive behaviors (Nikolaienko et al., 2023; A, Hansson et al., 2024). In addition, SI stress is associated with biological chan ge within the brain including altered dopamine signaling in the forebrain (Lam llai, Congiu et al. 2024), transcriptional change in the cortex and hippocamp(us (Musuroglu Keloglan et al., 2023; Goh et al., 2024), hypofunction of the serotonergic system (McElroy et al., 2025) and altered plasma levels of various neuropeptides (Dimonte et al., 2023). Based on these findings, and the well established role for the LC in the stress response, we sought to characterize how SI stress affects various forms of behavior as well as opioid receptor expression within LC.

While the behavioral roles of LC-expressed opioid receptors both within and outside the context of stress are not well explored, opioidergic regulation of behavior in other brain regions, and its sensitivity to stress, is more well characterized. Studies using broad opioid antagonists found that adaptations to repeated stress occur in an opioid-dependent fashion, with increased opioid signaling leading to a less depressive-like behaviors (Cancela et al., 1991; Cancela et al., 1995; Agrawal et al., 2011). This is supported by observations that systemic DOR agonism decreases immobility in the tail suspension and forced swim tests (Moriya et al., 2023), increases time in the open arms of the EPM (Saitoh et al., 2004; Vergura et al., 2008), promotes active coping and stress resilience during social defeat (Henry et al., 2018), and reduces and norepinephrine release in the prefrontal cortex after in response to noxious stimuli (Hudzik et al., 2011). Direct injections of a DOR agonist into the BLA also increases time in the open arms of the EPM and center of the OFT and also facilitates extinction learning (Sugiyama et al., 2018). However, KOR seems to show the opposite effect, where systemic agonism leads to more anxiety-like behaviors in adult male rats (Mague et al., 2003; Przybysz et al., 2020) and antagonism leads to antidepressant-like effects (Mague et al., 2003).

SI stress has also been shown to modulate expression of endogenous opioid peptides and their receptors. Specifically, SI has been shown to induce downregulation of proenkephalin levels in the nucleus accumbens (Angulo et al., 1991), and of μ-opioid receptor (MOR) and KOR levels in the hippocampus and amygdala (Haj-Mirzaian et al., 2019). We have previously shown that an acute stressor similarly leads to downregulation of DORs in LC (Tkaczynski et al., 2022). Given its importance in mediating the physiological and behavioral limbs of the stress response, in this study, we sought to determine how the three main opioid receptors (MOR, DOR, and KOR) were affected by six weeks of SI stress, and how this related to social, stress coping, and anxiety-like behaviors. We found that relative to group housed (GH) animals, SI rats spent less time interacting with social targets animals, neophobic responses to novel environments, and passive stress coping. We also found that DORs were downregulated and KORs were upregulated, with no difference in MOR expression. Therefore, the SI-induced behavioral dysregulation may be in part related to altered expression of opioid receptors in LC.

2. Materials and methods

2.1. Subjects

The experimental timeline is shown in Fig. 1. Twenty male and female Sprague-Dawley rats arrived at six weeks of age and were housed singly (socially isolated, SI) or in groups of 2–3 per cage (group housed, GH) for six weeks from six weeks to twelve weeks of age on a 12 h reverse light-dark cycle (lights on at 9:00pm) with access to standard rat chow and water ad libitum. Rats remained undisturbed under these housing conditions except for occasional handling to habituate them to experimenters until behavioral testing began at twelve weeks of age. Two additional male and two additional female Sprague-Dawley rats of the same age as experimental animals were housed in pairs and were used as target animals in the three-chamber sociability test (3CST). All animal protocols were approved by the Rowan University Institutional Animal Care and Use Committee and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All behavioral tests took place in a dimly lit and soundproof chamber and was filmed by an overhead camera connected to a Lenovo ThinkCenter M700 PC.

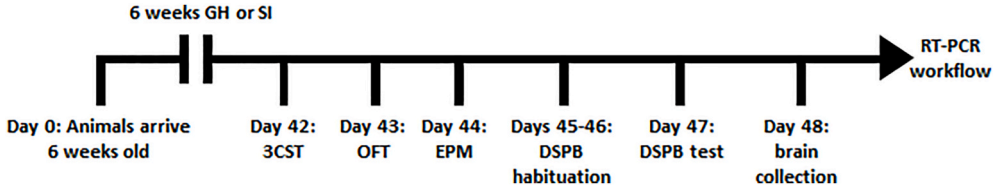

Fig. 1. The experimental timeline for both GH and SI animals from the time of arrival in the facility to RT-PCR experiments.

2.2. Three chamber sociability test

The 3CST took place inside of a gray plexiglass apparatus consisting of two 35 × 35 × 35 cm compartments joined in the middle by a single 35 × 10 × 35cm compartment. Removable doors allowed the rat to either be confined to the central compartment or explore the entire apparatus freely. Rats were allowed to freely explore the apparatus for 5 min and then were confined to the central compartment while one 10 × 10 × 18cm empty plastic cage was placed in the corner on one side of the apparatus and an identical cage containing a novel same-sex target rat was placed in the corner on the opposite side of the apparatus. The doors were then removed and the rat was allowed to freely explore for 10 min (phase 1). The rat was then confined again to the central compartment and a second, novel same-sex target rat was placed in the previously empty plastic cage. The doors were then removed and the experimental rat was again allowed to freely explore the apparatus for 10 min (phase 2). The side which contained the empty cup in phase 1 and the novel rat in phase 2 was counterbalanced between experiments. Animals were returned to their home cages after each test and the apparatus was cleaned with 10 % bleach and water between each experiment. The time each animal spent investigating each cage in each phase of the test was quantified using AnyMaze behavioral tracking software (Stoelting).

