



Early social isolation stress increases addiction vulnerability to heroin and alters c-Fos expression in the mesocorticolimbic system

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Abstract

Rationale Adverse psychosocial factors during early childhood or adolescence compromise neural structure and brain function, inducing susceptibility for many psychiatric disorders such as substance use disorder. Nevertheless, the mechanisms underlying early life stress-induced addiction vulnerability is still unclear, especially for opioids.

Objectives To address this, we used a mouse heroin self-administration model to examine how chronic early social isolation (ESI) stress (5 weeks, beginning at weaning) affects the behavioral and neural responses to heroin during adulthood.

Results We found that ESI stress did not alter the acquisition for sucrose or heroin self-administration, nor change the motivation for sucrose on a progressive ratio schedule. However, ESI stress induced an upward shift of heroin dose-response curve in female mice and increased motivation and seeking for heroin in both sexes. Furthermore, we examined the neuronal activity (measured by c-Fos expression) within the key brain regions of the mesocorticolimbic system, including the prelimbic cortex (PrL), infralimbic cortex (IL), nucleus accumbens (NAc) core and shell, caudate putamen, and ventral tegmental area (VTA). We found that ESI stress dampened c-Fos expression in the PrL, IL, and VTA after 14-day forced abstinence, while augmented the neuronal responses to heroin-predictive context and cue in the IL and NAc core. Moreover, ESI stress disrupted the association between c-Fos expression and attempted infusions during heroin-seeking test in the PrL.

Conclusions These data indicate that ESI stress leads to increased seeking and motivation for heroin, and this may be associated with distinct changes in neuronal activities in different subregions of the mesocorticolimbic system.

Keywords Heroin self-administration · Dose-response · Heroin seeking · Social isolation · Stress · c-Fos · Prefrontal cortex · Nucleus accumbens · Ventral tegmental area · Sex difference

Introduction

Substance use disorder is characterized by compulsive drug taking and enduring vulnerability to relapse. Stress is a well-known risk factor for both vulnerability and development of addiction (Sinha 2008). Chronic stress induces persistent homeostatic dysregulation to cause maladaptive behaviors such as addiction.

Adolescence or early adulthood is a critical period during which the brain is vulnerable to irreversible synaptic remodeling disruptions (Lupien et al. 2009; Paus et al. 2008).

Stress or life adversities during this time induce enduring alterations in neural plasticity that leads to long-term behavioral maladaptations. Negative life events during early life, such as isolation and low parental or social support, have all been associated with increased risk of drug use and abuse (Barrett and Turner 2006; Chassin et al. 1988; Costa et al. 1999; Newcomb and Bentler 1988; Newcomb and Harlow 1986; Sher et al. 1997; Wills and Cleary 1996; Wills et al. 1992). Additionally, social isolation stress during adolescence induces highly comorbid behavioral alterations (e.g., aggression (Mikics et al. 2018), anxiety (Caruso et al. 2018), social withdrawal (Liu et al. 2012)) related to anxiety disorders, depression, and schizophrenia, which are all risk factors for substance use disorders (Gregg et al. 2007; Krystal et al. 2006; Smith and Book 2008). Thus, early social isolation (ESI) stress model has been widely used in preclinical studies for psychiatric disorders.

Preclinical studies using a self-administration (SA) model for substance use disorder show that ESI stress enhances the

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initiation of SA for psychostimulants and opioids (Bozarth et al. 1989; Marks-Kaufman and Lewis 1984), while others show no effect (Boyle et al. 1991; Schenk et al. 1988) or decreased effect (Howes et al. 2000; Phillips et al. 1994) (reviewed in Lu et al. (2003) and Walker et al. (2019)). Meanwhile, ESI increases seeking or extinction resistance for drugs including cocaine (Fosnocht et al. 2019), amphetamine (Whitaker et al. 2013), and alcohol (Cortes-Patino et al. 2016) in different behavioral procedures. Yet, how ESI stress alters opioid seeking is still unknown. Therefore, the aim of the current study is to examine the impact of early life social isolation stress on drug-taking and drug-seeking behaviors using a heroin self-administration model. Compared to the extinction-reinstatement procedure, forced abstinence followed by cue-induced seeking better represents the human scenario, where relapse typically occurs after a drug-free withdrawal period rather than extinction training (Fuchs et al. 2008). As this model has been commonly used in studies of heroin-seeking behaviors in rodents (Reiner et al. 2019; Ren et al. 2009), a forced abstinence followed by cue-induced seeking procedure was chosen in the current study.

Here, we established that ESI stress does not alter the acquisition for sucrose or heroin self-administration, nor change the motivation for sucrose. However, ESI stress causes an upward shift of heroin dose-response curve in female mice and increases motivation for heroin and heroin seeking in both male and female mice. Then, using *c-Fos* as a proxy of neuronal activity (Bullitt 1990; Cruz et al. 2015), we examined neuronal activity patterns within the mesocorticolimbic reward pathway during abstinence and right after heroin-seeking test in control and ESI mice. Furthermore, we examined the correlation between *c-Fos* immunoactivity and heroin-seeking behavior. Our results indicate that ESI stress induces unique neuronal activity alterations that are associated with increased heroin seeking.

Materials and methods

Animals

The current study used both male and female C57BL/6J mice that were purchased from Jackson lab and maintained in the lab (stock number 000664, Bar Harbor, ME, USA). Animals were housed under the temperature and humidity controlled by animal care facility with 12-h light/dark cycle (lights on at 11:00 A.M. and light off at 11:00 P.M.). All the procedures are approved by the Institutional Animal Care and Use Committee, University of Kansas. All animals were maintained according to the National Institutes of Health guidelines in Association for Assessment and Accreditation of Laboratory Animal Care accredited facilities

Early social isolation stress

Based on previous publications (Cortes-Patino et al. 2016; Yamamuro et al. 2018), early social isolation (ESI) stress was carried out after weaning from postnatal day 21 (P21) to P56 (about 5 weeks). During this time, ESI mice were single housed, and control mice were group housed (4–5 mice per cage). Other housing conditions were the same to avoid environmental enrichment-induced effects.

Drug

Heroin hydrochloride, generously gifted from the NIDA drug supply program, was dissolved in 0.9% sterile saline. Heroin solutions were prepared on a weekly basis (0.1 mg/mL). Pump durations were adjusted according to the animals' body weights on a daily basis to ensure delivery of the correct dose of drug for each animal.

Self-administration test chambers

The experimental chambers have been described elsewhere (Gancarz et al. 2015; Wang et al. 2017; Wang et al. 2016) with modifications. Briefly, 16 standard Med Associates Inc. (St. Albans, VT) chambers containing two nose-poke holes each with infrared monitoring were used. Two stimulus lights were mounted within each nose-poke hole, with a house light in the center back wall of the test chamber. All chambers are housed in sound-attenuating boxes and controlled through a Med Associates interface.

Sucrose self-administration

At 8 weeks of age, animals were first trained to self-administer sucrose solution (10%). Responses in the active nose-poke holes resulted in the illumination of a discrete cue light (stimulus light, 5s) with the administration of sucrose, followed by 15-s time-out period with the chamber light off and no programmed consequences. Mice were trained for 8 days on a fixed ratio (FR) 1 schedule of reinforcement, which was increased daily to FR5 and maintained at this FR for the remainder of self-administration protocol. The mice were limited to a maximum of 100 infusions during the daily 90-min operant session. The following criteria for acquisition of operant responding were adopted from publications (Martin-Garcia et al. 2011; Soria et al. 2008; Wilkerson et al. 2017): mice will be included if they maintain stable responding with (1) less than 30% deviation from the mean of the total number of infusions earned in three consecutive sessions, (2) at least 65% responding on the reinforced nose-poke, and (3) a minimum of 5 reinforcers per session. Following 8 days of

sucrose training, mice were tested for 1 day on a progressive ratio (PR) schedule, where the response requirement for each sucrose infusion increased until the mouse did not fulfill the requirement and the session ended if the animal took longer than 60 min to meet the requirement. The response requirement was defined as $R(i) = [5e^{0.2i} - 5]$, rounded to the nearest integer (Gancarz et al. 2012; Martin et al. 2018; Richardson and Roberts 1996). The total numbers of infusions and active responses as well as the final ratio in effect (i.e., breakpoint) were recorded and analyzed.

