Adolescent social stress decrease of adult morphine-induced behavioral sensitization is dependent upon genetic background

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Running title: Social stress influences morphine sensitization

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Abstract

Deaths related to opioid use have skyrocketed in the United States, leading to a public health epidemic. Research has shown that both biological (genes) and environmental (stress) precursors are linked to opioid use. In particular, stress during adolescence – a critical period of frontal lobe development –influences the likelihood of abusing drugs. However, little is known about the biological mechanisms through which adolescent stress leads to long-term risk of opioid use, or whether genetic background moderates this response. Male and female C57BL/6J and BALB/cJ mice were exposed to chronic variable social stress (CVSS) or control conditions throughout adolescence (postnatal days, PND, 25-59) and then tested for morphine locomotor sensitization or morphine consumption in adulthood. To examine possible mechanisms that underlie stress-induced changes in morphine behaviors, we assessed physiological changes in response to acute stress exposure and miRNA gene expression in the prefrontal cortex. Male C57BL/6J mice exposed to adolescent CVSS had blunted morphine sensitization compared to control animals. No differences were observed in the acute locomotor response to morphine administration or morphine consumption in adult C57BL/6J mice. Stress did not influence morphine behaviors in BALB/cJ animals. C57BL/6J mice exposed to CVSS had a blunted corticosterone recovery following an acute stressor. Twelve miRNA were downregulated in the prefrontal cortex of C57BL/6J mice exposed to CVSS in adolescence, which suggests a biological mechanism through which stress may alter later morphine responses. The specificity of the effects for C57BL/6J versus BALB/cJ mice provides evidence of a gene by environmental interaction influencing opioid behaviors.

Introduction

The use of opioids is a widespread public health crisis. Between 1999 and 2018 opioid overdoses accounted for 440,000 deaths in the United States (Wilson *et al.* 2020). This number has been on the rise with over 46,000 Americans losing their lives to an opioid overdose in 2018 alone. These deaths arise from the 10.3 million individuals who report misusing opioids each year (Substance Abuse and Mental Health Services Administration 2019). Given the prevalence of opioid abuse in the United States, research into the mechanisms that increase use of opioids is important for combating this public health crisis.

Opioid abuse is influenced by multiple factors. Human genetics studies have demonstrated that genes influence opioid use and dependence with heritability estimates ranging from 0.52 – 0.76 (van den Bree *et al.* 1998; Kendler *et al.* 1999, 2000; Sun *et al.* 2012). In this work, the use of different types of opioids (e.g. morphine, oxycodone, fentanyl) is not differentiated. However, research with inbred panels of mice have confirmed a genetic influence for individual opioids, including morphine, with heritability estimates of morphine behavioral responses ranging from 0.26 - 0.44 (Bergeson *et al.* 2001; Kest *et al.* 2002; Liang *et al.* 2006). Although these findings support genetic influences on opioid use and responses, environmental factors also impact opioid use.

One environmental influence linked to opioid use is exposure to stress. Research in mouse models has demonstrated that exposure to social stressors influence opioid behaviors. Male C57BL/6J mice exposed to chronic social stress in adulthood increased morphine preference immediately after the stressor, but the effect was not long lasting (Cooper *et al.* 2017). Also, exposure to social defeat in adult male OF1 mice reinstated morphine conditioned place preference following extinction (Ribeiro Do Couto *et al.* 2006). Clearly, adult social stress can influence morphine behaviors; however, the influence of social stress during adolescence has not been examined. What has not yet been fully characterized during this developmental period are the mechanistic links that can lead to opioid use.

Adolescence is a critical time of brain and social development (Spear 2013) and stress during this time generally leads to increased substance use in both humans and in animal models (Dube *et al.* 2003; Andersen & Teicher 2009). The prefrontal cortex (PFC), which is responsible for executive functions, such as decision making, impulse control, and working memory has important connections with limbic structures involved in drug use. This brain region undergoes numerous structural and functional changes during adolescence. These dynamic changes open a window for environmental factors, particularly stress, to modulate processes associated with drug use (Jankord *et al.* 2011). One long-lasting molecular change found as a result of adolescent stress is miRNA expression patterns (Rao & Pak 2016; Liu *et al.* 2017; Xu *et al.* 2017). These small RNA molecules regulate the expression of messenger RNA molecules, and thus could lead to wide scale changes in gene expression profiles, neuronal function, and altered drug behaviors.

The current study aims to investigate both environmental and genetic influences on opioid behaviors in a mouse model. We examined the influence of adolescent social stress on opioid behaviors in two different inbred mouse strains. The C57BL/6J and BALB/cJ strains were chosen because they are sensitive to long-term alterations in drug behaviors following chronic social stress in adolescence (Caruso *et al.* 2018c, 2018b, 2018a). In prior research, adult social stress (isolation or social defeat) led to increased voluntary morphine consumption (Alexander *et al.* 1978; Raz & Berger 2010; Cooper *et al.* 2017). Thus, we hypothesized that chronic variable social stress (CVSS), a model of adolescent social stress that includes repeated cycles of social isolation and social re-organization, would increase morphine consumption or behavioral sensitization to morphine. To identify a potential mechanism by which adolescent stress alters morphine behaviors, we measured corticosterone (CORT) responses to an acute stressor and miRNA expression patterns in the PFC. As above, this brain region was chosen because it is known to be susceptible to stress manipulations and to influence drug behaviors

(Andersen & Teicher 2009), and in prior work we have shown long-term alterations in prefrontal cortex excitatory transmission following CVSS (Caruso *et al.* 2018a).

Methods

Animals

Male and female BALB/cJ and C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME, USA) were bred at The Pennsylvania State University. On postnatal day (PND) 21 offspring were weaned into groups of 2-3 same sex littermates housed in polycarbonate cages (28 cm × 17 cm × 12 cm) lined with corn-cob bedding. The animals were maintained on a 12-h light-dark schedule (lights on at 0700 h) in a temperature- and humidity-controlled facility with *ad libitum* food and water available. All procedures were approved by The Pennsylvania State University IACUC committee.

