Enduring disruption of reward and stress circuit activities by early-life adversity in male rats

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INTRODUCTION

Early-life adversity (ELA) related to poverty, trauma, and a chaotic environment affects over 30% of children in the United States [1]. In humans, ELA is associated with the development of emotional disorders such as depression and substance abuse. These disorders involve underlying disruption of reward circuits and likely vary by sex. Accordingly, we previously found that ELA leads to anhedonia for natural rewards and cocaine in male rodents, whereas in females ELA instead increases vulnerability to addiction-like use of opioid drugs and palatable food. While these findings suggest that ELA-induced disruption of reward circuitry may differ between the sexes, the specific circuit nodes that are influenced by ELA in either sex remain poorly understood. Here, in adult male Sprague-Dawley rats, we ask how ELA impacts opioid addiction-relevant behaviors that we previously tested after ELA in females. We probe potential circuit mechanisms in males by assessing opioid-associated neuronal activation in stress and reward circuit nodes including nucleus accumbens (NAc), amygdala, medial prefrontal cortex (mPFC), and paraventricular thalamus. We find that ELA diminishes opioid-seeking behaviors in males, and alters heroin-induced activation of NAc, PFC, and amygdala, suggesting a potential circuit-based mechanism. These studies demonstrate that ELA leads to behavioral and neurobiological disruptions consistent with anhedonia in male rodents, unlike the increased opioid seeking we previously saw in females. Our findings, taken together with our prior work, suggest that men and women could face qualitatively different mental health consequences of ELA, which may be essential for individually tailoring future intervention strategies.
Hedonic Setpoint ($Q_0$)

Price

Consumption

High Setpoint

Low Setpoint

Demand Curve Equation: $\ln Q = \ln Q_0 + k(e^{-Q_0/C} - 1)$

Fig. 1  Demand characteristics in sample curves. Schematic representation of sample demand curves generated from hypothetical response patterns reflecting differences in hedonic setpoint ($Q_0$) and demand elasticity ($\alpha$). Drug intake was determined at each response requirement and consumption data were modeled with an exponential demand equation: $\ln Q = \ln Q_0 + k(e^{-Q_0/C} - 1)$ [44, 60], where $Q =$ consumption, $C =$ unit cost, $k =$ a scalar constant for consumption range, $\alpha =$ demand elasticity, and $Q_0 =$ extrapolated intake at zero effort. This process yields a demand curve fitted to consumption at each price, from which variables corresponding to the hedonic setpoint ($Q_0$) and motivation ($\alpha$) are derived.

Parturition was checked daily, and the day of birth was considered postnatal day (PD) 0. On PD2, pups and dams were assigned to ELA or control (CTL) groups (10–12 pups per dam, sex balanced) and housed under these conditions through PD9, as described below. Rats were weaned at PD21 and housed by sex in groups of 2–3, under a 12-h reverse light cycle. Food and water were available ad libitum throughout all experiments. ELA ($n = 17$) and CTL ($n = 13$) males from six total dams and in roughly equal number from each litter ($n = 4–6$/litter) were used for these experiments. Sample sizes were chosen based on observed effect sizes in our prior reports [22, 26]. No clear differences due to the number of cagemates were seen on behavioral outcomes, though these studies were not designed to test this variable. These ELA and behavioral testing procedures are the same as those from our prior report in females [26]. Randomization of test order of behavioral tasks was not used in the design of these studies, and experimenters were not blind to experimental group during behavioral testing. All procedures were approved by the University of California Irvine Institutional Animal Care and Use Committee and conducted in accordance with the National Institutes of Health guide for the care and use of laboratory animals.

The limited bedding and nesting (LBN) model of early-life adversity

On PD2, pups from at least two litters at a time were gathered and assigned at random to each dam in equal numbers of male and female (10–12 pups per dam) to prevent the potential confounding effects of genetic variables, sex ratio, or litter size. Dams and pups were transferred to LBN or CTL cages, as described previously [21, 22, 40]. LBN rats were transferred to cages fitted with a plastic-coated aluminum mesh platform sitting ∼2.5 cm above the cage floor. Bedding only sparsely covered the cage floor under the platform, and one-half of a 24.2 cm 23.5 cm paper towel was provided for nesting material. CTL dams and pups were placed in cages containing a standard amount of bedding (∼0.33 cubic feet of corn cob) without a platform, and one full paper towel. Cages remained undisturbed during PD2–9. Throughout this epoch, maternal behaviors, which may constitute a source of stress in infant rats, were monitored as previously described [24, 40, 41]. On PD10, animals were all transferred to CTL condition cages, causing maternal behaviors to normalize rapidly, and for any stress to dissipate in the pups [41].