Jugular catheterization surgery

After sucrose self-administration, mice were anesthetized with 100 mg/kg ketamine and 5 mg/kg xylazine and then implanted with chronic indwelling jugular catheters as previously described (Wang et al. 2017; Wang et al. 2016) with modifications. Catheter was inserted into the right jugular vein and sutured up in place. The catheter was threaded subcutaneously over the shoulder blade and was connected to the harness (Instech, Plymouth Meeting, PA, USA). Following surgery, catheters were flushed daily with 0.05 ml of heparinized saline to preserve catheter patency. Before behavioral testing, each animal received an i.v. infusion of ketamine hydrochloride (1 mg/ml in 0.05 ml), and the behavioral response was observed to verify catheter patency. Decrease of muscle tone served as behavioral indicators of patency. During heroin self-administration, increased locomotor activity was used as an indicator for patency (Engeln et al. 2021).

Heroin self-administration

The heroin self-administration procedures were conducted as previously described (Gancarz et al. 2015; Martin et al. 2018; Wang et al. 2017; Wang et al. 2016) with modifications. Following the 7-day recovery from jugular catheter surgery, mice were assigned to acquisition of heroin self-administration. Mice were subjected to daily 3-h self-administration training, during which responses to the active alternative snout-poke hole resulted in i.v. infusions of heroin (0.05 mg/kg/infusion) according to a fixed ratio 1 (FR1) schedule of reinforcement, which was increased daily to FR3 and maintained at this FR for the remainder of self-administration protocol. Infusions were accompanied by a 5-s illumination of the stimulus light inside the active snout-poke hole followed by a 15-s time-out period, during which time the house light was extinguished. Responses to the inactive hole resulted in no programmed consequences. The criterion for acquisition of heroin self-administration was similar as described above.

Dose-response

After completing acquisition training, mice were subsequently trained on a within-session dose-response procedure, as previously described (Martin et al. 2018) with slight modifications. Briefly, the daily 3-h self-administration session was divided into four 45-min sessions, each preceded by a 2-min time-out period. Mice were exposed to four doses of heroin (0.00625, 0.0125, 0.025, and 0.05 mg/kg/inf) for 45 min. The order of the doses tested was pseudorandomized such that the same doses were never tested in the same order during training. We limited each of the four self-administration sessions to 45 min primarily to match the session duration during training (3 h) and to delineate drug intake at multiple doses of drug while minimizing drug side effects (i.e., sedation, stereotypy, etc.), which may be a confounding variable. The dose of heroin per infusion was regulated via adjusting the infusion volumes (i.e., pump-on durations). Following each test session, the catheters were flushed with saline and mice were returned to the colony room. The average number for earned infusions throughout the 5 days of dose-response training was calculated.

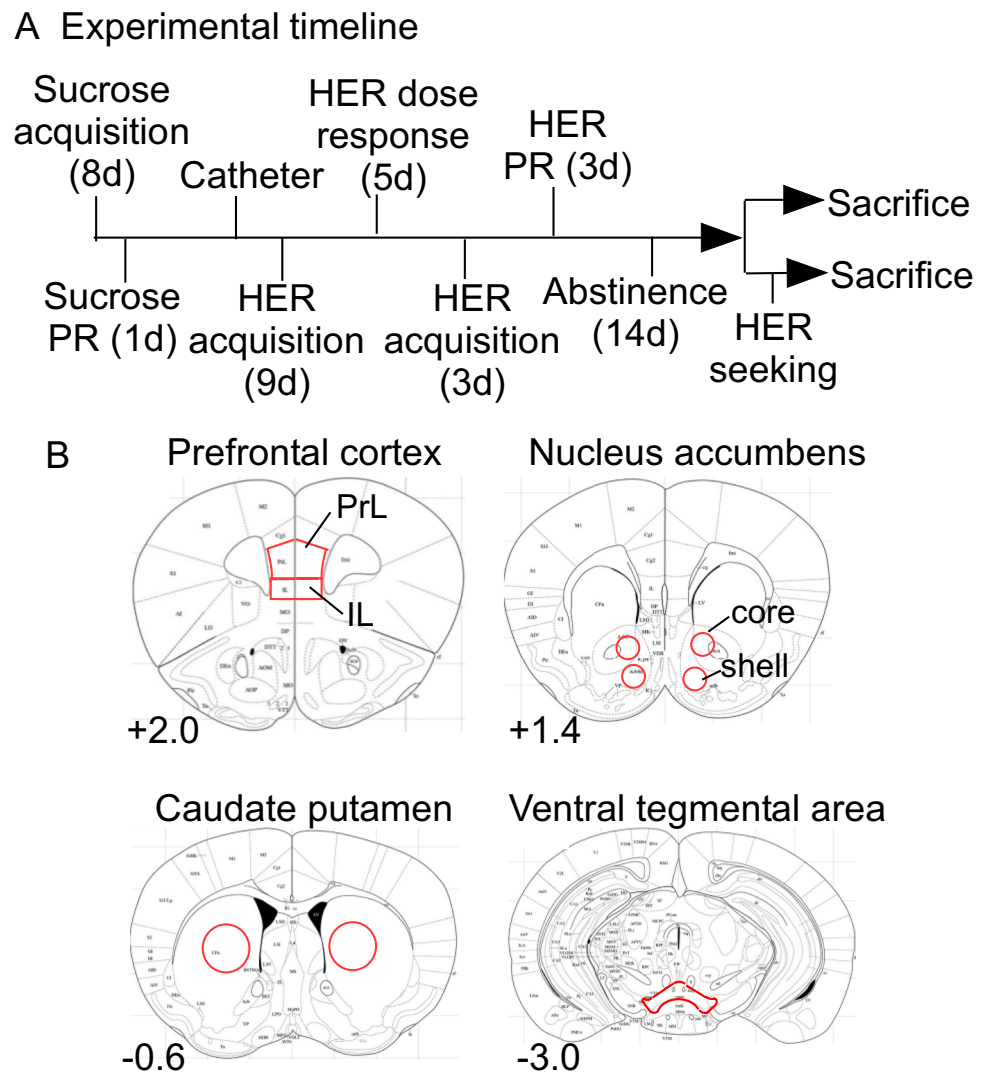
Progressive ratio

After completing dose-response training, mice underwent 3 days of re-acquisition of heroin self-administration as described above. After re-acquiring stable infusions, mice were tested for 3 days on a progressive ratio schedule, where the response requirement for each infusion increased until the mouse did not fulfill the requirement and the session ended if the animal took longer than 60 min to meet the requirement. The definition of response requirement is the same as described above.

Heroin-seeking test

After progressive ratio test, animals went through forced abstinence for 14 days (a time period showed high extinction responding for heroin (Shalev et al. 2001)), mimicking the real-life situation in which environmental cues precipitate relapse behavior following an extended period of abstinence. Then, mice were placed back in the same chambers for a 1-h context- and discrete cue-induced seeking test (thereafter named as heroin-seeking test). During heroin-seeking test, the active responses produced discrete cues previously paired with drug delivery and heroin is not available. Therefore, this active response-produced event during heroin-seeking test was termed as attempted infusions. The timeline for experimental protocol is illustrated in Fig. 1A.