Drugs and Solutions

For morphine consumption, morphine sulfate pentahydrate was obtained from the NIDA Drug Supply Program (Bethesda, MD), and saccharin sodium salt and quinine hemisulfate salt were obtained from Sigma-Aldrich (St. Louis, MO). Morphine, saccharin, and quinine were diluted in tap water for the consumption study (see below). For locomotor sensitization, morphine sulfate pentahydrate (Sigma-Aldrich, St. Louis, MO) was diluted in physiological saline (0.9% NaCl; Baxter) and injected intraperitoneally in a 10 ml/kg volume.

Chronic Variable Social Stress (CVSS)

To minimize litter effects, littermates were evenly distributed between the CVSS and control groups at weaning. CVSS mice underwent the stress protocol during adolescence (PND 25-59) as previously described (Caruso *et al.* 2017, 2018c, 2018b, 2018a). Following weaning mice sat undisturbed with littermates from PND 21 – 25. On PND 25, CVSS mice were

individually housed for 3 days followed by social rehousing with 1-2 unfamiliar cage mates for 4 days. This procedure was repeated for 5 consecutive weeks. On PND 59, CVSS mice were rehoused with their original cage mates from weaning until behavioral testing or sacrifice. For control mice, cage mates remained the same from weaning to the end of the study. To equate handling between the control and CVSS conditions, each time CVSS mice were transferred to a new cage for individual or social rehousing, control mice were also moved to new cages. Table 1 details the number of CVSS and control animals tested for each behavioral outcome described in detail below.

Outcomes

1. Morphine Sensitization

Approximately 1-2 weeks after CVSS was complete, adult mice (PND 65-72) were tested for morphine-induced locomotor sensitization using published methods (Phillips *et al.* 1994b; Kamens *et al.* 2005). This age was chosen because our prior work demonstrated changes in drug behavior and physiological responses at this time (Caruso *et al.* 2017, 2018c, 2018b, 2018a). Testing was conducted between 0900-1300 h. On test days, all animals were moved to the experimental room and allowed to acclimate for least 30 min. To acclimate mice to the test chambers, on days 1-2 all mice received an acute injection of saline immediately prior to being placed into a Superflex locomotor activity chambers (Omnitech Electronics, Inc., Columbus, OH). On day 3, animals were randomly assigned to the chronic saline (CS) or chronic drug (CD) group. CS mice received saline and CD mice received 100 mg/kg morphine on days 3, 5, 7, and 9 immediately before being placed in the activity chamber. On day 11, all animals received 100 mg/kg morphine (Koek 2014). On each day, locomotor activity was measured for 30 min and data were recorded as centimeters (cm) travelled. Three within-group scores were calculated to examine acute morphine locomotor stimulation and sensitization. Acute morphine stimulation was calculated as Day 3 – Day 2 locomotion in the CD group and

Day 11 – Day 2 locomotion in the CS group. Sensitization in the CD group was measured as Day 11 – Day 3 locomotion (Kamens *et al.* 2005). In each strain, 12-14 mice were tested in each of the four stress X drug groups (Control-CD, Control-CS, CVSS-CD, CVSS-CS), with approximately equal numbers of males and females in each group.

2. Morphine Consumption

Adult mice (starting PND 65-68) were tested for 2-bottle choice morphine consumption using published methods (Eastwood & Phillips 2014). Mice were individually housed and provided with two 25-ml graduate cylinders fitted with sipper tops. To acclimate mice to the new test environment, water was available for 2 days in both tubes. After acclimation, water tubes were replaced with one tube containing 0.2% saccharin plus morphine and another containing 0.2% saccharin plus quinine. Quinine was provided as the alternative solution to account for the bitter taste of morphine following published protocols (Eastwood & Phillips 2014). Drug solutions were available 24 hours a day. Morphine/quinine concentration increased every 4 days (morphine: 0.3, 0.7, 1 mg/ml; quinine: 0.2, 0.4, 0.55 mg/ml). Body weight was measured each time the drug concentration changed. The side of the cage that the morphine was presented was alternated every other day to prevent the development of a side preference. Morphine consumption (mg/kg), quinine consumption (mg/kg), and total fluid consumption (ml) were calculated and used as dependent variables. For each drug concentration, the average of day 2 and 4 consumption values were used to assess stable drug intake (Phillips *et al.* 1994a). For each strain, 22-28 mice were tested from each stress condition, split between males and females.

3. Acute Stress

Adult C57BL/6J mice (PND 81-83) were used to measure the physiological effect of an acute stressor. We used a standard restraint stress protocol administered between 0900-1100

h. Only C57BL/6J mice were tested as this strain was found to have long term differences in opioid behaviors (see results). Mice were removed from their home cage and placed in a broom-style restrainer. The tip of the tail (<1 mm) was removed and a baseline blood sample collected within 3 min of initial disruption. Mice remained in the restrainer for 15 min before being placed into individual holding cages. At 30 and 90 min after initial placement in the restraint tube, mice were briefly returned to restrainers for an additional blood sample collection. These time points were chosen to capture peak (30 min) and recovery (90 min) corticosterone (CORT) levels following an acute stressor (Romeo 2010). Stress reactivity was defined as 30 min CORT – baseline CORT and recovery was defined as 90 min CORT – 30 min CORT (Sapolsky *et al.* 1983). These two scores were used as dependent variables. Fourteen CVSS and 14 control animals were tested in this protocol including both sexes. Blood samples were collected into heparinized capillary tubes and immediately stored on ice. Plasma was obtain by centrifuging the samples for 15 min at 10,000g at 4°C then stored at -80°C. CORT was measured using a commercial [125I] radioimmunoassay kit (MP Biomedicals, Solon, OH, USA) following manufacturer guidelines (Caruso *et al.* 2018b, 2018a). Intra- and inter-assay coefficients of variation were 7.8 and 25.2 (for low control) and 7.5 and 7.5 (for high control).

4. Prefrontal Cortex miRNA Gene Expression

To examine adolescent stress-induced alterations in miRNA gene expression 11 male C57BL/6J mice were used. Following CVSS, these mice remained undisturbed (i.e. no behavioral testing) until PND 70 when they were euthanized by cervical dislocation and brains removed. A mouse brain matrix was used to obtain 1 mm slices. The prefrontal cortex was punched from the slice with a blunt needle that captured both hemispheres and immediately place into RNAlater. Tissue was homogenized and RNA was extracted using the RNeasy® Plus Universal Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturers' guidelines for the

isolation of total RNA including small RNAs. RNA quality was determined using the RNA Integrity Number (RIN) with an Agilent 2100 Bioanalyzer (Santa Clara, CA, USA).