Intravenous catheter surgery

At approximately PD70, rats were anesthetized with isoflurane (2–2.5%) and chronic indwelling catheters were inserted into the right jugular vein. Meloxicam (1 mg/kg, i.p.) for postsurgical analgesia, and prophylactic antibiotic cefazolin (0.2 mL, i.v.; 10 mg/0.1 mL) were given intraoperatively. After 5 days of recovery, catheters were flushed daily following each opioid self-administration session with cefazolin (10 mg/0.1 mL) and a heparin lock solution (10 U/0.1 mL) to maintain catheter patency.

Drugs

Heroin (diacetylmorphine) HCl was provided by the National Institute on Drug Abuse (NIDA) Drug Supply Program (Research Triangle Park, NC, USA) or Cayman Chemical Company (Ann Arbor, MI, USA), and remifentanil HCl was provided by the NIDA Drug Supply Program. All drugs were dissolved in sterile 0.9% saline for experimental use.

Behavioral/functional tests

Heroin self-administration, extinction, reinstatement, and remifentanil demand tests were all performed in the same rats ($n = 7$ ELA; 8 CTL). Two rats were excluded from remifentanil demand analyses: one (CTL) as a statistical outlier for the demand elasticity test ($\alpha$) (Grubbs outlier test Alpha = 0.05) and one (ELA) due to catheter failure leaving six ELA and seven CTL rats in remifentanil demand analyses.

Heroin self-administration. Self-administration training and testing took place in Med Associates operant chambers in sound-attenuating boxes, as described previously [26]. Rats received daily 2-h self-administration sessions, when pressing on the “active” lever (AL) yielded a heroin infusion of 0.1 mg/kg (acquisition; days 1–3) or 0.05 mg/kg (training; days 4–17). Heroin infusions were accompanied by concurrent 2.9-kHz tone and lever light illumination for 3.6 s. A 20 s timeout period (signaled by turning off the house light) followed each infusion/cue presentation, during which additional lever presses were recorded but had no consequence. Pressing on the second “inactive” lever (IL) was recorded but had no consequence.

Heroin extinction and reinstatement. Following heroin self-administration, rats received extinction training for a minimum of 7 days, or until extinction criterion (2 consecutive days <20 AL presses) was met. Lever presses were recorded but had no consequence. Upon meeting the extinction criterion, rats underwent a 2-h cue-induced reinstatement test, during which one presentation of the drug-paired cues was delivered 10 s after the start of the session, then AL presses yielded additional cue presentations. Rats then underwent a minimum of 2 extinction training days until the extinction criterion was re-attained, after which they underwent drug/vehicle-primed reinstatement tests. Rats received an experimenter-administered injection of heroin (0.25 mg/kg, s.c [26, 42]) or saline immediately before the start of the 2-h session. Lever presses were recorded but did not yield additional heroin or cues. All animals received both heroin and saline on separate days in counterbalanced order. Again, 2+ additional extinction training days occurred between primed reinstatement tests to re-establish the extinction criterion. Following reinstatement testing, catheter patency was confirmed using intravenous methoxetinal (0.1–0.2 mL, 5 mg/mL). Rats with catheter failure ($n = 1$) were re-catheterized in the left jugular vein and allowed to recover for five days before starting the behavioral economic procedures.

Behavioral economic thresholding procedure. Rats were trained on a previously described within-session economic thresholding procedure.
This behavioral tool, variations of which can be implemented in both rodents and humans [44, 47–50], allows for simultaneously measuring both consummatory and motivational aspects of reward by systematically increasing the cost of a particular commodity or substance, such as a drug or food reinforcer. By mathematically generating a demand curve based on lever press responses at increasing costs (and subtracting percentages), tissue was removed and postfixed overnight, then cryoprotected in a 20% sucrose-azide solution. Frozen 40-µm coronal sections were collected on a cryostat and stored in phosphate-buffered saline (PBS) with sodium azide at 4°C for subsequent immunohistochemistry analyses.