Fig. 1 Experimental design. **(A)** Timeline for the experimental design. HER, heroin; PR, progressive ratio. **(B)** Schematic representations of brain regions analyzed for c-Fos expression. Red color indicates the areas where cells were counted. Numbers in the lower-left corner of each brain section represent the distance from bregma. PrL, prelimbic cortex; IL, infralimbic cortex



Tissue collection and immunostaining

To examine the effect of ESI stress on heroin responses in the brain, we performed immunofluorescent staining for c-Fos as previously described with some modifications (Cruz et al. 2015; Wang et al. 2017; Wang et al. 2015). Four mice per group were sacrificed after 14 days of forced abstinence, and five mice per group were sacrificed right after the heroin-seeking test. Mice were sacrificed via transcardial perfusion of PBS followed by 4% formaldehyde. Whole brains were immediately removed and post-fixed at 4 °C for 24 h and then immersed in 30% sucrose in 0.01 M PBS (pH 7.4) at 4 °C. Coronal sections encompassing the prelimbic area (PrL), infralimbic area (IL), nucleus accumbens core (NAcc) and shell (NAcSh), caudate putamen (CPU), and ventral tegmental area (VTA) were cut at a thickness of 40 μm using a vibratome (Leica VT1000s, Buffalo Grove, IL, USA).

For immunostaining, brain sections were rinsed in 0.01 M PBS (pH 7.4) for 3 times then blocked in 3% normal donkey serum with 0.3% Triton-X for 2 h at room temperature. Sections were then incubated in c-Fos (1:500, Abcam, ab190289, Cambridge, MA, USA) primary antibody overnight at 4 °C. On the second day, sections were incubated with Alexa Fluor 488-conjugated secondary antibody at 1:800 (Thermo Fisher, Waltham, MA, USA) for 2 h at room temperature. Then, slides were mounted with DAPI-containing mounting media (Vector lab, Burlingame, CA, USA).

Cell counting

C-Fos immunoactivity was assessed via imaging on a Leica BM4000 microscope under 20× magnification. c-Fos-immunolabeling was bilaterally quantified from at least 3–4 sections per mouse and averaged to determine the profile of each brain region. The experimenter quantifying was blind

to group assignments. c-Fos images were quantified using ImageJ software. The numbers of Fos-positive nuclei in regions of interest were counted with a point counter tool. This tool simultaneously marked and counted each cell so that no cells could be counted twice. The levels chosen corresponded to the following distances from bregma (based on Paxinos and Watson (Paxinos et al. 1980), Fig. 1B): PrL and IL, +2.2 mm; NAcc and NAcSh, +1.4 mm; CPU, -0.6 mm; VTA, -3.0 mm.

Statistical analysis

Multi-factor repeated ANOVA was performed on the number of infusions and nose-poke responses during acquisition for sucrose or heroin self-administration, as well as the number of infusions in heroin dose-response curve. All the other comparisons of dependent variables were analyzed by two-way ANOVA. Post hoc analysis was performed by Tukey's comparisons. The correlation between c-fos numbers and heroin-seeking behavior was assessed using Pearson's correlation analysis. All data were analyzed using SPSS software (IBM Corp., Armonk, NY, USA) or Graphpad (GraphPad Software, San Diego, CA), and are represented as the mean \pm SEM, with $P < 0.05$ indicating significance. Statistical details are provided in Supplementary table 1.

Results

ESI stress does not alter sucrose acquisition and the motivation for sucrose

To find out whether ESI stress alters the behavioral responses to sucrose, male and female mice underwent sucrose self-administration and followed by progressive ratio test. Using multi-factor repeated ANOVA analysis, we did not find any significant main effect for stress in sucrose infusions (Fig. 2A, $F_{1,45}(\text{stress}) = 3.178$, $P = 0.081$), total active responses (Fig. 2B, $F_{1,45}(\text{stress}) = 1.577$, $P = 0.216$), and total inactive responses (Fig. 2C, $F_{1,45}(\text{stress}) = 1.921$, $P = 0.173$) during the fixed ratio self-administration phase, suggesting that ESI stress does not alter sucrose acquisition. Furthermore, we found that the infusions (Fig. 2D, $F_{1,45}(\text{stress}) = 0.019$, $P = 0.89$) and total active responses (Fig. 2E, $F_{1,45}(\text{stress}) = 0.006$, $P = 0.936$) for sucrose on a progressive ratio schedule showed no difference across groups. Additionally, ESI did not change the progressive ratio breakpoint for sucrose (Fig. S1, $F_{1,45}(\text{stress}) = 0.305$, $P = 0.583$; $F_{1,45}(\text{sex}) = 0.039$, $P = 0.843$; $F_{1,45}(\text{interaction}) = 0.031$, $P = 0.861$). These data indicate that the motivation for sucrose was similar among the tested groups.

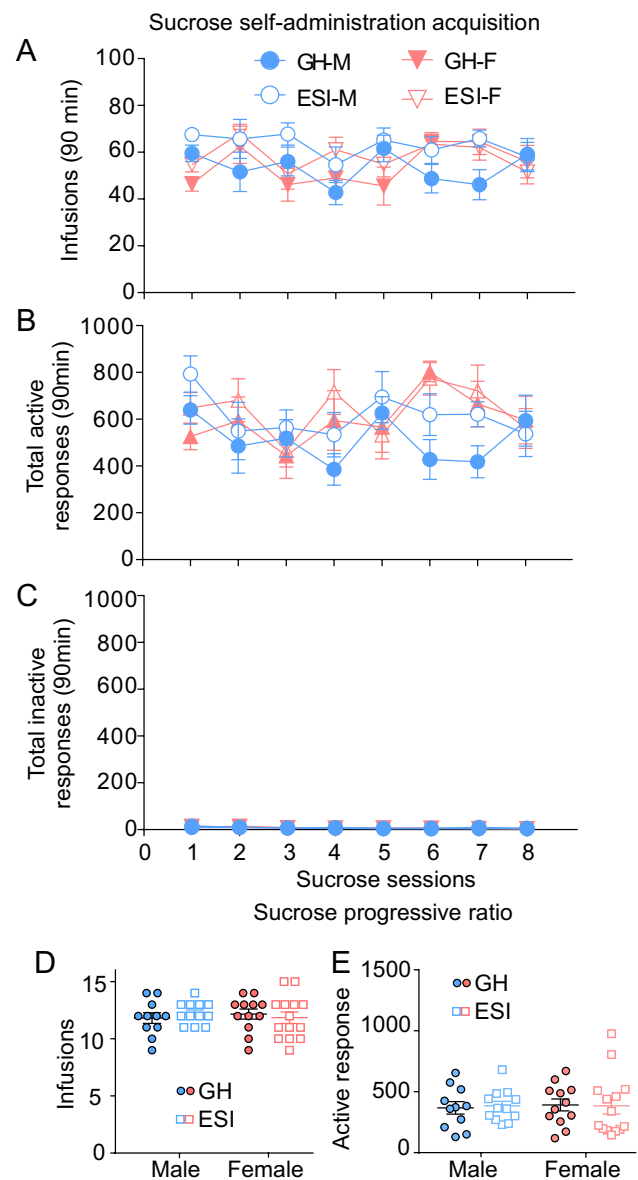


Fig. 2 Early social isolation stress does not affect sucrose self-administration. Mean numbers of (A) infusions, (B) total active responses, and (C) total inactive responses per session during sucrose self-administration training. Mean numbers of (D) earned infusions and (E) total active responses during the progressive ratio test for sucrose. Data are expressed as mean \pm SEM, $n = 11$ –14/group. GH, group house; ESI, early social isolation

ESI stress induces an upward shift of heroin dose-response curve in females and increases heroin motivation and potentiates heroin seeking in both sexes

Heroin self-administration acquisition

To examine whether ESI stress affects heroin addiction-like behaviors, male and female mice underwent heroin

self-administration at the dose of 0.05 mg/kg/infusion. Multi-factor repeated ANOVA did not reveal any significant main effect for stress in the number of infusions (Fig. 3A, $F_{1,45}(\text{stress}) = 2.525$, $P = 0.12$) and total active responses per session across days of training (Fig. 3B, $F_{1,45}(\text{stress}) = 1.147$, $P = 0.29$), suggesting that ESI stress does not affect heroin acquisition. Moreover, the total active responses were increased over the course of the 9 training sessions (Fig. 3B, $F_{8,360}(\text{session}) = 9.617$, $P < 0.0001$), implying the