RNASeq libraries were prepared using the TruSeq Small RNA Library Construction Kit (Illumina, San Diego, CA, USA). The resulting libraries were quantified and pooled for sequencing on an Illumina HiSeq to obtain 50 bp reads. On average we obtained 7 million reads per sample. Library preparation and sequencing was conducted in the Penn State Genomics Core Facility, University Park, PA. Sequencing data are available from the NCBI GEO database (experimental series accession number: to become available upon publication).

Sequencing reads were mapped to the mouse genome (*Mus musculus* mm10) using ShortStack (Johnson *et al.* 2016). miRNA annotations were downloaded from miRBase and reads mapped to miRNAs were counted in ShortStack using the default settings. Differential expression between groups was performed using DESeq2 (Love *et al.* 2014). *P*-values were corrected for multiple comparisons using a Benjamini-Hochberg false discovery rate of 5% (Benjamini & Hochberg 1995).

Differentially expressed miRNAs were analyzed for predicted mRNA targets and associated biological pathways using the DIANA toolkit (available at http://diana.imis.athena-innovation.gr/DianaTools/) and Ingenuity Pathway Analysis (IPA) software (content version 52912811; Krämer, Green, Pollard, & Tugendreich, 2014). The number of miRNA-gene relationships was predicted using the DIANA microT-CDS tool, v.5 (Paraskevopoulou et al., 2013), which computes miRNA-mRNA interaction scores for predicted targets in mRNA 3′-UTR and coding sequence (CDS) transcript regions. The microT-CDS score threshold was set to the default 0.8, which is recommended for optimal analysis sensitivity and stringency.

Enrichment analyses were run using two different methods. First, an exploratory assessment, IPA Core Expression analysis was run using IPA microRNA target filter output. The IPA microRNA target filter queries the TargetScan, TarBase, and miRecords databases, which contain predicted and experimentally validated miRNA-mRNA interactions at all prediction

confidence levels. Enriched Canonical Pathways and Diseases/Biological Functions were identified using an uncorrected significance threshold of 0.05. Full IPA analysis settings are available in supplementary materials (File S1). Second, a more stringent approach, the DIANA miRPath tool, v.3 (Vlachos et al., 2015) was used to identify enriched KEGG pathways from transcripts identified by the microT-CDS algorithm. miRPath was run using the “Pathways Union” algorithm, with “Unbiased Empirical Distribution” selected as the enrichment method. In contrast to the commonly used miRPath “Genes Union” setting, which is similar to the approach taken by the IPA Core analysis, Pathways Union utilizes an FDR corrected, merged p-value that represents the likelihood a certain biological pathway is targeted by at least one of the input miRNAs. Further, use of an empirical distribution, which includes all transcripts predicted to be targets of miRNAs (vs. the entire murine transcriptome), has been suggested to reduce algorithm bias and increase prediction accuracy (Bleazard, Lamb, & Griffiths-Jones, 2015).

Statistical Analysis

Behavioral data were analyzed in SPSS (IBM, Armonk, NY) with factorial analysis of variance (ANOVA). Independent factors included stress (Control vs CVSS), strain (C57BL/6J vs BALB/cJ), sex (male vs female), day (1-11), concentration (0.3/0.7/1 or 0.2/0.4/0.55 mg/ml), and drug group (CS vs CD) where appropriate. Interactions that included multiple variables were interpreted with successive ANOVAs with fewer factors. Fishers LSD *post hoc* test was used. The alpha level set at 0.05.

Results

1. Morphine Sensitization

The impact of adolescent social stress on morphine sensitization varied by strain. Adult C57BL/6J mice exposed to adolescent social stress had blunted morphine sensitization (Fig 1) however BALB/cJ mice did not show this effect. In the overall analysis with strain, stress, sex,

drug group, and day as factors, we observed many significant effects involving strain ranging from a main effect of strain to 4-way interactions involving this factor, thus we conducted further analyses split by strain. In C57BL/6J mice, we observed significant main effects of day ($F_{6,264}$ = 85.9, $p < 0.001$) and drug group ($F_{1,44}$ = 152.8, $p < 0.001$), and interactions of day X drug group ($F_{6,264}$ = 55.1, $p < 0.001$), day X stress X drug group ($F_{6,264}$ = 2.6, $p < 0.05$), and day X stress X sex X drug group ($F_{6,264}$ = 2.5, $p < 0.05$). To decompose the 4-way interaction including sex, follow up analyses were conducted in each sex separately. In male C57BL/6J mice, we observed a significant main effect of day ($F_{6,132}$ = 38.4, $p < 0.001$), drug group ($F_{1,22}$ = 88.2, $p < 0.001$), day X drug group ($F_{6,132}$ = 22.5, $p < 0.001$), and day X stress X drug group interactions ($F_{6,132}$ = 4.3, $p < 0.01$). The interactions between day and group are consistent with the experimental design and suggest that the pattern of activity across days is dependent on the experimental drug group. Thus, further analyses were limited to the key days that examine between-group acute locomotor stimulation (Day 3) and sensitization (Day 11) (Gubner *et al.* 2014; Miller & Kamens 2019). On Day 3, C57BL/6J mice in the CD group showed increased locomotion following acute morphine injection relative to the CS group that received saline (main effect of group: $F_{1,22}$ = 24.4, $p < 0.01$), but there were no main effects or interactions with stress. On Day 11, there was a significant main effect of drug group ($F_{1,22}$ = 11.7, $p < 0.01$) and a stress X drug group interaction ($F_{1,22}$ = 7.6, $p < 0.05$). *Post hoc* analysis demonstrated that among control animals there was a significant difference between the CD and CS groups ($p < 0.05$), indicative of between-groups sensitization, but there was no significant difference between the CD and CS groups among CVSS animals. The within-group analysis confirmed this pattern. Control male C57BL/6J mice exposed to chronic morphine exhibited robust behavioral sensitization that was attenuated in CVSS animals (t_{11} = 3.8, $p < 0.01$; Fig 1B). When the acute response was examined with a between-groups analysis there was a significant group difference in the CS mice, where stressed male C57BL/6J mice had a more robust acute locomotor response to morphine compared to control animals (Day 11 – Day 2; t_{11} = -2.6, p <

0.05; Fig 1B). There was no significant difference between stress and control mice for the CD group acute locomotor response.