2.3. Open field test

The next day, anxiety-like behavior was assessed in the open field test (OFT), consisting of a 90 cm × 90 cm x 30 cm black plexiglass box. Rats were allowed to explore the apparatus for 10 min, during which their activity was filmed by the overhead camera. After each test, rats were returned to their home cages and the apparatus was cleaned with 10 % bleach and water between each test. Center time, corner time, and time freezing were scored using AnyMaze behavioral tracking software. The onset of freezing episodes was defined by a period of 1s without motion, and were terminated when motion was again detected.

2.4. Elevated plus maze

The next day, the elevated plus maze (EPM) took place in a plus shaped black plexiglass apparatus elevated 76 cm off the ground with two sets of opposing arms (each arm = 40 cm in length) meeting in a central 10 × 10 cm area. Two opposing arms have vertical walls extending 30 cm from the floor of the maze, while the other two arms do not have walls. Rats were allowed to explore the maze for 10 min while their activity was filmed by the camera. At the conclusion of each test, rats were returned to their home cages and the maze was cleaned with 10 % bleach and water. Open arm time, closed arm time, and time freezing, were scored using AnyMaze behavioral tracking software (Stoelting). The onset of freezing episodes was defined by a period of 1s without motion, and were terminated when motion was again detected.

2.5. Defensive shock probe burying

The day after EPM, rats were first habituated to the defensive shock probe burying (DSPB) behavioral apparatus, a modified polycarbonate rat home cage 46 × 25 × 20 cm (L x W x H) with a 1.25 cm hole drilled in the center of one of the short sides 7 cm off the bottom and filled with 5 cm of bedding, for 15 min a day for two days. On the third day, a plastic probe 10 cm in length and 1 cm in diameter with two bare copper wires coiling around it was inserted through the hole on the short side of the apparatus. The copper wires were coupled to a Coulbourn Instruments H13-15 precision animal shocker. When an animal touches the probe, it closes the circuit between the two copper wires and receives a small 2 mA shock. The probe is then de-electrified to prevent subsequent shocks, and the animal remains in the behavioral apparatus for 15 min. Videos of each trial are recorded using the overhead camera. Behaviors such as burying, rearing, and freezing are scored manually by a trained observer. After each test, rats were returned to their home cages, bedding was discarded, the apparatus was cleaned with 70 % ethanol, and new bedding was added to a height of 5 cm for the subsequent test.

2.6. Real time PCR

All tools, materials and instruments were autoclaved and treated with RNAse Zap (Invitrogen) prior to use to prevent degradation of RNA. The day after the final behavioral test, rats were deeply anesthetized with 4 % isoflurane and rapidly decapitated. Brains were extracted and blocked coronally to a piece of tissue containing cerebellum and pons <0.5 mm in length. This piece of tissue was placed in 1.8 ml RNALater (Invitrogen) at 4 °C for 24 h, then placed in a new dry vial followed by long term storage at −20 °C. Brain blocks containing LC were attached to a cryostat mounting block with tissue freezing medium (Triangle Biomedical Sciences; Durham NC) at −30 °C and placed in a Leica CM1860 cryostat. The brain was trimmed to approximately 1.5 mm in length to contain the full rostrocaudal extent of LC. A 1 mm trephine was then used to collect bilateral punches of the area directly lateral to the fourth ventricle to collect LC. LC punches were collected in 350 μL RLT lysis buffer (Qiagen) and homogenized using a glass Dounce homogenizer. Total RNA was extracted from each LC tissue punch using a Qiagen RNeasy Micro Kit according to manufacturer instructions to produce 14 μl samples (n = 5/group). RNA concentration and purity within each sample was assessed by using 1 μl from each sample in a NanoDrop spectrophotometer (Thermo Scientific). A TaqMan Reverse Transcription Reagent kit (Invitrogen) was used according to manufacturer instructions to produce and amplify ten 50 μl samples of cDNA for each animal in a BioRad T100 DNA Engine. Samples were stored at −20 °C until further use in RT-PCR experiments. In each experiment individual wells contained 20 μl of reaction mixture (consisting of 10 μl TaqMan 2X Master Mix, 8 μl DEPC water, 1 μl 20x primers, and 1 μl cDNA per sample). Individual ΔΔCt experiments were carried out using an Agilent AriaMax RT-PCR thermal cycler and software to quantify relative expression of *oprd1*, *oprm1*, and *oprk1* (the genes for DOR, MOR, and KOR, respectively) mRNA, with *gapdh* used as a housekeeping gene. Fluorescence baselines and thresholds were manually set for each experiment. Threshold cycle (Ct) values were measured from each sample, and a mean Ct value was calculated from all samples. This same mean value was then subtracted from each individual ΔCt value obtained from each sample to produce an individual ΔΔCt value for each sample. These values were then used to generate relative quantity = $2^{-\Delta\Delta Ct}$ for each sample. This relative quantity for each sample was then divided by the mean control population relative quantity such that the mean of the control group was equal to 1. Therefore, each relative quantity for each sample represents a fold-change from the mean control population.

2.7. Experimental design and statistical analysis

Data from male and female animals in these experiments were pooled to preserve statistical power and because t-tests did not reveal any major sex differences when data were analyzed according to sex instead of housing condition. Statistical analyses were performed with GraphPad Prism version 10.3.1. All data sets were tested for normality and homogeneity of variance. Normal data sets with equal variance were analyzed using unpaired t-tests. Normal data sets with unequal variance were analyzed using unpaired t-tests with Welch's correction applied. Non-normally distributed data sets were analyzed using non-parametric Mann-Whitney U tests. Data sets were screened for outliers by determining how many standard deviations were from the mean with a threshold of three applied for exclusion. No data points met this criterion and thus none were removed for analysis. Data in all figures are presented as mean ± SEM. Males are represented by squares, and females are represented by circles.