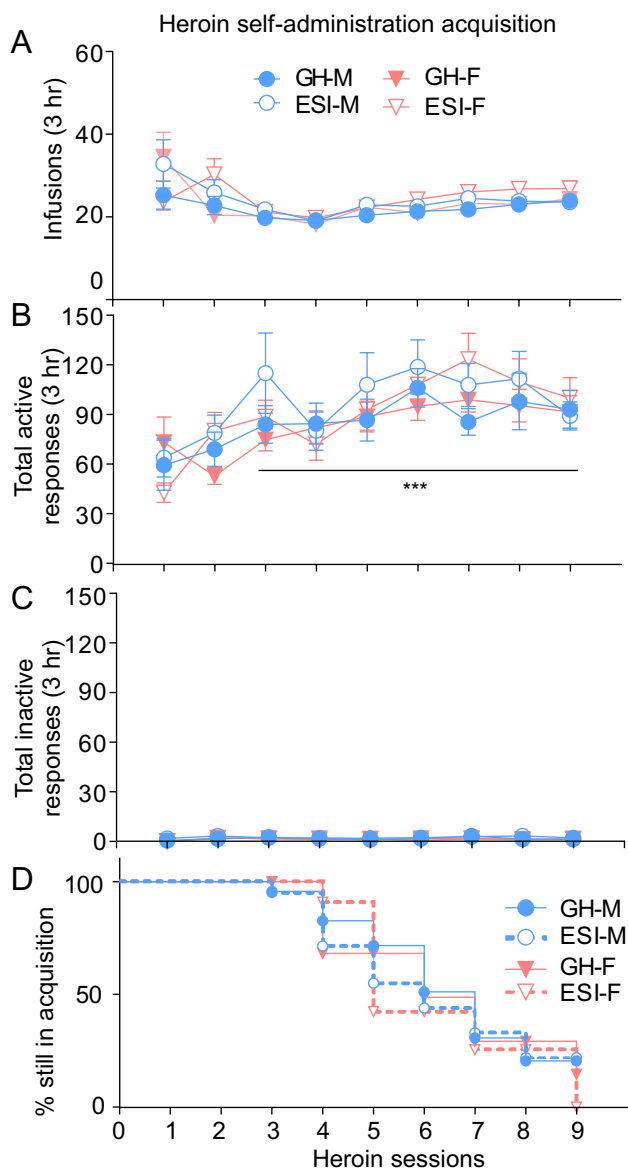


Fig. 3 Early social isolation stress does not change the acquisition of heroin self-administration. Mean numbers of (A) infusions, (B) total active responses, and (C) total inactive responses per session during heroin self-administration training. (D) Survival distributions when meeting acquisition criteria. Data are expressed as mean \pm SEM. *** $P < 0.001$ vs session 1, $n = 11$ – 14 /group. GH, group house; ESI, early social isolation

overall increased acquisition of operant responding for heroin. Additionally, ESI stress did not change the total inactive responses (Fig. 3C, $F_{1,45}(\text{stress}) = 0.286$, $P = 0.595$). Furthermore, a similar number of mice in each group failed to meet acquisition criteria for heroin operant conditioning across 9 sessions, and a log-rank analysis comparing survival distributions of groups to meeting criteria did not show any significant difference (Fig. 3D). Interestingly, there was a significant main effect for session or for the interaction of stress, sex, and session in infusion numbers across the 9 training sessions (Fig. 3A, $F_{8,360}(\text{session}) = 8.915$, $P = 0.0001$; $F_{8,360}(\text{interaction}) = 3.1$, $P = 0.002$). Post hoc analysis indicated that the infusion numbers for session 1 were significantly higher than those for sessions 5–9 ($P < 0.001$), and the infusion numbers for sessions 5–9 were notably higher than those for session 4 ($P < 0.001$). This high responding for heroin at the beginning of acquisition phase may be due to the influence of sucrose extinction, as these mice were trained to respond for sucrose prior to heroin self-administration.

Heroin dose-response

As ESI stress increases the vulnerability for the development of addiction and relapse, we next sought to examine the role of ESI stress in regulation of other addiction-like behaviors. We first turned to a within-session dose-response paradigm, which can measure sensitivity to drug (in case of horizontal shift) or predict drug vulnerability (in case of vertical shift) (Martin et al. 1996; Piazza et al. 2000). Multi-factor ANOVA analysis revealed a significant main effect for heroin dose (Fig. 4A, $F_{3,135}(\text{dose}) = 111.875$, $P < 0.0001$), indicating that the number of infusions is dependent on heroin doses. A Tukey's post hoc analysis showed that there were significant differences in the number of earned infusions between any two selected doses ($P < 0.001$). In addition, there was a significant main effect for stress (Fig. 4A, $F_{1,45}(\text{stress}) = 10.864$, $P = 0.002$) and sex (Fig. 4A, $F_{1,45}(\text{sex}) = 8.285$, $P = 0.006$). A Tukey's post hoc analysis revealed that ESI stress increased the earned infusions at the dose of 0.025 mg/kg/infusion (Fig. 4A, $P < 0.05$), demonstrating increased drug vulnerability in ESI female mice.

Heroin progressive ratio

Next, we moved on to determine whether ESI stress alters the motivation for heroin. To this end, animals were tested in a progressive ratio schedule of reinforcement, which is considered a model for measuring reinforcing efficacy and motivation for obtaining a reinforcer (Hodos 1961; Richardson and Roberts 1996). Two-way ANOVA analysis showed a significant main effect for stress in the number of infusions (Fig. 4B, $F_{1,45}(\text{stress}) = 105.1$, $P < 0.0001$) and in active

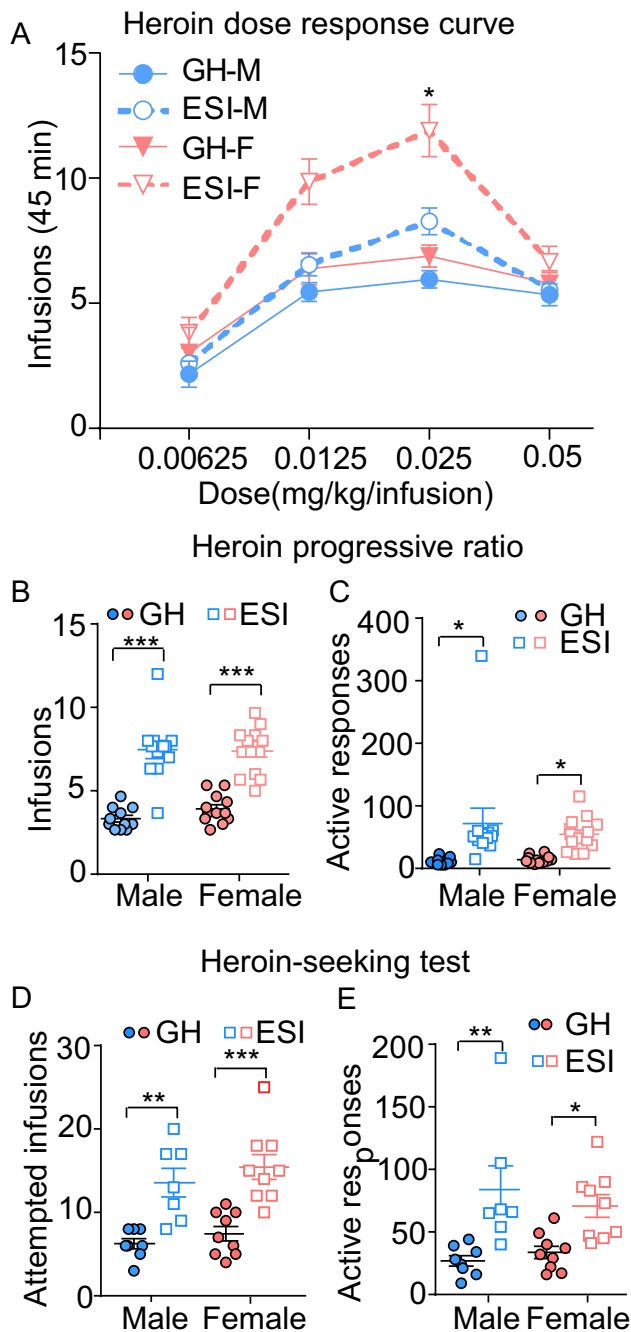


Fig. 4 Early social isolation stress causes an upward shift of heroin dose-response curve in female mice and increases motivation for heroin and heroin seeking in both sexes. **(A)** Within-session heroin self-administration dose-response (mg/kg/infusion [inf]) in group-housed (GH) and early social-isolated (ESI) mice ($n = 11\text{--}14/\text{group}$, $*P < 0.05$ vs GH-F). Mean numbers of **(B)** earned infusions and **(C)** total active responses during the progressive ratio test for heroin in GH and EIS mice ($n = 11\text{--}14/\text{group}$). Mean numbers of **(D)** attempted infusion and **(E)** total active responses during heroin-seeking test in GH and ESI mice ($n = 7\text{--}9/\text{group}$). Data are expressed as mean \pm SEM, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$