Since stress attenuated morphine sensitization in male C57BL/6J mice, we next analyzed the female C57BL/6J animals. In female C57BL/6J mice, we observed a significant main effect of day ($F_{6,132} = 48.7$, $p < 0.001$), group ($F_{1,22} = 68.4$, $p < 0.001$), and a day X drug group interaction ($F_{6,132} = 34.3$, $p < 0.001$). Importantly, in female C57BL/6J mice the main effect of stress and interactions with this variable were not significant. When examining days indicative of acute stimulation (Day 3) and sensitization (Day 11) there were significant main effects of drug group ($t_{24} = -5.5$, $p < 0.001$, $t_{24} = -2.4$, $p < 0.05$, respectively). Compared to CS mice, CD mice exhibited greater locomotor response on Day 3 (mean ± SEM, 5541 ± 258, 15732 ± 1863, respectively), indicative of the acute stimulant response, and a greater response on Day 11, indicative of between-groups sensitization (mean ± SEM, 17994 ± 1463, 24929 ± 2475, respectively). In the within-group analysis, no significant stress group differences were observed for the acute locomotor response in either the CS or CD groups. When behavioral sensitization was examined, there was a statistical trend ($p = 0.055$) where female C57BL/6J mice exposed to chronic adolescent social stress had blunted morphine sensitization compared to control animals (Fig 1D).

In BALB/cJ mice, the 100 mg/kg dose of morphine did not result in locomotor stimulation or sensitization (Fig 1E-H). The overall analysis within this strain demonstrated a main effect of day ($F_{6,252} = 4.9$, $p < 0.001$) and a day X drug group interaction ($F_{6,252} = 6.6$, $p < 0.001$). *Post hoc* analysis found no significant effects on Day 3 or Day 11. There were no significant effects observed in the within-group analysis for either acute stimulation or sensitization in BALB/cJ mice.

2. Morphine Consumption

Five C57BL/6J mice (spread among stress conditions and sex) died during the consumption study. While we followed a published 24-hour morphine access consumption paradigm (Eastwood & Phillips 2014), future work should consider limiting drug availability to 18 h to decrease the chance for over dosing or drug-induced anorexia (Kamens *et al.* 2005).

Adolescent social stress did not influence adult morphine consumption in either C57BL/6J or BALB/cJ (Fig 2) mice. In an overall analysis of morphine consumption (mg/kg) there was a significant main effect of strain ($F_{1,95} = 180.9$, $p < 0.001$) and a strain X concentration interaction ($F_{2,190} = 43.5$, $p < 0.001$), thus in further analyses C57BL/6J and BALB/cJ mice were analyzed independently. In C57BL/6J mice, morphine consumption increased in a concentration dependent manner (Fig 2A; main effect of concentration: $F_{2,90} = 348.8$, $p < 0.001$, all *post hoc* $p < 0.001$), but no other significant main effects or interactions were observed. There were no significant effects on quinine consumption (Fig 2C). For total fluid intake, while there was a significant sex X concentration interaction ($F_{2,90} = 7.4$, $p < 0.01$), *post hoc* comparison suggest this was driven by a trend ($p = 0.06$) for the males to consume more fluid compared to females (8.9 ± 0.6, 7.7 ± 0.3, respectively), but only when the middle morphine concentration (0.7 mg/ml) was available.

Similar to the results observed in C57BL/6J mice, in BALB/cJ mice drug concentration influenced intake, but no main effects or interactions with stress condition were observed. BALB/cJ mice consumed significantly more morphine when the 0.7 and 1 mg/ml morphine concentrations were available compared to the 0.3 mg/ml concentration (Fig 2B, main effect of concentration: $F_{2,100} = 19.8$, $p < 0.001$, all *post hoc* $p < 0.05$). Importantly, there were no main effects or interactions with stress condition. In BALB/cJ mice, there was a significant main effect of concentration on quinine consumption ($F_{2,100} = 6.0$, $p < 0.01$). Here, quinine consumption was lower when the middle quinine concentration was available relative to the low or high concentrations (Fig 2D, *post hoc* ps < 0.05). Finally, when total fluid intake was examined there was a significant main effect of concentration ($F_{2,100} = 27.2$, $p < 0.001$) and sex ($F_{1,50} = 4.9$, $p <$

0.05). BALB/cJ mice drank significantly more fluid when the 0.3 mg/ml (6.8 ± 0.2) morphine concentration was available relative to both the 0.7 (5.5 ± 0.1) or 1 (5.6 ± 0.2) mg/ml concentrations (Fig 2F, *post hoc* ps < 0.05). Overall, male BALB/cJ mice consumed more fluid than female BALB/cJ mice (6.2 ± 0.1, 5.7 ± 0.2, respectively).

3. Acute Stress

Adult C57BL/6J mice exposed to adolescent social stress had a slower recovery of CORT levels following an acute restraint stress compared to control mice (Fig. 3). There were no significant effects when reactivity (30 min CORT – baseline CORT) was examined. In contrast, when recovery (90 min CORT – 30 min CORT) was examined there was a significant main effect of stress ($F_{1,24}$=12.1, p<0.01) and sex ($F_{1,24}$=4.9, p<0.05), but the interaction of these variables was not significant. Mice exposed to CVSS had a slower CORT recovery compared to control animals (3.3 ± 32.5, -88.7 ± 32.2, respectively) and females recovered slower than males (45.1 ± 26.9, -99.5 ± 28.5, respectively).