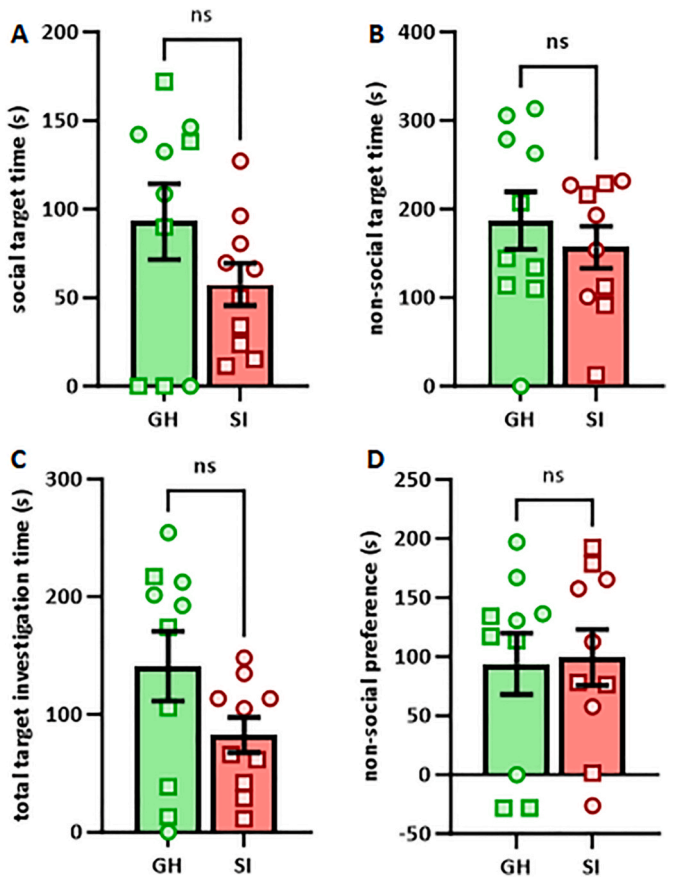

Fig. 2. Social behavior in phase 1 of the 3CST is unaffected by SI stress. No differences exist between SI and GH animals in time spent investigating a novel social target (A), a non-social target (B), total time investigating both targets (C), or preference for the non-social versus social target (D) in phase 1 of the 3CST.

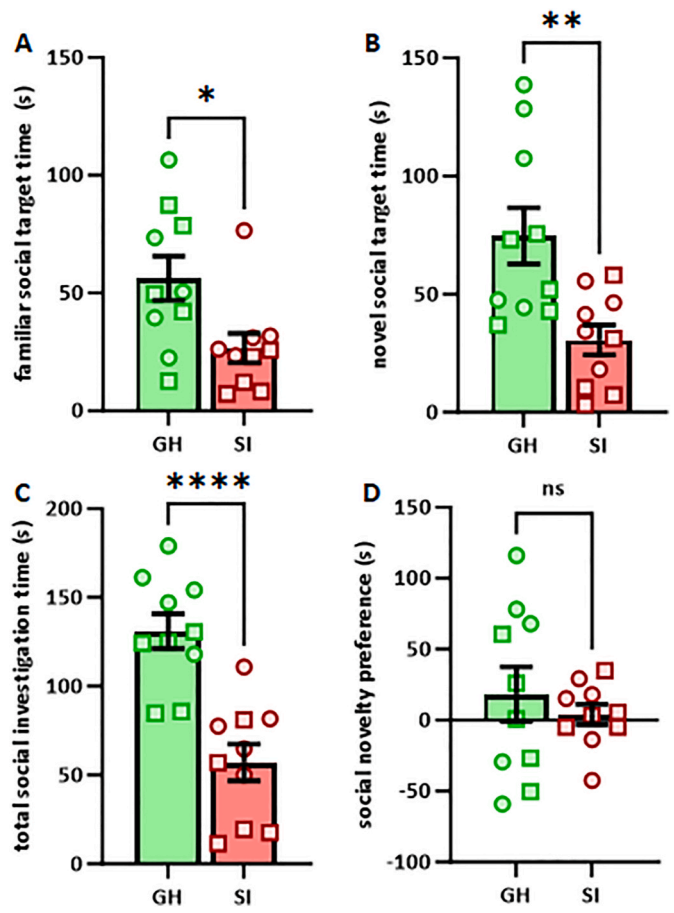

Fig. 3. Investigation of social targets in phase 2 of the 3CST is reduced by SI stress. Rats that underwent six weeks of SI stress spent significantly less time investigating a familiar social target (A), a novel social target (B), and total time investigating both social targets (C).

3. Results

3.1. Three chamber sociability test

Unpaired t-tests did not reveal effects of SI stress on investigation of the social target ($t = 1.45$, $df = 18$, $p = 0.1641$, Fig. 2A), investigation of the non-social target ($t = 0.7476$, $df = 18$, $p = 0.4643$, Fig. 2B), total target investigation ($t = 1.763$, $df = 18$, $p = 0.0949$, Fig. 2C), or non-social target preference (non-social target time - social target time; $t = 0.1541$, $df = 18$, $p = 0.8792$, Fig. 2D) or in phase 1 of the task. In phase 2 of the task, SI animals spent significantly less time investigating the familiar rat (Mann-Whitney $U = 19$, $p = 0.0185$, Fig. 3A), less time investigating the novel rat ($t = 3.277$, $df = 18$, $p = 0.0042$, Fig. 3B), and less cumulative time investigating both social targets ($t = 5.203$, $df = 18$, $p < 0.0001$, Fig. 3C) than GH rats. An unpaired *t*-test with Welch's correction applied failed to find a significant effect of SI stress on novel social preference (novel social time - familiar social time) in phase 2 of the task ($t = 0.707$, $df = 11.44$, $p = 0.4937$, Fig. 3D). Collectively, these findings indicate that in the second phase of the 3CST investigating motivation for social novelty, overall socialability was decreased by SI stress.