**Immunohistochemistry.** An avidin-biotin complex (ABC)-amplified, diaminobenzidine (DAB) reaction was conducted to visualize heroin-induced c-Fos and CTb expression. Endogenous peroxidase was blocked with 0.3% H2O2, then 3% normal donkey serum (Vector Laboratories, Burlingame, CA) in PBS containing 0.3% Triton-X. Sections were incubated for 16 h at room temperature polyclonal rabbit anti-c-Fos, washed, then incubated for 2 h in biotinylated donkey anti-rabbit IgG (1:500, Jackson Laboratories, West Grove, PA). After ABC amplification (90 min), Fos was visualized in blue/black with DAB in Tris buffer with 0.01% H2O2 and Nickel Ammonium Chloride (0.04%; Vector Laboratories). Sections from brains with appropriate CTb placement were further processed to visualize somatic CTb in brown/purple and DAB in black. Tissue was incubated for room temperature in goat anti-CTb (1:10,000, Millipore) for 16 h, followed by biotinylated donkey anti-goat (1:500; Jackson Laboratories), ABC amplification, and DAB reaction. Sections were mounted and coverslipped in Permamount medium.

**CTb injection sites were visualized using fluororescent immunohistochemistry (ABC amplification).** Sections were stained for C-fos antibody (Millipore polyclonal rabbit anti-c-Fos, #ABE457; 1:5,000), however due to stock shortages, later rounds of ABC were conducted using Abcam polyclonal rabbit anti-c-Fos (AB2393718-1, 1:10,000). The two antibody combinations were compared head-to-head in neighboring NAc sections from the same brains (n = 8 brains; two sections per brain per antibody), and both qualitative appearance and the cell counts did not differ significantly between the two antibodies (Fig. S1; total NAc cell density Millipore vs. Abcam 0.3442, P = 0.7331; Pearson correlation between antibody tested on tissue from the same rats: r = 0.6637, P = 0.0051), so data from both antibodies were combined.

Of the opioid-experienced rats, one CTL brain was excluded due to incomplete exsanguination during perfusion for a total of seven ELA and seven CTL rats for Fos analysis. An additional two CTL and one ELA rat were excluded from CTb analyses due to CTb infusion localized outside NAc. Of the opioid-naïve rats, one CTL and three ELA rats were excluded from CTb analyses due to misplaced infusion outside NAc. One additional CTL rat was excluded from only PFC CTb analyses due to tissue damage during processing.

**Imaging and Fos analysis.** Images of structures quantified for Fos/CTb were taken at 10X magnification on a Leica DM400B microscope with stage automation, and stitched using Stereo Investigator (SI) software (MicroBrightfield). Three to four coronal sections per structure from comparable regions in each animal were quantified bilaterally by a trained, blinded observer. Brain region borders were delineated based on a brain atlas (Paxinos and Watson, 2007). The coordinate range sampled from each structure is as follows (mm relative to bregma): PFC: +3.24 to +3.00; NAc: +2.28 to +1.44; CeA: −2.28 to −2.76; BLA: +2.28 to −2.92; PVT: −2.16 to −2.82. Most injections spread to both shell and core of NAc. Brains with misplaced CTb injections or leakage beyond NAc borders were not used for CTb quantification, and were only used for quantifying heroin-induced c-Fos expression. Initial rounds of Fos IHC were conducted using Millipore polyclonal rabbit anti-c-Fos (AAB457; 1:5,000), however due to stock shortages, later rounds of IHC were conducted using Abcam polyclonal rabbit anti-c-Fos (AbR2393718-1, 1:10,000). The two antibodies were compared head-to-head in neighboring NAc sections from the same brains (n = 8 brains; two sections per brain per antibody), and both qualitative appearance and the cell counts did not differ significantly between the two antibodies (Fig. S1; total NAc cell density Millipore vs. Abcam 0.3442, P = 0.7331; Pearson correlation between antibody tested on tissue from the same rats: r = 0.6637, P = 0.0051), so data from both antibodies were combined.