4. miRNA Expression

In adult C57BL/6J male mice, twelve miRNA (miR-429-3p, miR-200a-3p, miR-96-5p, miR-141-3p, miR-200b-3p, miR-183-5p, miR-200a-5p, miR-182-5p, miR-200c-3p, miR-141-5p, miR-183-3p, miR497b) were downregulated following adolescent social stress (for full results see supplementary File S2). The DIANA microT-CDS algorithm, run with the 12 differentially expressed miRNA, predicted 5433 total miRNA-mRNA relationships, with 2820 unique mRNA targets. Five significant KEGG pathways were identified by miRPath: MAPK signaling, Glycosphingolipid biosynthesis (lacto and neolacto series), AMPK signaling, Glycosphingolipid biosynthesis (globo series), and Gap junction (see also Table 3). At a significance threshold of 0.05, IPA Core Expression analysis identified 500 enriched Diseases/Biological Functions and 267 enriched Canonical Pathways. See Table 4 for the top five enrichment terms in each category and supplementary Files S3-S4 for full IPA output.

Discussion

In the present study we examined the effect of adolescent social stress on adult morphine behaviors in two inbred strains of mice. We further sought to identify biological mechanisms altered by stress exposure that may account for differences in morphine sensitization. We found that adolescent social stress blunted morphine sensitization in adult C57BL/6J, but not BALB/cJ, mice, an effect that was more robust in male mice. This behavioral effect of stress emerged after repeated drug administration, as there was no evidence of a change in the acute locomotor response to morphine. Adolescent stress did not influence adult voluntary morphine consumption in either strain. We observed long-term changes in both the recovery of the CORT response following an acute stressor and in the expression of adult miRNA in the prefrontal cortex. It is possible that these changes reflect mechanisms linked to the altered morphine response. These data indicate that a gene-environment interaction may influence opioid behavioral responses that are indicative of morphine sensitization in adulthood.

Our results indicate that male C57BL/6J mice are particularly vulnerable to lasting effects of adolescent stress. In both the within- and between-groups analysis we observed that control animals developed normal morphine-induced behavioral sensitization, but this was blunted in animals that were exposed to social stress in adolescence. A similar statistical trend ($p = 0.055$) was observed in female C57BL/6J mice with the within-group analysis. Due to the complex design of this experiment, there were 5-8 mice per sex, stress condition, and drug group; thus, it is possible we were underpowered to detect significant effects. The fact that there is a known statistical power advantage of within-subject design may explain why we observed a trend in the female C57BL/6J mice using this design (Keppel 1991).

Our finding that adolescent social stress attenuates adult morphine-induced behavioral sensitization adds to literature on the role of stress in this behavioral response, although prior results are mixed. Rats who received morphine paired with restraint stress had augmented

morphine behavioral sensitization (Shaham *et al.* 1995). In contrast, adult C57BL/6J mice showed no lasting effect of adult restraint stress on morphine sensitization (Xu *et al.* 2014). Unfortunately, there are too many factors (species, type of stressor, age of exposure) that differ among these studies to make strong conclusions on what may be driving these differences.

The differences in morphine behavioral responses appear to emerge after repeated drug exposures as we saw little indication that stress altered the acute locomotor response to morphine. We observed that CVSS increased morphine stimulation in the male C57BL/6J CS group, but not in the CD group or with the between-groups analysis. These findings are consistent with a previous study that demonstrated that 14 days of restraint stress in adult C57BL/6J mice had no effect on acute morphine locomotor stimulation (Xu *et al.* 2014). However, these results differ from prior research in adult rats that have demonstrated that stress (restraint, handling, social defeat, foot shock, or social isolation) potentiates morphine-induced locomotor stimulation (Leyton & Stewart 1990; Deroche *et al.* 1994; Stöhr *et al.* 1999). The stressors in these studies varied in duration and number of exposures. Stöhr and colleagues demonstrated that one 60-min restraint stress was not enough to potentiate morphine locomotor stimulation, but 3 exposures to restraint stress, handling or social defeat did. The stressors in the other papers were longer with 5 sessions of 20 min foot shock or 17 days of continuous social isolation in adulthood. These procedural differences make it difficult to draw strong conclusions, but it is interesting to note that while significant effects of stress exposure on morphine stimulation have been observed in rats, there is currently little evidence in C57BL/6J mice.

In the present work, we observed no difference in morphine consumption following adolescent social stress. Our findings align with work in adult male C57BL/6J mice. Exposure to chronic social stress increased morphine preference immediately (1 day) after stress exposure, but the effect was no longer present when tested 14 days post-stress exposure (Cooper *et al.* 2017). In the current experiment, mice were given access to morphine beginning 8-11 days

following the stress exposure. We originally hypothesized that stress during adolescence would result in long-lasting differences in drug consumption because of the critical time in brain development, but that was not observed with morphine consumption. Instead the effects of stress on opioid consumption may be time-dependent and only evident immediately after stress exposure. This is important when considering factors that could lead to drug use initiation.

In both the morphine sensitization and consumption experiments, we found pronounced differences between C57BL/6J and BALB/cJ mice. These differences align with prior research demonstrating strain differences in morphine behaviors (Belknap *et al.* 1993; Semenova *et al.* 1995; Kest *et al.* 2002; Kennedy *et al.* 2011). For example, consistent with the prior literature C57BL/6J mice consumed significantly more morphine than BALB/cJ mice (Belknap *et al.* 1993). In the current study our paradigm produced robust behavioral sensitization in C57BL/6J mice, but not in BALB/cJ mice. It is possible that the dose of morphine (100 mg/kg) we chose was too high for this strain. While we found no effects of stress in BALB/cJ mice, it is critical to recognize that different effects may be observed on other morphine behaviors or in response to other doses of morphine used in our paradigms.

Here we show that exposure to chronic social stress in adolescence changes the physiological response to an acute stressor in adulthood. In particular, we found that it took stressed mice longer to recover following a 15 min restraint stress. This finding is in line with research suggesting that animals chronically exposed to one type of stressor beforehand have an altered hormonal response to a novel stressor (Romeo 2013). For example, adolescent rats exposed to 7 days of a cold room stressor then restraint stress (a heterotypic stressor) had a delayed recovery of CORT to the restraint stress. In contrast, this effect was not observed in adult rats or in adolescent rats exposed to 8 days of restrain stress (a homotypic stressor) (Lui *et al.* 2012). In this prior study, all stress exposures occurred during adolescence, but the current findings suggest that this altered physiological response to an acute stressor may last into adulthood.