3.2. Open field test

Time spent freezing in the OFT (Fig. 3) was significantly greater in SI animals than GH animals ($t = 2.666$, $df = 18$, $p = 0.0158$). Center time ($U = 49$, $p = 0.9705$), corner time ($t = 1.215$, $df = 18$, $p = 0.2405$), total distance traveled ($t = 0.4616$, $df = 18$, $p = 0.65$) and time mobile ($t = 0.8295$, $df = 18$, $p = 0.4177$) were not significantly affected by SI stress. These findings indicate that a neophobic behavioral phenotype may be induced by SI stress without affecting locomotion.

3.3. Elevated plus maze

An unpaired *t*-test with Welch's correction applied found that time spent freezing in the EPM (Fig. 4) was significantly increased by SI stress ($t = 3.386$, $df = 12.51$, $p = 0.0051$). Time in the open arms ($t = 0.5299$, $df = 18$, $p = 0.6027$) time in the closed arms ($t = 0.3978$, $df = 18$, $p = 0.6954$), total distance traveled ($t = 0.8459$, $df = 18$, $p = 0.4087$) and time mobile ($t = 1.3904$, $df = 18$, $p = 0.2088$) were not affected by SI stress. These findings similarly indicate that a neophobic behavioral phenotype may be induced by SI stress without affecting locomotion.

3.4. Defensive shock probe burial

A Mann-Whitney *U* test found that total time burying the shock probe (Fig. 5) was significantly decreased in SI animals compared to GH animals ($U = 23$, $p = 0.0186$). In addition, an unpaired *t*-test found that SI animals spent significantly more time rearing than group housed animals ($t = 2.137$, $df = 18$, $p = 0.0466$). A Mann-Whitney *U* test failed to detect a significant effect of SI stress on time freezing in the DSPB task ($U = 44$, $p = 0.6842$). These findings indicate the adoption of a passive coping strategy in response to the noxiousness of the shock by SI animals.

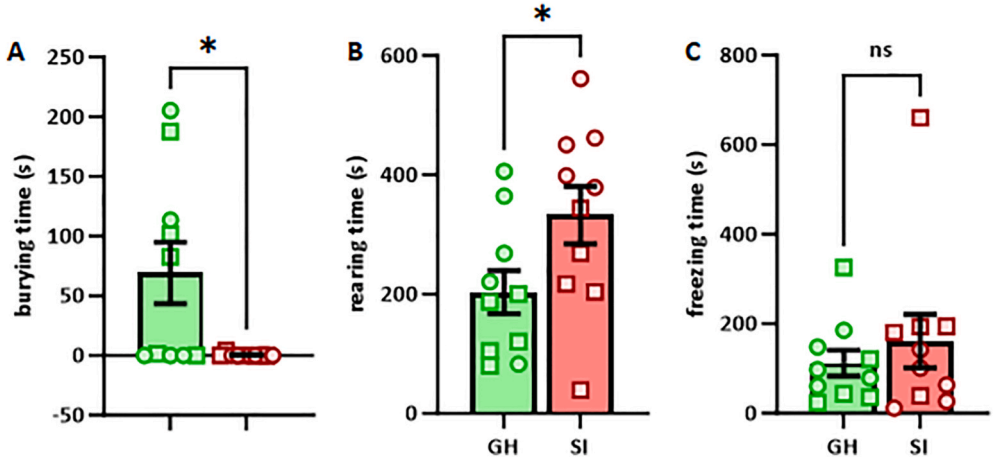

Fig. 4. Rats that underwent six weeks of SI stress freeze significantly more in the OFT than GH controls (A). Time spent in the center (B), time spent in the corners (C), total distance traveled (D) and total time mobile (D) were all unaffected by housing status. Heat maps show average activity for each group in the OFT.