To pinpoint if ELA-induced changes in heroin-induced neuronal activation depended upon a prior history of opioid exposure, a separate group of handled, age-matched, but otherwise experimentally naive rats (n = 10 ELA, 5 CTL) underwent the same CTb surgeries, a single acute heroin challenge, and sacrifice.

**c-Fos & CTb studies**

**Tissue preparation.** Rats were transcardially perfused with chilled 0.9% saline followed by 4% paraformaldehyde under deep anesthesia. Brains were removed and postfixed overnight, then cryoprotected in a 20% sucrose-azide solution. Frozen 40-µm coronal sections were collected on a cryostat and stored in phosphate-buffered saline (PBS) with sodium azide at 4°C for subsequent immunohistochemistry analyses.

**Analysis approach.** Statistical analyses were performed using GraphPad Prism software. Independent samples t-tests were used to determine the effects of ELA on heroin consumption, extinction persistence (days until extinction criterion), and demand characteristics for remifentanil (Q0). Extinction persistence was further analyzed for “survival” of seeking behavior as a percentage of rats in each group failing to meet extinction criterion on each training day), and Kaplan–Meier plots were compared using the log-rank test [57]. For analyses of ELA effects on within-subjects variables (e.g., saline/heroin-primed reinstatement), two-way repeated-measures ANOVA were used. Between-subjects two-way ANOVAs were used to test interactions of ELA with opioid experience on Fos.
Fig. 2  ELA reduces heroin self-administration but not does not alter extinction or relapse.  A Average daily heroin consumption. B There was a trend towards reduced consumption of heroin during the high-dose acquisition phase among ELA-reared animals. ELA led to reduced consumption during the training phase (C) and overall consumption (D). Despite reduced intake, ELA did not alter extinction (E, F) or reinstatement to heroin seeking (G, H). * ELA vs CTL *P < 0.05.  n = 8 CTL/7 ELA.

Intra-session heroin self-administration timecourse. To assess whether the above findings may be a result of differences in drug sensitivity, malaise/sedation, development of pharmacological tolerance or sensitization, or other potential non-hedonic factors, we analyzed the pattern of heroin self-administration behavior within the 2 h training sessions, following establishment of stable responding (training days 3–14). Rats showed characteristic “drug loading” in the first 5 min of each session, followed by slower “maintenance” responding the remainder of the session (Fig. 3C). Average drug consumption during the loading phase was significantly lower in ELA than control rats (Fig. 3A; *t*13 = 2.321; *P* = 0.0372, *η2* = 0.2931), an effect that was constant across stable training days (main effect of training day: *F*11,143 = 5.389, *P* = 0.0372, *η2* = 0.2931; main effect of day: *F*11,143 = 0.97, *P* = 0.4765; interaction: *F*11,143 = 0.971, *P* = 0.8288). There was no effect of ELA on the average inter-infusion-interval (I-I) during the remainder of the session (Fig. 3B; main effect of training condition across stable training days: *F*11,143 = 1.499, *P* = 0.2426). In addition, there was a significant ELA x day interaction, whereby the I-I tended to decrease as training progressed in ELA rats but increased over training in controls (days 9–14; main effect of training day: *F*11,143 = 0.9357; *P* = 0.5082; rearing x day interaction: *F*11,143 = 2.241, *P* = 0.0086, *η2* = 0.1470). Finally, there was no effect of ELA on inactive lever presses (main effect of rearing condition: *F*11,143 < 0.01; *P* = 0.99; main effect of training day: *F*11,143 = 0.4227, *P* = 0.9756; interaction: *F*11,143 = 1.268, *P* = 0.2204), suggesting no overt ELA-induced locomotor deficits, as expected [22, 24, 58, 59].