responses during the progressive ratio schedule (Fig. 4C, $F_{1,45}(\text{stress}) = 15.61$, $P = 0.0003$). A Tukey's post hoc analysis determined that ESI stress increased the number of infusions (Fig. 4B, $P < 0.001$) and active responses (Fig. 4C, $P < 0.05$) in both male and female mice. Additionally, there was no significant difference in inactive responses (Fig. S2A), suggesting that the increased operant responding for heroin is not due to overall increased nose-poking behavior. We also found that ESI stress increased the progressive ratio breakpoint for heroin (Fig. S2B, $F_{1,45}(\text{stress}) = 15.48$, $P = 0.0003$; $F_{1,45}(\text{sex}) = 0.226$, $P = 0.6369$; $F_{1,45}(\text{interaction}) = 0.5558$, $P = 0.4598$) in both male ($P < 0.01$) and female ($P < 0.05$) mice. These data indicate that ESI stress increases the motivation for heroin.

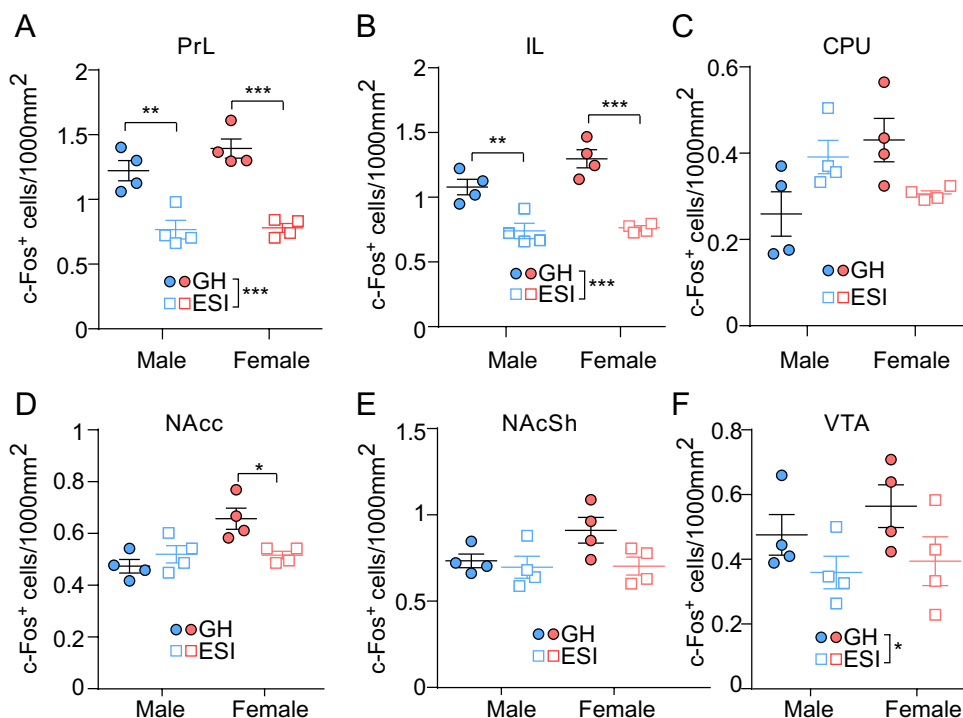
Heroin seeking

To examine whether ESI stress affects heroin-seeking behavior, mice went through a context- and cue-induced heroin seeking test. Using two-way ANOVA analysis, we found a significant main effect for stress in attempted infusions (Fig. 4D, $F_{1,29}(\text{stress}) = 38.6$, $P < 0.0001$), as well as in total active responses (Fig. 4E, $F_{1,29}(\text{stress}) = 21.9$, $P < 0.0001$). A Tukey's post hoc analysis discovered that ESI stress increased the attempted infusions (Fig. 4D) and total active responses (Fig. 4E) in both males ($P < 0.01$) and females (attempted infusions: $P < 0.001$, total active response: $P < 0.05$). Importantly, ESI stress did not change the inactive responses (Fig. S3) across groups.

ESI stress dampens c-Fos expression in PrL, IL, and VTA after heroin abstinence

To better understand the neurobiological mechanisms underlying the ESI-dependent behavioral responses to heroin, we used c-Fos expression as a representative marker for neuronal activity to examine the neuronal activity changes in several brain regions within the mesocorticolimbic system. Some mice were sacrificed after 14 days of forced abstinence for c-Fos immunostaining (Fig. 5, Fig. S4). Two-way ANOVA analysis revealed a significant main effect for stress in the PrL (Fig. 5A, $F_{1,12}(\text{stress}) = 62.88$, $P < 0.0001$), IL (Fig. 5B, $F_{1,12}(\text{stress}) = 62.82$, $P < 0.0001$), and VTA (Fig. 5F, $F_{1,12}(\text{stress}) = 4.996$, $P = 0.045$). A Tukey's post hoc analysis showed that the number of c-Fos-positive cells in the PrL (Fig. 5A) and IL (Fig. 5B) from ESI mice was significantly lower comparing to GH mice in both sexes (males: $P < 0.01$; females: $P < 0.001$). Interestingly, there was a significant main effect for sex or for the interaction of stress and sex in NAcc (Fig. 5D, $F_{1,12}(\text{sex}) = 8.858$, $P = 0.01$; $F_{1,12}(\text{interaction}) = 9.55$, $P = 0.009$), with post hoc analysis showing reduced c-Fos expression specifically in ESI female mice comparing

Fig. 5 c-Fos immunoreactivity is reduced in early social-isolated mice after forced abstinence from heroin self-administration. (A–F) Number of c-Fos-positive cells in the following brain regions after 14 days of forced abstinence from heroin self-administration: prelimbic cortex (PrL), infralimbic cortex (IL), nucleus accumbens core (NAcc) and shell (NAcSh), caudate putamen (CPU), and ventral tegmental area (VTA). Data are expressed as mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, $n = 4$ mice/group. GH, group house; ESI, early social isolation