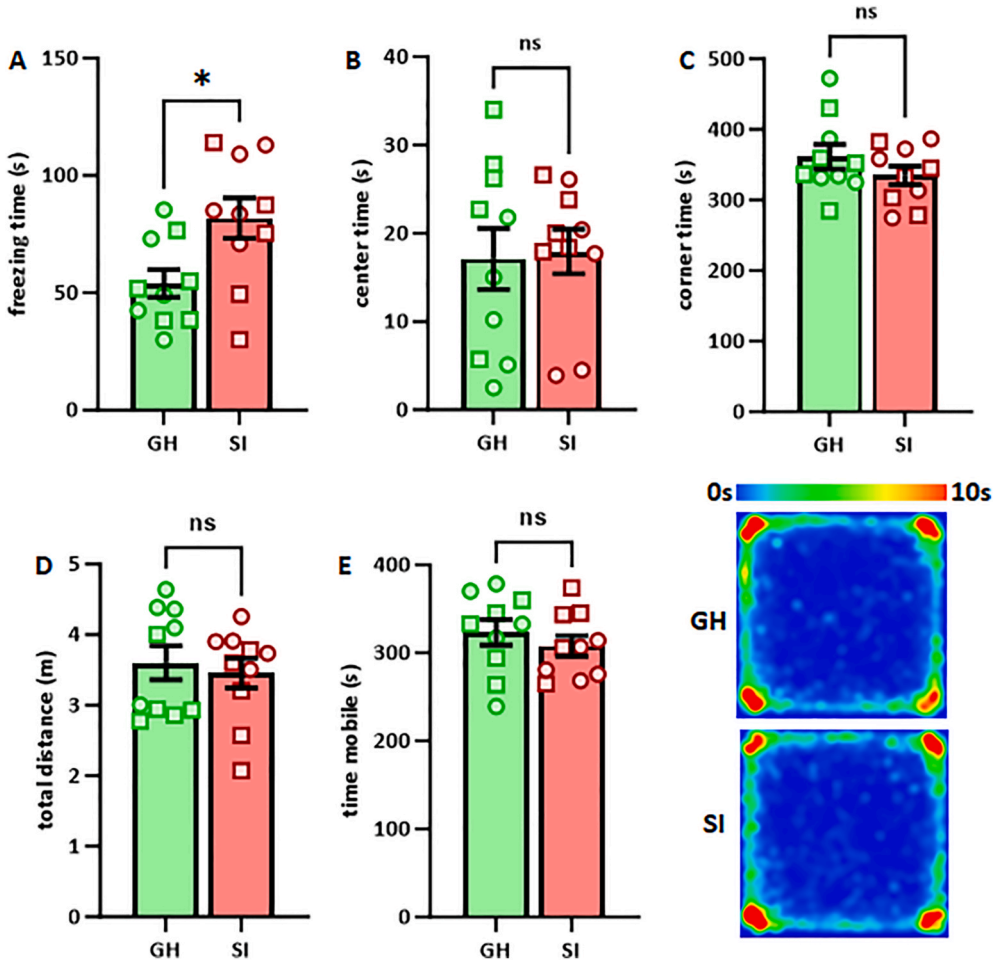

Fig. 5. Rats that underwent six weeks of SI stress freeze significantly more in the EPM than GH controls (A). Time spent in the open arms of the maze (B), time spent in the closed arms (C), total distance traveled (D) and total time mobile (E) were all unaffected by housing status. Heat maps show average activity for each group in the EPM with closed arms being vertical.

3.5. LC opioid receptor expression

Unpaired t-tests found that *oprd1* expression ($t = 2.247$, $df = 18$, $p = 0.0374$) and *oprk1* expression ($t = 2.105$, $df = 18$, $p = 0.0496$) were significantly decreased and increased, respectively, in SI versus GH animals. A Mann-Whitney *U* test failed to detect a significant effect of SI stress on *oprm1* expression ($U = 43$, $p = 0.6305$, Fig. 6)). These findings indicate that some classes of opioid receptors in LC are affected by SI stress.

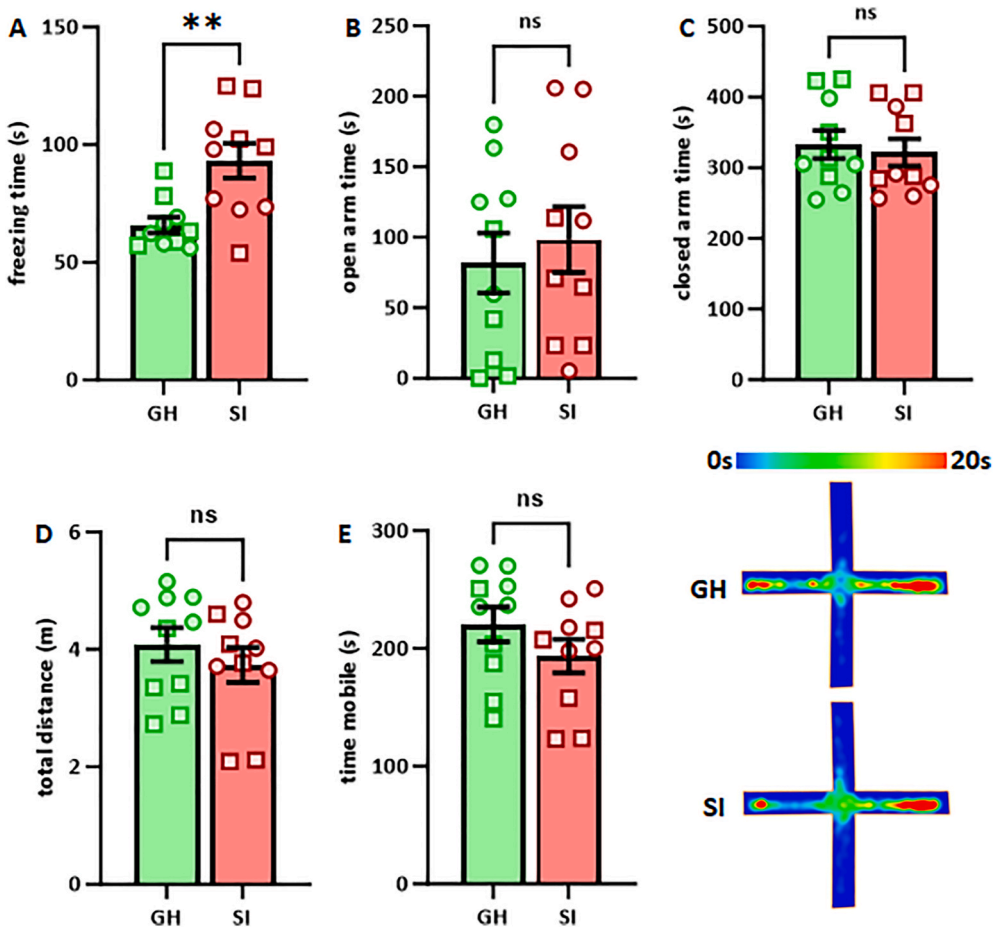

Fig. 6. Six weeks of SI stress reduces time spent burying the probe in the DSPB task (A) and increases the time spent rearing (B). Time spent freezing in this task is unaffected by housing status.