Demand for remifentanil. Analysis of stable responses for remifentanil revealed that ELA males had a reduced “hedonic setpoint” for the drug (Q0). Q0, a value extrapolated from the animal’s calculated demand curve, is a parameter that reflects consumption at low cost or when the drug is essentially “free” [44, 50] (Fig. 4A; *t*13 = 2.213, *P* = 0.0490, *η2* = 0.3080), in accord with our published finding for cocaine in male ELA rats [22]. In contrast to the hedonic setpoint, ELA did not alter demand elasticity (α) for remifentanil (Fig. 4B; *t*13 = 0.9560, *P* = 0.3596), indicating that ELA and control males were similarly sensitive to
increasing “price” of the drug, as an increasing effort was required to maintain preferred blood levels. Notably, we previously found this indicator of motivation for remifentanil was robustly enhanced by ELA in female rats [26].

Whereas these experiments were not designed to detect sex differences, we performed a two-way ANOVA directly comparing our previously published data in females [26] with the current data in males. For hedonic setpoint (Q₀), there was a main effect of sex (F₁,₂₇ = 24.43, P < 0.001, η₀² = 0.3851) and of rearing condition (F₁,₂₇ = 7.051, P = 0.0114, η₀² = 0.1531), and a trend toward interaction of these variables (F₁,₂₇ = 3.420; P = 0.0720; η₀² = 0.0806). Notably, ELA reduced the hedonic setpoint only in males (ELA vs CTL: males P = 0.0207; females P = 0.9341). For demand elasticity (α), there was a main effect of rearing condition across the sexes (F₁,₂₇ = 4.328, P = 0.0441, η₀² = 0.0999), though this effect survived post hoc multiple comparison correction only in males (ELA vs CTL: females P = 0.0105; males P > 0.999). There was no significant effect of sex on α (F₁,₂₇ = 2.064; P = 0.1588) and no sex x rearing condition interaction (F₁,₂₇ = 1.356; P = 0.2513). These comparisons are thus consistent with, though do not prove, the testable hypothesis that ELA differentially affects opioid reward in males and females.

**Heroin-induced neuronal activation**

**Effects of ELA.** ELA led to several changes in the pattern of heroin-induced Fos expression, when analyzed regardless of prior opioid experience. Specifically, ELA blunted heroin-induced c-Fos expression in NAc core (Fig. 5C; t₂₇ = 3.108, P = 0.0044, η² = 0.2634) but not shell (Fig. 5C; t₂₇ = 0.8425, P = 0.4069). In contrast, ELA increased heroin-induced Fos expression in CeA (Fig. 5A; t₂₇ = 3.188, P = 0.0036, η² = 0.2734) and PFC (Fig. 5C; t₂₇ = 2.675, P = 0.0125, η² = 0.2095). The selectivity of these effects of ELA are apparent from the lack of changes in BLA (Fig. 5C; t₂₇ = 0.4239, P = 0.6750) or PVT (Fig. 5C; t₂₇ = 0.4901, P = 0.6280).

**Influence of prior opioid exposure.** Next, we examined the impact of prior chronic opioid self-administration history on heroin-induced Fos in ELA or control rats. In opioid-experienced rats, heroin-induced NAc core Fos expression was higher than in previously opioid-naïve rats (Fig. 5B; main effect of opioid experience: F₁,₂₅ = 11.28, P = 0.0025, η₀² = 0.3109). There was no experience x rearing condition interaction (F₁,₂₅ = 0.1024, P = 0.7516). The main effect of rearing condition described above persisted in this analysis: F₁,₂₅ = 8.868, P = 0.0064, η₀² = 0.2618. In NAc shell, ELA rats trended toward having lower Fos when

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**Fig. 3** ELA specifically blunts loading phase heroin consumption. **A** ELA rats consumed fewer infusions during the first 5 min “loading period” of the 2 h session across stable training days (days 3–14). **B** Average inter-infusion-interval was not different between ELA and CTL, but there was a significant ELA x training day interaction on stable training days (days 3–14). **C** Average consumption across the 2 h session. * ELA vs CTL P < 0.05. n = 8 CTL/7 ELA.