In this study we observed 12 miRNA in the adult prefrontal cortex that were downregulated following adolescent social stress. We chose to examine differential gene expression in the prefrontal cortex because of our prior work which demonstrated long-lasting changes in neuronal excitability in this region (Caruso *et al.* 2018a). A differentially expressed miRNA of interest that we identified was miR-183. This finding is consistent with a prior study that showed chronic immobilization stress in adulthood decreased the expression of this gene in the amygdala and CA1 region of the hippocampus (Meerson *et al.* 2010). Interestingly, in that study an upregulation was identified after acute immobilization stress, suggesting that the duration of stress exposure is important. Time-dependent effects of stress exposure on other miRNAs have also been observed. For example, miR-200a-3p was shown to be down regulated in the prefrontal cortex of rats exposed to 4 weeks of chronic unpredictable stress in adulthood; a finding similar to our own. In contrast, after 12 weeks of stress exposure this same miRNA was upregulated (Satyanarayanan *et al.* 2019). We believe that this investigation produced the first known data to examine the global patterns of miRNA expression following adolescent stress. Given that these small RNA molecules regulate mRNA expression, our findings could contribute to the altered behavioral responses we observed, but additional work is necessary to confirm this hypothesis. Further, these data provide additional support that repeated exposure to stress in adolescence may compromise typical development of the PFC with this altered development resulting in impaired cognitive, emotional, and behavioral regulatory functions.

Functional enrichment analysis of the differentially expressed miRNA and their predicted mRNA targets found proCancer-related pathways among the top enrichment terms in both miRPath and IPA analyses. Cancer-related signaling tends to involve ubiquitous molecules and pathways that often also participate in neurological functioning. For example, the top enriched miRPath KEGG pathway was MAPK signaling, a biological pathway well known to mediate cancer formation as well as learning and memory (Atkins *et al.* 1998; Wagner & Nebreda 2009). Further, in addition to their known role in tumor formation (Inokuchi *et al.* 1987),

glycosphingolipids have recently been implicated in addiction (Kalinichenko *et al.* 2018). IPA core analysis identified overall top enrichment terms related to cancer and synaptogenesis. It is also notable that the top IPA Diseases/Functions terms under the subcategory “Physiological System Development and Function” was “Nervous System Development and Function.” Thus, adolescent stress appears to alter expression of adult miRNAs broadly regulating signaling pathways related to cancer and nervous system development/functioning. These specific miRNA and pathways serve as mechanisms by which adolescent stress my change later opioid behaviors.

The current work demonstrates a gene-environmental interaction that leads to opioid behaviors. Specifically, adult C57BL/6J mice exposed to adolescent social stress had attenuated morphine sensitization compared to control animals, whereas BALB/cJ mice did not. It remains possible adolescent stress may impact morphine sensitization in BALB/cJ if a more optimal dose of this drug was used in this strain. This is not the first time we have observed such an strain X stress dependent effects with this stressor as a similar finding was observed when we looked at long-term effects on nicotine behaviors (Caruso *et al.* 2018b, 2018a). Given that the majority of addiction research utilizes C57BL/6J mice it is important to recognize that we may miss gene-environment interactions if utilizing only a single mouse strain.

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Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request. Sequencing data are available from the NCBI GEO database (experimental series accession number: to become available upon publication acceptance).

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Figure Legends

Figure 1: Morphine-induced behavioral stimulation and sensitization in adult C57BL/6J and BALB/cJ mice. Daily mean locomotor distance (cm) ± SEM in C57BL/6J male (A), C57BL/6J female (C), BALB/cJ male (E), and BALB/cJ female (G) mice. With-subject (mean ± SEM) initial stimulant response to morphine of chronic saline animals (left side) and chronic drug treated mice (right side) in C57BL/6J male (B), C57BL/6J female (D), BALB/cJ male (F), and BALB/cJ female (H) mice. With-subject sensitization in C57BL/6J male (B), C57BL/6J female (D), BALB/cJ male (F), and BALB/cJ female (H) mice chronic drug treated mice is shown on the right side. N = 5-8 per stress and drug condition. *$p<0.05$ for stress X drug group interaction; #$p<0.05$ for the main effect of drug group; $$p<0.05$ for the main effect of stress; &$p=0.055$ for the main effect of stress

Figure 2: Adolescent stress did not influence morphine or quinine consumption in adult C57BL/6J or BALB/cJ mice. Morphine consumption and quinine consumption in C57BL/6J and BALB/cJ mice. Mean (±SEM) morphine consumption in C57BL/6J (C) and BALB/cJ (D) mice. Mean (±SEM) quinine consumption in C57BL/6J (A) and BALB/cJ (B) mice. Mean (±SEM) fluid intake in C57BL/6J (E) and BALB/cJ (F) mice. N =22–28 per strain and stress condition

Figure 3: Change in corticosterone following 15 minutes of restraint stress in adult C57BL/6J mice. Mean corticosterone (ng/ml) ± SEM in C57BL/6J male and female mice N = 14 per stress condition. *$p<0.05$ for main effect of sex; $$p<0.05$ for main effect of stress group

Table 1. Overview of experimental design.

Group	Subjects		Outcome
1	C57BL/6J CON =	12 M & 15 F	Morphine Sensitization
	C57BL/6J CVSS =	14 M & 11 F	
	BALB/cJ CON =	11 M & 14 F	
	BALB/cJ CVSS =	11 M & 14 F	
2	C57BL/6J CON =	11 M & 11 F	Morphine Consumption
	C57BL/6J CVSS =	15 M & 12 F	
	BALB/cJ CON =	15 M & 11 F	
	BALB/cJ CVSS =	14 M & 14 F	
3	C57BL/6J CON =	9 M & 5 F	Acute Stress CORT Response
	C57BL/6J CVSS =	8 M & 6 F	
4	C57BL/6J CON =	6 M	Prefrontal Cortex miRNA
	C57BL/6J CVSS =	5 M	

CON = control; CVSS = chronic variable social stress; M = male; F = female

Table 2. Treatment and testing protocol for morphine sensitization protocol.

Group	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11
Chronic Saline											
Injection	Saline	Saline	Saline	None	Saline	None	Saline	None	Saline	None	MO
Test	Yes	Yes	Yes	No	Yes	No	Yes	No	Yes	No	Yes
Chronic Drug											
Injection	Saline	Saline	MO	None	MO	None	MO	None	MO	None	MO
Test	Yes	Yes	Yes	No	Yes	No	Yes	No	Yes	No	Yes

MO: Morphine.