3.6. Correlations

Pearson correlation coefficients and p-values (Fig. 7) were calculated to identify potential relationships between LC opioid receptor expression and various behaviors. Significant positive correlations were identified between *oprd1* relative quantity and social investigation time ($r^2 = 0.29$, $p = 0.015$) and social preference ($r^2 = 0.271$, $p = 0.019$) in phase 1 of the 3CST. A significant negative correlation was also identified between *oprd1* relative abundance and freezing time in the OFT ($r^2 = 0.27$, $p = 0.018$). In addition, there was a trend towards a significant positive correlation between *oprd1* levels and total social investigation time in phase two of the 3CST ($r^2 = 0.13$, $p = 0.119$). KOR expression showed the opposite trends, with *oprk1* expression levels trending towards a negative correlation with total social investigation time in phase two of the 3CST ($r^2 = 0.12$, $P = 0.14$) towards a significant positive correlation with freezing time in the EPM ($r^2 = 0.14$, $p = 0.09$). These findings indicate that SI stress-induced changes in opioid receptors in LC may be contributing to behavioral change.

4. Discussion

The LC plays an important role in the stress response by enhancing NE release throughout the central nervous system to promote a behavioral phenotype characterized by hypervigilance and anxiety-like behavior (McCall et al., 2015; Hirschberg et al., 2017; McCall et al., 2017; Borodovitsyna et al., 2020; Tkaczynski et al., 2022). This effect is primarily mediated through CRF signaling which not only depolarizes LC cell bodies to elicit NE release in terminals but also through genetic, cellular, and neuroplastic regulation (Mamalaki et al., 1992; Rusnak et al., 2001; Swinny and Valentino, 2006; Salim et al., 2007; Swinny et al., 2010; Fan et al., 2013; George et al., 2013). Consistent with prior reports of the impact of various other forms of stress on LC neurobiology and its dependent behaviors (Curtis et al., 2012; George et al., 2013; Chaijale et al., 2015; Reyes et al., 2015; Sabban et al., 2015; Wood et al., 2015; Wang et al., 2017; Borodovitsyna et al., 2018; Sabban et al., 2018; Reyes et al., 2019; Serova et al., 2019; Borodovitsyna et al., 2020; Giustino et al., 2020; Arikawe et al., 2021; Tillage et al., 2021; Borodovitsyna et al., 2022; Tkaczynski et al., 2022; Kelberman et al., 2023), here we found that six weeks of SI stress is associated with down-regulation of *oprd1* (Fig. 7A) mRNA and upregulation of *oprk1* (Fig. 7B) mRNA in LC and behavioral change. Many of our behavioral findings are consistent with other investigations of the impact of SI stress on brain and behavior (Nikolaienko et al., 2023; A, Hansson et al., 2024; Goh et al., 2024; Lallai et al., 2024; Mohammadkhanizadeh et al., 2024). Notably, some, but not all social behaviors were affected by SI stress. Specifically, in the first phase of the 3CST, which measures social approach/motivation, experimental animals have access to a novel social rat and a non-social target. During this phase, there were no significant differences between groups in time investigating the novel rat (Fig. 2A), the non-social target (Fig. 2B), time spent investigating either target (Fig. 2C) or preference for the non-social target over the novel rat (Fig. 2D). However, in the second phase, which is classically more a measure of preference for social novelty, SI rats spent significantly less time investigating both a familiar rat (Fig. 3A), a novel rat (Fig. 3B), total time investigating both social targets (Fig. 3C). Preference for the novel versus familiar social target was notably unaffected by SI stress (Fig. 3D). This indicates that these animals may have a reduced affinity for social stimuli due to lack of exposure to other animals. This is

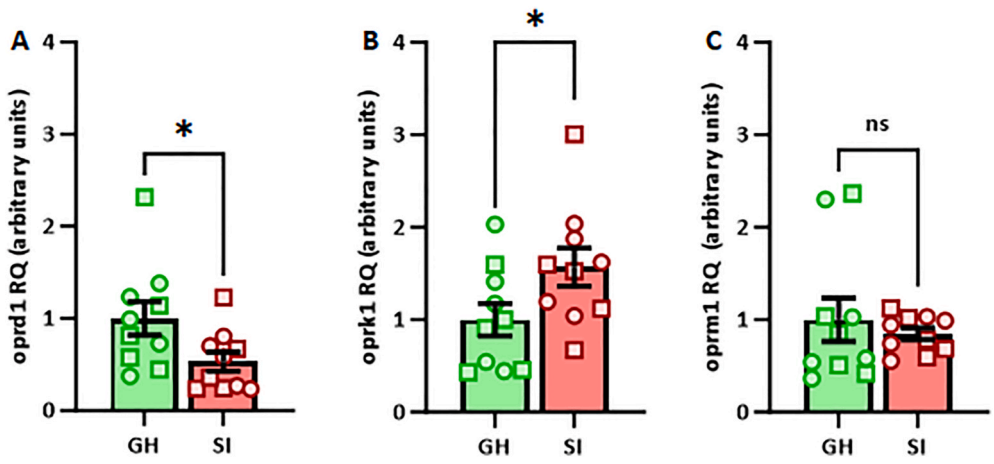

Fig. 7. SI stress was associated with downregulation of *oprd1* (A) and upregulation of *oprk1* (B) in LC relative to GH control animals. *Oprm1* expression was unaffected by SI stress (C).

consistent with other studies where other stressors or HPA-axis activators (single prolonged stress (Liu et al., 2024), maternal separation stress (Papadakakis et al., 2019; Ohta et al., 2020), CRFR1 receptor agonist (Zhao et al., 2007)) reduced sociability.