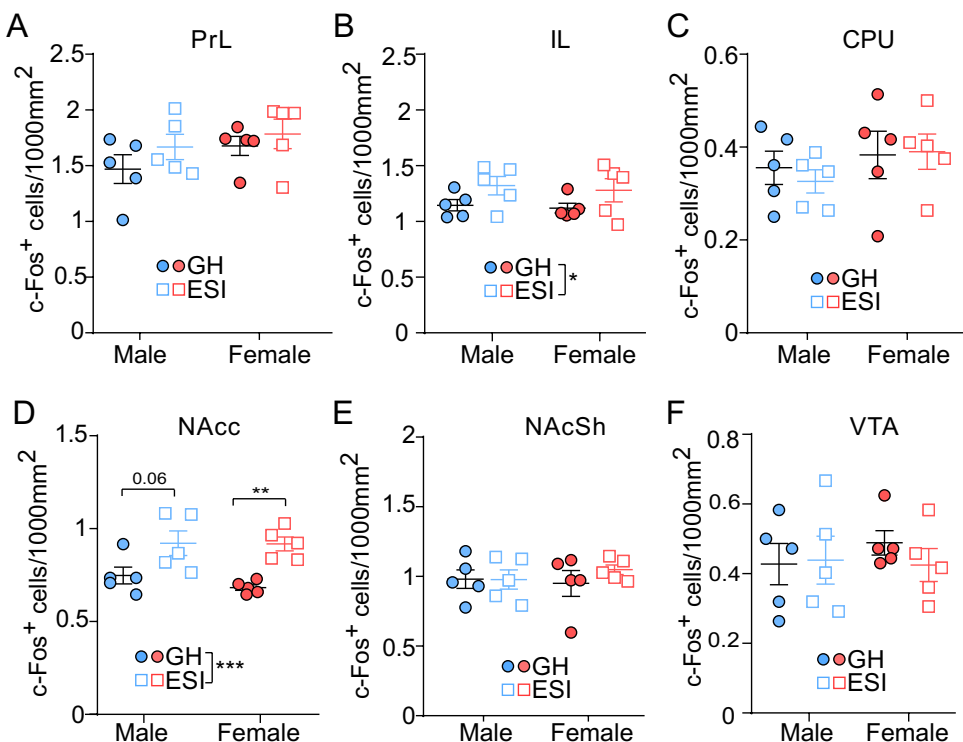


to GH females ($P < 0.05$). Although there was a significant main effect for interaction in CPU ($F_{1,12}(\text{interaction}) = 9.781$, $P = 0.009$), no significant difference between any two groups was revealed by post hoc analysis. Additionally, there was no statistical difference in the main effect for neither stress nor sex in CPU and NAcSh.

ESI stress enhances the c-Fos activation in IL and NAcc after heroin-seeking test

Some animals were sacrificed right after the 1-h heroin-seeking test (Fig. 6, Fig. S5). Two-way ANOVA analysis showed a significant main effect for stress in the IL (Fig. 6B,

Fig. 6 c-fos immunoreactivity after heroin-seeking test is enhanced in early social-isolated mice. (A–F) Number of c-Fos-positive cells in the following brain regions after heroin-seeking test: prelimbic cortex (PrL), infralimbic cortex (IL), nucleus accumbens core (NAcc) and shell (NAcSh), caudate putamen (CPU), and ventral tegmental area (VTA). Data are expressed as mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, $n = 5$ mice/group. GH, group house; ESI, early social isolation



$F_{1,16}(\text{stress}) = 5.144, P = 0.0375$) and NAcc (Fig. 6D, $F_{1,16}(\text{stress}) = 20.72, P = 0.0003$). A Tukey's post hoc analysis showed that c-Fos expression right after heroin-seeking test in NAcc (Fig. 6D) was increased in ESI female mice ($P < 0.01$), and showed a trend toward increase in ESI male mice ($P = 0.06$).

To assess the effect of heroin-predictive context and cue re-exposure on c-Fos expression in different brain regions, we analyzed the number of c-fos-positive cells regardless of the sex conditions (Table 1), as there was no sex difference in heroin-seeking behavior (Fig. 4C). Using two-way ANOVA analysis, we found a significant main effect for context and cue re-exposure in the PrL ($F_{1,32}(\text{context and cue}) = 70.11, P < 0.0001$), IL ($F_{1,32}(\text{context and cue}) = 22.95, P < 0.0001$), NAcc ($F_{1,32}(\text{context and cue}) = 73.63, P < 0.0001$), and NAcSh ($F_{1,32}(\text{context and cue}) = 76.63, P < 0.0001$). Additionally, there was a significant main effect for stress in the PrL ($F_{1,32}(\text{stress}) = 6.87, P = 0.013$), IL ($F_{1,32}(\text{stress}) = 7.39, P = 0.011$), NAcc ($F_{1,32}(\text{stress}) = 6.2, P = 0.019$), and VTA ($F_{1,32}(\text{stress}) = 4.46, P = 0.043$), which was consistent with previous results (Figs. 5 and 6). A Tukey's post hoc analysis indicated that re-exposure to heroin-associated context and cue resulted in a significant increase of c-Fos expression in the PrL ($P < 0.05$) and NAcc ($P < 0.01$) from both GH and ESI mice, and enhanced c-fos immunoactivity in the IL ($P < 0.001$) and NAcSh ($P < 0.001$) from ESI mice.

ESI stress decouples the correlation between PrL c-Fos expression and heroin-seeking behavior

To examine the degree of association between the neuronal activity (i.e., c-Fos immunoactivity) and heroin-seeking behavior, we calculated Pearson's correlation coefficient (r) between the number of c-fos-positive cells and the attempted infusions during heroin-seeking test. We found a significant positive correlation between the number of PrL

c-Fos-positive cells and the number of attempted infusions ($r = 0.64, P = 0.048$) from GH mice, whilst in ESI mice, this positive correlation was disrupted (Fig. 7A, $r = -0.34, P = 0.34$). Of note, we found overall significant positive correlations between the c-Fos numbers and attempted infusions during heroin-seeking test in the IL ($r = 0.47, P = 0.03$) and NAcc ($r = 0.83, P < 0.0001$) without considering stress and sex variables (Table 2). Taken together, these data suggest that ESI stress alters the neuronal responses (c-Fos as proxy) to heroin-associated context and cue in several key brain regions (including PrL, IL, NAcc, and VTA), and decouples the association between the neuronal activation and heroin-seeking behavior in the PrL.

Discussion

Early social isolation stress increases addiction vulnerability for heroin

Stress during early lifetime causes long-term behavioral maladaptations. Clinical studies have shown that early life adversities such as maltreatment, isolation, or low social support are associated with increased risk for the development of substance use disorders and relapse vulnerability (Barrett and Turner 2006; Chassin et al. 1988; Costa et al. 1999; Newcomb and Bentler 1988; Newcomb and Harlow 1986; Sher et al. 1997; Wills and Cleary 1996; Wills et al. 1992). Preclinical studies using an early social isolation (ESI) model to mimic early life stress have found that ESI stress enhances the initiation of self-administration for cocaine and opioids (Bozarth et al. 1989; Marks-Kaufman and Lewis 1984; Yajie et al. 2005). However, different findings show that social isolation does not affect cocaine or amphetamine self-administration (Boyle et al. 1991; Schenk et al. 1988), or even impairs cocaine self-administration

Table 1 Numbers of C-Fos positive cells comparison without and with heroin-associated cue exposure

	Without context and cue exposure (abstinence) (cells/1000 mm ²)				With context and cue exposure (after heroin-seeking test) (cells/1000 mm ²)			
	GH		ESI		GH		ESI	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
PrL ^{#^}	1307.871	59.661	773.725	37.196	1573.611*	80.861	1726.389*	84.351
IL ^{#^}	1188.078	59.359	750.289	28.554	1133.333	31.803	1293.75***	67.162
CPU	344.908	46.518	348.379	24.337	369.444	29.731	358.333	23.927
NAcc ^{#^}	565.393	41.384	517.94	16.908	715.972**	24.898	920.139**	35.88
NAcSh [#]	822.336	51.456	700.233	37.964	965.278	54.307	1013.194***	38.337
VTA [^]	519.964	45.242	376.736	42.378	458.333	33.958	431.944	39.417

[#]p<0.05 for main effect of context and cue exposure (i.e., abstinence vs after heroin-seeking test).

[^]p<0.05 for main effect of stress.

* p<0.05, ** p<0.01, *** p<0.001 *post hoc* analysis between without and with cue exposure (i.e., abstinence vs after heroin-seeking test) within GH (group house) or ESI (early social isolation) group.