Table 3. miRPath output.

KEGG pathway	p-value	#genes	#miRNAs
MAPK signaling pathway	0.015	5	1
Glycosphingolipid biosynthesis – lacto and neolacto series	0.018	2	1
AMPK signaling pathway	0.027	4	1
Glycoshingolipid biosynthesis – globo series	0.027	3	2
Gap junction	0.048	2	1

Table 4. IPA output.

IPA top 5 canonical pathways	p-value
Molecular mechanisms of cancer	3.98E-11
BMP signaling pathway	1E-10
Synaptogenesis signaling pathway	2.75E-10
Cardiac hypertrophy signaling	3.24E-09
GNRH signaling	1.66E-08

IPA disease/function annotation	p-value	#molecules
Non-hematological solid tumor	7.44E-208	4133
Nonhematologic malignant neoplasm	1.25E-204	4118
Cancer	3.74E-190	4164
Malignant solid tumor	1.4E-189	4151
Non-melanoma solid tumor	5.4E-189	4079

Figure 1

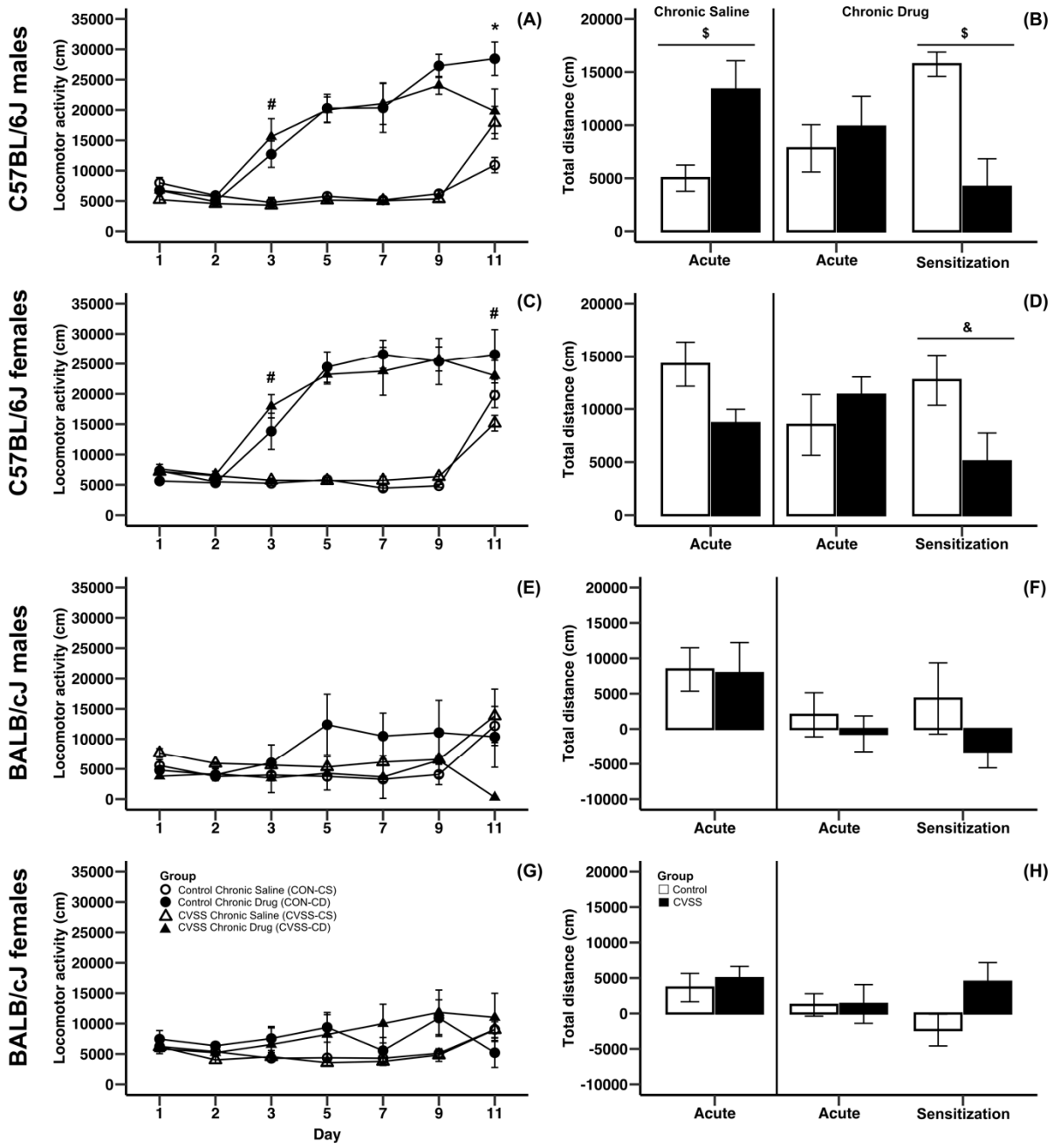

C57BL/6J males
C57BL/6J females
BALB/cJ males
BALB/cJ females
Locomotor activity (cm)
Total distance (cm)
(A)
(B)
(C)
(D)
(E)
(F)
(G)
(H)
Chronic Saline
Chronic Drug
Acute
Sensitization
Day
Group
Control Chronic Saline (CON-CS)
Control Chronic Drug (CON-CD)
CVSS Chronic Saline (CVSS-CS)
CVSS Chronic Drug (CVSS-CD)
Control
CVSS