Notably, SI animals spent significantly less time investigating both the familiar and novel targets, and not just the novel. This indicates reduced sociability overall, but this was not the case in phase 1. One possible explanation is that both GF and SI animals had not yet habituated to the interaction chamber during phase 1, reducing their social motivation. By phase 2, however, their familiarity with the environment increased to the point that differences in social motivation had emerged. This is corroborated by the observation that the overall social investigation time in phase 1 was similar to the total social investigation time in phase 2 for both GH and SI animals. The lack of a significant effect in phase 1 may have been due to fear of the novel environment of the testing chamber. Thus, SI stress may reduce sociality overall without effects on social novelty preference, evidenced by a lack of significant effect in this measure. Aniother important technical detail that needs to be considered in interpreting these tests of sociability is the fact that only two male and two female animals were used as target rats. Target rats may have habituated to experimental animals and become less responsive to those investigating them throughout a day of testing. This could have impacted the behavior of experimental animals. However, we did not find any significant correlations between the order in which animals were tested and how much time they spent investigating the target animals (data not shown).

In the OFT (Fig. 4) and EPM (Fig. 5), SI animals spent significantly more time freezing than GH animals, although they did not spend less time in the open portions of each apparatus than GH controls. Although the lack of an effect on time in the open portions of the maze make it difficult to conclude that an anxiety-like phenotype developed in SI animals, the increased freezing, which is used as a marker of unconditioned fear in other behavioral tasks, indicates that these animals may be in a state of heightened vigilance in response to the novel environments of the mazes. This is supported by observations that total distance traveled and total time mobile in both of these tasks did not differ between groups. In addition, the number of freezing bouts was very nearly significantly increased in both the OFT ($p = 0.0575$) and EPM ($p = 0.056$, data now shown). SI animals also displayed significantly lower time burying the probe in the DSPB task (Fig. 6A), which is considered a measure for passive stress coping (Bondi et al., 2007; Fucich and Morilak, 2018). The shock at the beginning of this task has been shown to increase norepinephrine release in the lateral septum (Bondi et al., 2007), implicating LC involvement in this task. The SI-induced decrease in burying is indicative of a passive coping response, possibly as a consequence of dysregulated opioid receptor expression in LC. This is also consistent with others' findings which showed that rats that experienced social defeat less time burying in adulthood (Bingham et al., 2011). Somewhat counterintuitively, rearing behavior was greater in SI animals (Fig. 4B). Rearing is typically lower following chronic stress in larger open environments such as the OFT (Jiang et al., 2021; Shen et al., 2022; Xia et al., 2022). Rearing is less commonly studied in the DSPB task, but there is similar evidence that intracerebroventricular CRFR2 agonist administration, and subsequent HPA axis activation, decreases rearing in the DSPB as well (Zhao et al., 2007). Within the context of this study, we hypothesize that the increased rearing may be indicative of animals seeking out escape routes from the environment with the shock probe, however, additional studies are needed to confirm this. Interestingly, freezing behavior did not differ between groups in the DSPB task (Fig. 4C), although it was significantly greater in SI animals in both the OFT and EPM. This disparity may be due to the fact that these mazes were novel for all animals, while they had previously been habituated to the DSPB apparatus. Thus, the increased freezing in the SI group may be indicative of a neophobic response to the novel environments of the EPM and OFT.

Importantly, significant and nearly-significant correlations between *oprd1* and *oprk1* expression levels and various behaviors were identified (Fig. 8). Specifically, positive correlations were identified between *oprd1* relative quantity and social investigation time and social preference in phase 1 of the 3CST, while a significant negative correlation was found between *oprd1* relative abundance and freezing time in the OFT. In addition, there was a trend towards a significant positive correlation between *oprd1* levels and total social investigation time in phase two of the 3CST ($r^2 = 0.13$, $p = 0.119$). KOR expression showed the opposite trends, with *oprk1* expression levels trending towards a negative correlation with total social investigation time in phase two of the 3CST ($r^2 = 0.12$, $P = 0.14$) towards a significant positive correlation with freezing time in the EPM ($r^2 = 0.14$, $p = 0.09$). These correlations indicate that LC-expressed DORs may have important roles in driving social interaction and reducing neophobic responses to novel environments, while KORs promote the opposite. The present findings are also consistent with some of our prior observations, in which we found that *oprd1* levels are decreased in the LC one week after a single episode of combined restraint and predator odor stress as well (Tkaczynski et al., 2022). Notably, in that study, *oprm1* and *oprk1* expression levels were both unaffected by stress. In the present study, although *oprm1* levels were also unaffected by SI stress, the upregulation of *oprk1* indicates that stressors of varying modalities that induce similar behavioral changes may have variable impacts on LC gene expression. In addition, although we did not quantify the expression of the nociceptin opioid receptor in

LC, this was another potential target of the stress induced by SI that might impact behavior (Neal et al., 1999; Bodnar, 2013; Ulugol et al., 2016). We also did not identify any sex differences in these studies, while others in the past have found not only sex differences in anxiety-like behavior but also differences among females across the estrous cycle. (Borodovitsyna et al., 2022; Costa et al., 2024; Pestana and Graham, 2024). Thus, future studies should focus specifically on whether any of the outcome measures in this study vary according to sex but also the estrous cycle as well.