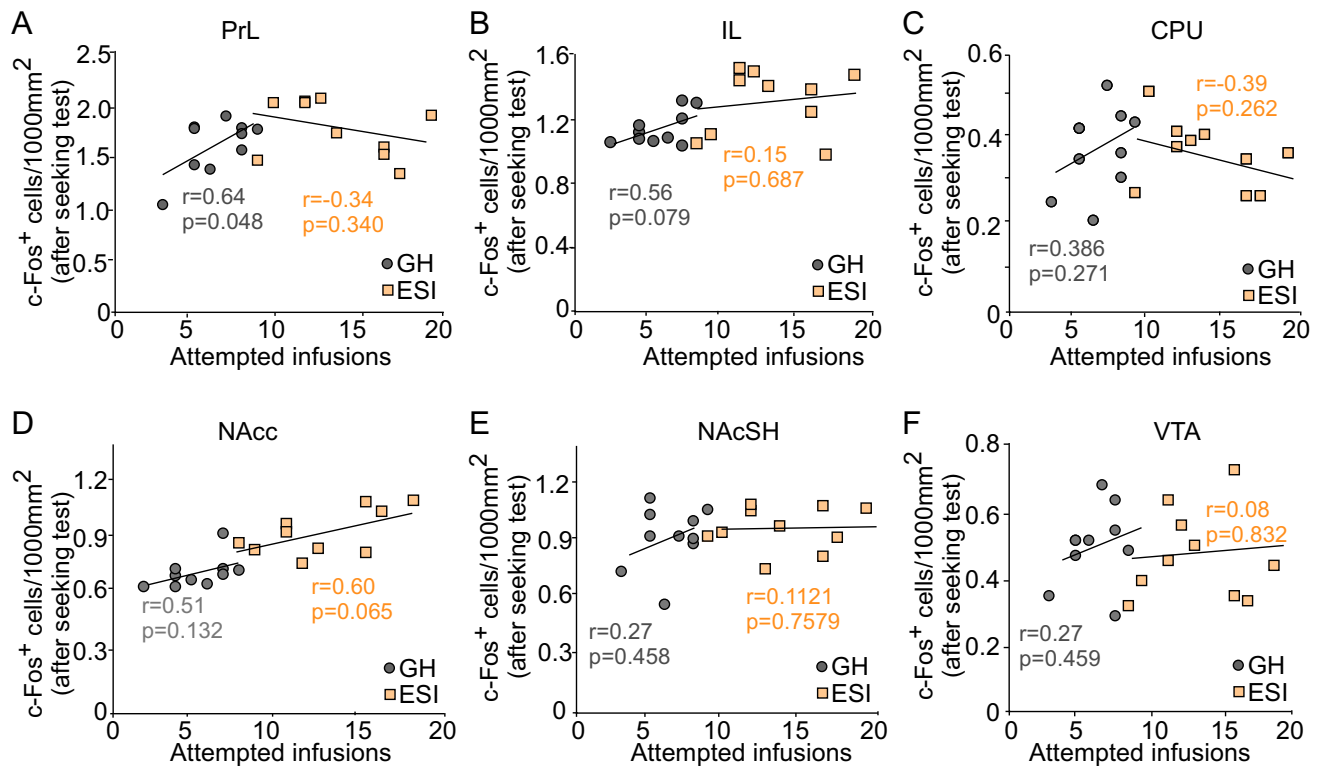


Fig. 7 The correlation between c-fos immunoreactivity and heroin-seeking behavior. (A–F) The correlation between the number of attempted infusions during heroin-seeking test and the number of c-Fos-positive cells (right after heroin-seeking test) in the following

brain regions: prelimbic cortex (PrL), infralimbic cortex (IL), nucleus accumbens core (NAcc) and shell (NAcSh), caudate putamen (CPU), and ventral tegmental area (VTA). GH, group house; ESI, early social isolation

Table 2 Correlation between numbers of c-Fos positive cells and heroin-seeking behavior

		PrL	IL	NAcc	NAcSh	CPU	VTA
Heroin-seeking test	r	0.228	0.5177	0.827	0.2263	-0.1016	-0.0213
(Attempted infusions)	p	0.3336	0.0194	<0.0001	0.3373	0.67	0.9289

(Howes et al. 2000; Phillips et al. 1994). These discrepancies reflect an ESI stress-induced shift of dose-response curve for drugs (Howes et al. 2000; Piazza et al. 2000). In the current study, we found that ESI stress did not change the acquisition of heroin self-administration at the dose of 0.05 mg/kg/infusion (Fig. 3). Additionally, ESI stress increased heroin infusions at the dose 0.025 mg/kg/infusion in females but not males, resulting in an upward shift of heroin dose-response curve (Fig. 4), and indicating an increased heroin vulnerability (Piazza et al. 2000) in ESI female mice. This result reflects the sex difference in the etiology of opioid use disorders (Anglin et al. 1987; Hser et al. 1987), and the sex-specific responses to social stress (Hodes et al. 2015; Pena et al. 2019; Walker et al. 2021). Surprisingly, we did not find sex difference in heroin infusions in control (GH) mice at any given doses; yet Towers et al. reported females showed increased heroin intake at 0.03 (but not 0.06) mg/kg/infusion as compared to males (Towers et al. 2019).

This inconsistency may be due to the different experimental protocols. First, Towers et al. used a between-session dose-response paradigm for two doses (0.03 and 0.06 mg/kg/infusion) without preceding heroin acquisition. Second, in our experimental protocol, the prior heroin exposure (0.05 mg/kg/infusion, for 9 days) may alter the behavioral responses during the within-session dose-response tests.

Although ESI stress did not change heroin intake in male mice, it significantly augmented the motivation for heroin and potentiated heroin seeking in both males and females (Fig. 4). This is in concert with previous reports showing that social isolation stress increases seeking or extinction resistance for several drugs in different behavioral procedures. For example, isolation stress increases cocaine seeking in a mouse self-administration model (Fosnocht et al. 2019) and a conditioned place preference (CPP) model (Ribeiro Do Couto et al. 2009); isolation stress also delays extinction in an amphetamine-induced CPP model (Whitaker

et al. 2013) and in a self-administered alcohol drinking model (Cortes-Patino et al. 2016). We also found that the ESI stress-augmented motivation is specific to heroin, as the motivation for other natural rewards such as sucrose was unaffected (Fig. 2), which is consistent with other reports (Fosnocht et al. 2019). Interestingly, studies that use different early life stressors have found that short-term (postnatal days 2–9) exposure to limited bedding and nesting promotes resilience to opioid addiction-related phenotypes in male rats (Ordonez Sanchez et al. 2021). These controversial results highlight that stress-induced behavioral adaptations are sensitive to different types of stressors and stress timing, and are dependent on species and sex.

It is noteworthy that in our experimental design, ESI female mice had higher total heroin intake during dose-response training comparing to other groups (Fig. 4). As drug exposure-induced neuronal adaptations are associated with relapse propensity (Shaham et al. 2003), the increased heroin seeking in ESI female mice may be not only due to isolation stress-induced maladaptation, but also due to prior history of increased heroin exposure. Future studies are needed to tease apart these two different effects.

Early social isolation stress alters the neuronal activity during heroin abstinence

Adolescence is a critical developmental stage characterized by heightened sensitivity to stress. During this time, the maturation of brain network occurs, such as the glutamatergic and dopaminergic systems in the prefrontal cortex and mesolimbic regions (Spear 2000; Walker et al. 2019). Therefore, chronic stress during this vulnerable period induces irreversible neural plasticity (Caruso et al. 2018; Hermes et al. 2011; Melendez et al. 2004; Yamamuro et al. 2018), which is associated with susceptibility for mental disorders including substance use disorders. For example, clinical imaging studies showed that children who were maltreated exhibited altered prefrontal cortex size/volume (Gratton and Sullivan 2005). Preclinical studies also show that ESI stress leads to prefrontal cortex dysfunction (by diminishing glutamatergic synaptic function (Hermes et al. 2011; Yamamuro et al. 2018)), as well as enhances VTA synaptic plasticity (by potentiating the NMDAR receptor-mediated glutamatergic transmission (Shepard and Nugent 2020; Whitaker et al. 2013)). Maladaptations in these brain regions contribute to the susceptibility for relapse (Luscher and Malenka 2011; Nestler and Luscher 2019).