Figure 2

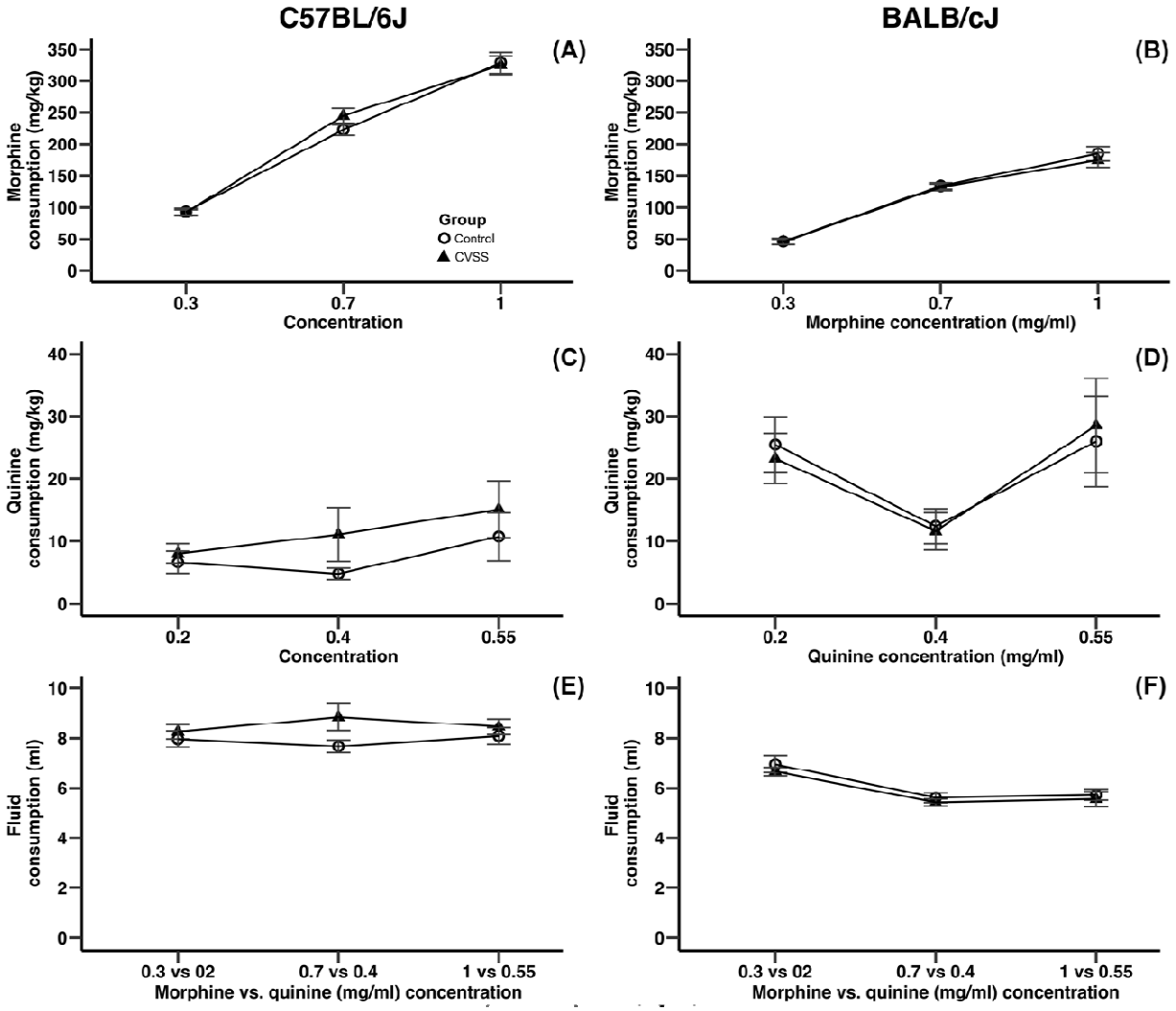
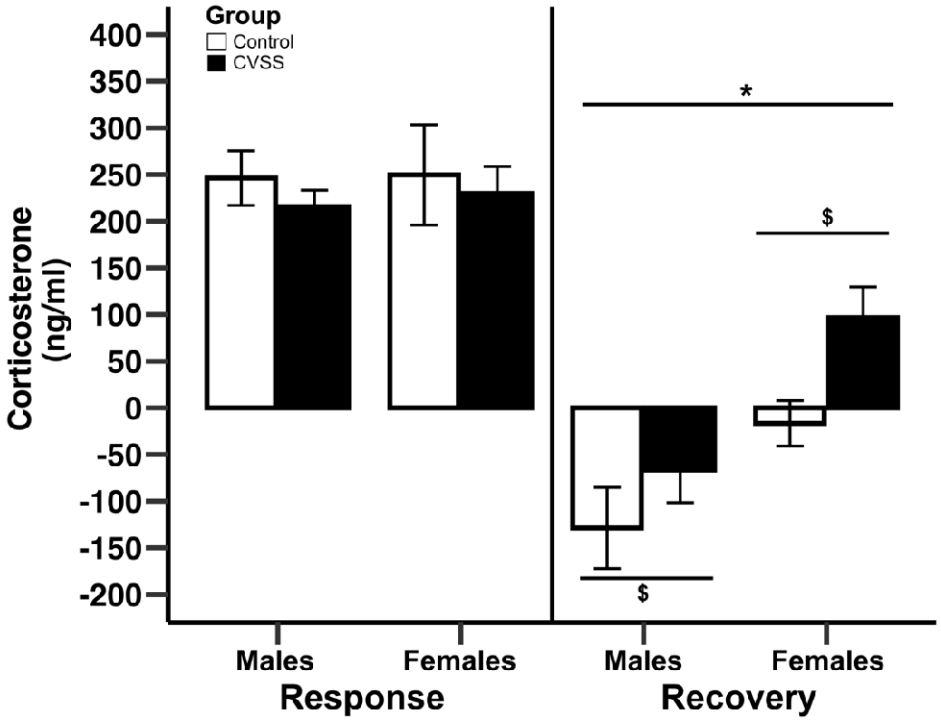

C57BL/6J
BALB/cJ
(A)
(B)
(C)
(D)
(E)
(F)
Morphine consumption (mg/kg)
Quinine consumption (mg/kg)
Fluid consumption (ml)
Group
Control
CVSS
Concentration
Morphine concentration (mg/ml)
Quinine concentration (mg/ml)
0.3
0.7
1
0.2
0.4
0.55
0.3 vs 02
0.7 vs 0.4
1 vs 0.55
Morphine vs. quinine (mg/ml) concentration

Figure 3


Group
Control
CVSS
Corticosterone (ng/ml)
400
350
300
250
200
150
100
50
0
-50
-100
-150
-200
*
$
$
Males
Females
Males
Females
Response
Recovery