It is also worth noting that in addition to the modality of stress that animals are exposed to, variations in the duration of stress may have an impact on both gene expression and behavior. Other investigations into SI have used varying durations of isolation including one week (Ortelli et al., 2023; Pitcairn et al., 2024), five weeks (Harding et al., 2024), or eight weeks (Valdez et al., 2023; Hernandez Carballo et al., 2024; Xu et al., 2024). It may be worth investigating shorter or longer isolation times in the future to determine how varying durations of SI stress affect opioid receptor expression. For example, from this study alone, it is not clear if expression levels worsen with time, or if there are initial dramatic alterations that then gradually restore to baseline levels with longer durations through a habituation-like process. Relatedly, while animals will display behavioral habituation to various forms of stress over time upon continuous or repeated exposure (Hajos-Korcsok et al., 2003; Girotti et al., 2006; Kearns and Spencer, 2013), the fact that there were clear behavioral differences between SI and GH animals in this study indicates that behavioral habituation does not occur in response to this type of stress, even if isolation-induced biological changes do wane with time. Housing status may also introduce other confounds as well. For example, the core body temperatures of SI animals has been shown to be significantly different than GH (Kaneda et al., 2021), which could lead to metabolic differences between groups that manifest as behavioral change. In contrast to habituation, it is possible that the addition of other stressors on top of SI stress may have affected our outcomes in this study. For example, other groups have combined SI stress with additional stress models including chronic unpredictable stress and single prolonged stress to produce a greater depression-like or anxiety-like effect (Liu et al., 2024; Pitcairn et al., 2024; Yue et al., 2024). Future studies will investigate how opioid receptors are altered in socially isolated animals that have also undergone exposure to secondary stressors.

Age is another factor that may have affected our results. We previously found that adolescent (PND 30–35) rats were more susceptible to combined restraint and predator odor stress, both in terms of behavioral change and electrophysiological adaptation within LC, than adult (PND 77–82) rats (Tkaczynski et al., 2022). In the present study, social isolation began during a later adolescence point (PND 42) and continued into adulthood (PND 84), at which point behaviors were recorded. Therefore, rather than just the possibility of habituating to the stressor, the animals may be more resilient due to age, which could explain why only freezing in the OFT and EPM is affected by housing status, but not the time spent in the open portions of either maze. Other labs have also shown age-related changes in LC in response to stress, such as social defeat stress during adolescence causing elevated LC spontaneous discharge, while the same stress had no effect in adult animals (Bingham et al., 2011). Another limitation of this s tudy is that gene expression was assayed in crude LC tissue punches and did not take into account the fact that LC is a heterogeneous nucleus with anatomically and functionally distinct neurons (Chandler et al., 2014, 2019; Borodovitsyna et al., 2020; Poe et al., 2020). We previously showed that LC cells with discrete terminal fields vary in their gene expression patterns (Chandler et al., 2014), and that acute stress promotes different types of physiological adaptation based on whether they innervate the prefrontal cortex or central nucleus of amygdala (Borodovitsyna et al., 2020). Future experiments will investigate if unique changes in the expression of opioid receptors occurs in each of these populations of cells and how that relates to SI stress-induced behavioral change. Collectively, these data show that six weeks of social isolation stress produces neophobic, anxiety-like and passive coping behavior and social avoidance. The LC opioid system was investigated for its potential role in these behaviors, and it was found that KOR was upregulated and DOR was downregulated, with no effect on MOR. These results provide further evidence that the LC opioid system is important in the behavioral impact of SI stress, and be a potential therapeutic target for mood disorders and social deficits brought on by feelings of loneliness and social isolation.

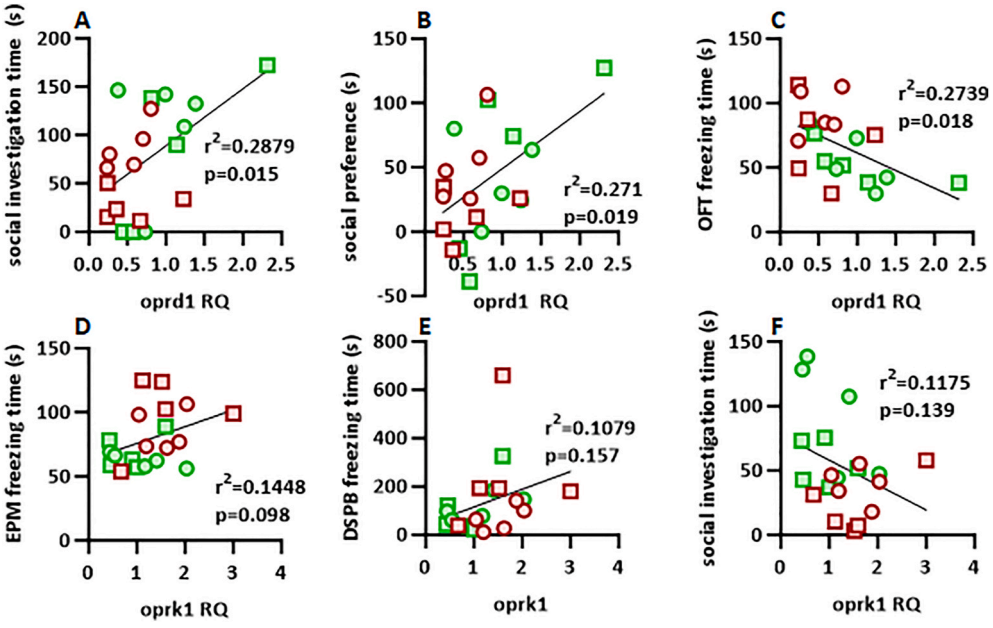

Fig. 8. Significant positive correlations were found between *oprd1* relative quantity in the LC and social investigation time (A) and social preference (B) in phase 1 of the 3CST. A significant negative correlation was found between LC *oprd1* relative quantity and time spent freezing in the OFT. Nonsignificant correlational trends were identified between *oprk1* abundance in the LC and time freezing in the EPM (D), time freezing in the DSPB (E), and time investigating social targets in phase 2 of the 3CST (F).

CRediT authorship contribution statement

John Tkaczynski: Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Formal analysis. **Jordan Riser:** Investigation. **Maya Patel:** Investigation. **Nicole Shellenbarger:** Investigation. **Jin Park:** Project administration, Investigation. **Daniel Manvich:** Resources, Methodology, Funding acquisition. **Daniel J. Chandler:** Writing – review & editing, Writing – original draft, Visualization, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

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Declaration of competing interest

None.

Data availability

Data will be made available on request.

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