In our study, we found that neuronal activity (measured as c-Fos immunoactivity) in the prefrontal cortex (both PrL and IL) during forced abstinence following heroin self-administration was decreased in ESI mice (Fig. 5). Human imaging studies have identified structural and functional deficits in the prefrontal cortex of subjects with opioid use

disorder (Goldstein and Volkow 2011). Preclinical studies also reported hypoactive pyramidal neurons in the PrL after long-term opioid abstinence (Anderson et al. 2020), and reduced neuronal activity marker zif268 expression in the IL after extinction training followed by heroin self-administration (Schmidt et al. 2005). Our results indicate that ESI stress lowers neuronal activity in the prefrontal cortex during abstinence, which may potentiate prefrontal cortex dysfunction and contribute to increased relapse susceptibility. Future studies using methods with temporal resolution (such as in vivo electrophysiology or calcium imaging) will be needed to determine whether ESI stress-induced prefrontal cortex hypofunction during heroin abstinence is correlated with ESI-potentiated heroin seeking.

It is noteworthy that ESI stress also significantly decreased c-Fos expression in VTA regardless of sex (Fig. 5). It has been reported that early life stress alters the transcriptional modifications in VTA and induces susceptibility to chronic stress (Pena et al. 2019). Additionally, chronic stress during early lifetime alters the morphology and synaptic transmission in VTA (Shepard and Nugent 2020; Whitaker et al. 2013). These transcriptional and synaptic modifications induced by early life stress may alter the neural activity (c-Fos expression) in VTA after heroin abstinence in ESI group (Fig. 5). Considering the role of VTA in opioid tolerance (Harvey et al. 2007; Russo et al. 2007) and heroin seeking (Bossert et al. 2004), these ESI stress-induced maladaptations in VTA may contribute to ESI-induced heroin vulnerability.

Interestingly, we found that numbers of c-Fos-positive cells are selectively decreased in the NAc core in isolated females but not in males (Fig. 5), which is in concert with studies showing that early life stress exerts sex-specific impact in NAc (Chang et al. 2019; Ordonez Sanchez et al. 2021) and that stress induces distinct sex-dependent transcriptome profiles in NAc (Hodes et al. 2015). For example, neonatal predator odor exposure upregulates the mu- and delta-opioid receptor mRNA levels in NAc from female rats (Chang et al. 2019); limited bedding and nesting induces distinct transcriptomic profiles in NAc from male and female rats (Ordonez Sanchez et al. 2021). Moreover, studies have shown that social isolation stress disrupts sex-specific transcriptional response to cocaine in brain reward circuit including NAc (Walker et al. 2021). These studies together with our data suggest that NAc may play important roles in mediating the early life stress-induced sex-specific neural adaptations.

It is important to mention that genes like c-fos not only serve as markers of neuronal activation but also act as transcription factors to regulate the expression of other genes. The reduced c-Fos expression in ESI mice in reward pathway (Fig. 5) may potentially alter the molecular adaptations induced by heroin abstinence. As abstinence-induced

neuroadaptations in reward pathway contribute to the incubation of heroin craving (Fanous et al. 2012; Pickens et al. 2011; Theberge et al. 2013; Theberge et al. 2012), future studies are needed to reveal the ESI-induced unique adaptations contributing to ESI-potentiated heroin seeking.

Early social isolation stress alters the neuronal responses to heroin-predictive context and cue, and decouples the correlation between neuronal activity and heroin seeking

Re-exposure to the environmental context and/or discrete cues that are associated with previous drug reward can trigger drug relapse in humans (Wikler 1973). Preclinical studies revealed that several key brain regions within the reward circuit are required for cue-induced heroin seeking, including the prefrontal cortex, NAc, and VTA (Bossert et al. 2016; Bossert et al. 2004; Bossert et al. 2011; Bossert et al. 2012; Rogers et al. 2008; Schmidt et al. 2005). For example, studies have found that neuronal activity (measured by c-Fos or zif268 immunoactivity) in the prefrontal cortex (Bossert et al. 2011; Bossert et al. 2012; Schmidt et al. 2005) and NAc (Schmidt et al. 2005) is upregulated after heroin-seeking test. Consistently, we also found that c-Fos immunoactivity in the PrL, IL, NAc core, and shell are all increased after context- and cue-induced heroin-seeking test (Table 1). We also found an overall positive correlation between c-Fos expression and heroin-seeking behavior in the IL and NAc core (Table 2). Our data together with previous publications suggest that neuronal activation in these brain areas is involved in heroin seeking.

Remarkably, the functional role of the PrL and IL in heroin seeking is inconsistent in literatures. Some studies suggest that inactivation of the PrL enhances cue-induced heroin reinstatement (Schmidt et al. 2005); other studies have found that inactivation of the PrL (Rogers et al. 2008) or IL (Bossert et al. 2011; Bossert et al. 2012; Rogers et al. 2008) attenuates cue-, heroin-, or context-induced heroin reinstatement. In our study, we found a positive correlation between PrL c-Fos activation and heroin seeking in control mice (Fig. 7). However, ESI disrupted this positive correlation, suggesting that ESI stress remodels the role of PrL in heroin-seeking behavior. Additionally, we found an increased IL c-Fos immunoactivity after heroin-seeking test in ESI mice (Fig. 6), which agrees with previous studies showing that inactivation of the IL attenuates reinstatement of heroin seeking after extinction training (Bossert et al. 2011; Bossert et al. 2012; Rogers et al. 2008). Future studies are needed to investigate the exact role of the PrL and IL in mediating ESI-potentiated heroin seeking.

Intriguingly, we found that ESI stress enhances c-Fos expression in the NAc core in male and female mice (Fig. 6). The NAc receives glutamatergic projections from

the prefrontal cortex (PrL to NAc core, IL to NAc shell) and dopaminergic projections from the VTA, and plays critical roles in mediating cue-elicited drug seeking (Fuchs et al. 2004; Ito et al. 2004). As ESI stress alters the neuronal activity during abstinence in the PrL and VTA (Fig. 5), the synaptic transmission in the NAc core may be affected. These ESI-induced “predispositions” may alter the neuronal reactivity to heroin-associated cues in the NAc core. Moreover, early life stress changes the NAc transcriptional response to cocaine (Walker et al. 2021) and heroin (Ordonez Sanchez et al. 2021). These potentially altered synaptic transmission and transcriptional profiles in the NAc core may lead to the ESI-augmented c-Fos reactivity to heroin-associated cues. Although we speculate that NAc core overactivation may contribute to ESI-potentiated heroin seeking, the causative factors for ESI-potentiated heroin seeking as well as the underlying molecular mechanisms need further exploration.

Of note, we did not find CPU neuronal activation (Table 1) after heroin-seeking test as others reported (Schmidt et al. 2005). This inconsistency may be due to the distinctive experimental design (e.g., with or without extinction training, with or without a prior history of sucrose exposure) and different neuronal activity markers that were used (c-Fos or zif268). Additionally, we did not find altered c-Fos expression in the VTA after heroin-seeking test (Table 1). Studies have shown that c-Fos activities in the VTA and CPU are increased after cocaine priming prior to cocaine-seeking test (Neisewander et al. 2000) and that the VTA is involved in contextual cue-induced reinstatement of heroin seeking (Bossert et al. 2004). Future studies may be needed to investigate whether ESI stress alters VTA and CPU neuronal reactivity profiles in heroin-primed seeking behaviors using both forced abstinence model and extinction-reinstatement model.

Conclusion

Taken together, our data indicate that early social isolation stress causes an upward shift of heroin dose-response curve in female mice, enhances motivation for heroin (but not sucrose) in both males and females, and potentiates heroin seeking in both sexes. Meanwhile, early social isolation stress alters the neuronal activity during abstinence as well as neuronal reactivity in response to heroin-predictive cues in key brain reward pathway including the prefrontal cortex (PrL and IL), NAc core, and VTA. Additionally, early social isolation stress decouples the association between heroin-seeking behavior and neuronal activation in PrL. These results provide neural mechanisms for early life stress-induced vulnerability for opioid addiction, as well as provide

a clearer understanding of the neurobiological substrates of opioid addiction.

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Author contribution A.S. performed experiments, analyzed data, and wrote part of the Methods; Y. X. performed experiments and analyzed data; A. D. performed experiments; and Z.-J. W. designed experiments, performed experiments, supervised the project, and wrote the manuscript.

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Declarations

Conflict of interest The authors declare no competing interests.

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