**Fig. 4** ELA reduces hedonic setpoint for opioids, but demand elasticity is preserved. **A** Hedonic setpoint or consumption at low effort is reduced by ELA. **B** Demand elasticity or motivation to obtain drug at high cost, is not changed by ELA. **C** Average consumption at each price. * ELA vs. CTL P < 0.05. n = 7 CTL/6 ELA.
Effect of ELA on heroin-induced c-Fos expression in select nodes of reward and limbic circuit, so we aimed to increase the resolution of our stress circuits. Yet these nodes functionally communicate as a network, and we needed to increase the resolution of our assessments by determining the effects of ELA specifically on circuit projections from these regions to NAC. Specifically, we found that ELA had no direct effect on heroin-induced activity in NAC-projecting BLA, PVT, or PFC neurons (Fig. 6B: BLA: $t_{12} = 1.136, P = 0.2695$; PVT: $t_{12} = 0.7545, P = 0.4598$; PFC: $t_{12} = 0.09062, P = 0.9287$). However, activation of some of these populations tended to be influenced by prior opioid exposure. For example, in NAC-projecting BLA cells, there was a main effect of opioid experience (Fig. 6C: $F_{1,18} = 4.378, P = 0.05, \eta^2 = 0.1956$), and a near-significant interaction with ELA ($F_{1,18} = 3.961, P = 0.0620; \eta^2 = 0.1803$) such that there was a lower percentage of heroin-activated NAC-projecting BLA cells after ELA in previously opioid-naive rats, which tended to be reversed by chronic opioid exposure. There was no main effect of rearing condition on this cell population ($F_{1,18} = 1.393, P = 0.2533$). In PVT there was a trend towards reduced activity in NAC-projecting cells following opioid experience (Fig. 6C: main effect of opioid experience: $F_{1,18} = 3.491, P = 0.0781, \eta^2 = 0.1624$; main effect of rearing condition: $F_{1,18} = 0.4052, P = 0.5324$; interaction: $F_{1,18} = 0.5323, P = 0.4750$). In NAC-projecting PFC neurons, Fos was not influenced by prior opioid experience or ELA (Fig. 6C: main effect of rearing condition: $F_{1,17} < 0.01, P = 0.9559$; main effect of opioid experience: $F_{1,17} = 0.4589, P = 0.5073$; interaction: $F_{1,17} = 0.1241, P = 0.7290$). These results remained when infralimbic and prelimbic cortices were analyzed separately ($F < 1, P > 0.2$ for all main effects and interactions).

**DISCUSSION**

Here, we show that ELA in males reduces opioid consumption behaviors, and alters heroin-induced neural activity in specific brain stress- and reward-related structures. Our principal findings are that: (1) ELA diminishes behavioral reward responses to opioids in males, apparent from blunted heroin and remifentanil taking, (2) the mechanism of the ELA-induced reduction of opioid self-administration involves altered activation of reward and stress circuit nodes including NAC, PFC, and specific amygdala nuclei, and (3) chronic exposure to opioids alters the impact of ELA on neuronal activation after acute heroin, raising intriguing speculation that this exposure might mitigate aspects of the anhedonia-like phenotype provoked by ELA in male rats.

**ELA blunts opioid self-administration and hedonic value**

We found that in males, ELA produced behavioral changes consistent with anhedonia for opioids, in accordance with our prior findings of ELA-induced anhedonia for natural and cocaine rewards [21, 22] and with the recent work of Ordóñez Sanchez et al. [29], who also found that ELA imposed by LBN reduced opioid (morphine) intake in male rats. Specifically, we found that ELA males self-administered less heroin than controls, and had a lower hedonic setpoint in a within-session remifentanil economic

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**Fig. 5** ELA alters heroin-induced neuronal activation in a manner independent of prior opioid experience. ELA leads to blunted activation of NAC core but not the shell and leads to aberrant activation in CeA and PFC. ELA did not alter heroin-induced activity in BLA or PVT. A Representative images of c-Fos expression in NAC and CeA. B Heroin-induced c-Fos expression (cell density) in all animals separated by opioid history. C Heroin-induced c-Fos expression in all animals collapsed across opioid exposure conditions. *ELA vs. CTL P < 0.05; **ELA vs. CTL P < 0.005; # opioid-naive vs. opioid-experienced P < 0.05. experienced n = 7 CONTROL/ELA; naïve n = 5 CONTROL/10 ELA.
ELA does not substantially alter the neuronal response to heroin in NAc-projecting cells. A Diagram of NAc CTb injection placements and example of cell appearance. Black arrow = Fos+; clear arrow = CTb+; white arrow = Fos+CTb+ cell. B Percentage of total CTb-labeled cells that are also Fos+ in all animals collapsed across opioid exposure conditions. There was no effect of ELA on the activation of NAc-projecting cells in BLA, PVT, or PFC. C Percentage of total CTb-labeled cells that are also Fos+ in all animals separated by opioid history. # opioid-naive vs. opioid-experienced P = 0.05. experienced n = 5 CTL/6 ELA; naïve n = 4 CTL (3 CTL for PFC/7 ELA).
not designed to directly demonstrate sex differences, our observations in each individual sex, together with the trending interactions between the effects of sex and ELA on economic demand characteristics (α and Q_0) even in our underpowered comparison, support the notion that ELA might lead to different addiction-relevant outcomes in males and females, in accord with epidemiological observations in humans [7, 10, 14, 70–72]. The basis for these differences likely involves both intrinsic functional differences in the organization and function of the reward circuit across sexes [73], as well as the potential that ELA affects brain development differentially in males and females [74, 75]. In both cases, future studies aimed at understanding how ELA alters distinct types of reward-related behaviors across sexes would provide clues into the developmental effects of ELA on reward circuitry and subsequent risk for psychiatric illness, and how these may differ by sex.

**ELA alters the balance of stress and rewards circuit activation by heroin**

The nucleus accumbens is a central node of the circuit that governs reward-seeking behaviors and can be segmented into anatomically and functionally distinct regions including the shell and core [76–79]. We found that ELA leads to blunted activation of the nucleus accumbens core, but not shell in response to acute heroin, suggesting that ELA-experienced males may be less sensitive to the rewarding or reinforcing effects of opioids. This finding is again consistent with Ordoñes Sanchez et al. [29], who also observed reduced excitatory activity in NAc core of LBN-reared males. Although the NAc shell (rather than core) has been classically associated with opioid-dependent hedonic processing [80, 81], others have observed reduced c-Fos expression in the NAC core of anhedonic rats after reward consumption [82]. Therefore, inadequate activation of the NAc core in response to pleasurable stimuli like heroin may be a feature of anhedonia induced by ELA [83]. Notably, c-Fos expression in NAc following social play and cocaine is not altered by ELA [21, 22], suggesting that ELA alters brain responses to different rewards in a distinct manner [15].

In addition to blunted NAC activation, we observed aberrant activation of CeA and PFC in ELA males compared to controls. Interestingly, whereas activation of other brain structures is altered by ELA in a reward-specific manner, CeA is the only structure we observed with elevated activation in response to opioids as well as social play and cocaine [21, 22], three distinct types of rewards. This suggests that dysfunction of CeA, an important node involved in encoding and processing stress [84] as well as reward [85, 86], may represent an important mechanism by which ELA causes global reward-related deficits in males. Among the studied amygdala nuclei, these findings were specific to CeA; they were not identified in BLA. Rather, in BLA, ELA and chronic opioid experience tended to interact to affect later response to heroin, as discussed below. In PFC, ELA enhanced heroin-induced Fos overall, without affecting activity in NAC-projecting cells. Although projections from PFC into NAC mediate reward-seeking behaviors, including for opioids [87–89], this finding suggests that the PFC→NAc pathway is not overtly altered by ELA. ELA may thus instead alter other PFC neurons such as those targeting other brain regions—a possibility that should be investigated in future studies.

We did not observe any effects of ELA on heroin-associated PVT activation, nor on activation of its NAC-projecting cells, suggesting that ELA does not alter PVT control over opioid reward, despite its role in a variety of reward-related behaviors [39], including drug-seeking [35].

We note that these changes in heroin-induced c-Fos expression were observed following exposure to a novel environment, which likely has its own effects on neuronal activation, particularly in stress-related regions. While we cannot dissociate the effects of the novel environment from the effects of heroin in this study, we have previously observed no difference in anxiety-like behaviors in male rats following ELA using elevated plus maze and open field tests [22, 24, 58, 59]. Accordingly, the effects of ELA observed here are likely due to differences in response to heroin rather than an anxiety response to a novel environment per se.

**Chronic opioid exposure might counteract some ELA-induced reward deficits**

An intriguing trend in our data, though requiring replication in a larger sample, suggests that chronic opioid experience may counteract some of the effects of ELA on reward circuit responses to acute heroin, particularly in BLA. For example, ELA tended to alter overall BLA activation only in opioid-naïve rats. Likewise, ELA reduced activation of NAc-projecting BLA neurons in naive rats, but this deficit was recovered following chronic opioid experience. This is consistent with this pathway’s reported reward-promoting function, and the fact that reduced activity in it results in anhedonia [90–93]. Such findings generally support the idea that anhedonia, such as that produced by ELA, may be “self-medicated” with opioid drugs [94, 95], and further work should be conducted to test this hypothesis.

Notably, the effects of ELA on heroin-induced BLA activity also align with evidence from humans and animals showing that ELA alters amygdala function and connectivity, particularly via projections to PFC [21, 96–101]. Considered alongside our current findings, the BLA-PFC pathway would be an intriguing target for future studies of ELA effects on an opioid reward.

**Experimental limitations and caveats**

These studies delineate an ELA-induced behavioral phenotype consistent with anhedonia in male rats, as indicated by reduced heroin self-administration, and reduced remifentanil “hedonic setpoint.” These findings align with our prior work on the effects of ELA on intake and pursuit of other rewards [21, 22], whereas others showed that ELA via LBN may instead augment reward-seeking in male mice, including excessive alcohol intake [102]. Additionally, ELA imposed via other methods such as maternal separation tends to result in enhanced drug-seeking behaviors in males, as reviewed elsewhere [15, 103], thus highlighting the critical impact of adversity type and timing when it comes to influencing reward-related or emotional-like outcomes [104, 105]. Whereas we identified ELA-induced changes in neuronal activation in several reward and stress circuit nodes, we did not observe robust effects of ELA specifically on the predominantly glutamatergic inputs to NAC we studied. This may stem in part from the low number of subjects in some experiments. It is also possible that ELA preferentially affects non-NAc-projecting neurons in these regions (such as the BLA→PFC pathway known to be altered by ELA), or other glutamatergic or non-glutamatergic NAc inputs from stress or reward regions not included in the present study, such as dopaminergic inputs from ventral tegmental area. Additionally, only one subcutaneous dose of heroin was tested for these experiments; assessing effects of multiple doses administered either subcutaneously or intravenously (to more closely mimic the animal’s self-administered dosing) might reveal additional effects and would be instructive components of future studies.

It is also plausible that ELA differentially affects distinct cell types within a region. For example, our prior work showed that ELA alters CeA neuronal responses to rewards specifically in corticotropin-releasing hormone (CRH)-expressing neurons [21], and influenced gene expression selectively even in certain subpopulations of CRH-expressing neurons in the hypothalamus [106]. Alternatively, ELA may alter the abundance or survival of specific cell types [23, 58, 107]. These findings point to several important next steps for understanding the mechanisms by which disrupted reward-seeking occurs after ELA.

**CONCLUSIONS**

Here we demonstrate that ELA alters the balance of stress and reward-related neuronal responses to heroin, and leads to...
behavioral disruptions consistent with anhedonia. Intriguingly, chronic exposure to heroin tends to mitigate some of the ELA-induced dampening of opioid-seeking behaviors and neuronal reward responses, leading to speculation that consumption of pro-hedonic opioids may counteract processes underlying the anhedonia-like phenotype provoked by ELA. Taken together with our previous work, our findings demonstrate that the effects of ELA on reward-seeking behaviors and neuronal responses are both sex- and reward-type specific. Further studies on how ELA alters the developmental trajectories of reward and stress circuits in both males and females will be critical for more completely understanding the relative risks for the myriad mental health consequences of ELA among men and women, how they may differ, and developing more effective and tailored interventions.

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AUTHOR CONTRIBUTIONS
SCL, TZB, and SVM conceived of the project and designed the analysis. SCL, MTB, JLB, CRP, and JSM collected the data. SCL performed the analysis. SCL, TZB, and SVM wrote the manuscript.

COMPETING INTERESTS
The authors declare no competing interests.

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