The long-term effects of repeated heroin vapor inhalation during adolescence on measures of nociception and anxiety-like behavior in adult Wistar rats

Arnold Gutierrez, Ph.D., Eric L. Harvey, Ph.D., Kevin M. Creehan, B.S., Michael A. Taffe, Ph.D.

Department of Psychiatry, University of California, San Diego; La Jolla, CA, USA

Running Head: Adolescent Heroin Vapor Inhalation

Address Correspondence to: Dr. Michael A. Taffe, Department of Psychiatry, Mail Code 0714; 9500 Gilman Drive; University of California, San Diego; La Jolla, CA 92093; USA; Email: mtaffe@ucsd.edu

ABSTRACT

The United States continues to experience a public health crisis related to the nonmedical use of opioid drugs. Adolescents represent a vulnerable group due to increased experimentation with illicit substances that is often associated with the adolescent period, and because adolescent drug use can result in long-term effects that differ from those caused by drug use initiated during adulthood. The present study examined the effects of repeated heroin inhalation, a common route of administering opioids, during adolescence, on measures of nociception and anxiety-like behavior in adulthood. Rats were exposed twice daily to 30-minutes of heroin vapor from post-natal day (PND) 36 to PND 45. At 12 weeks of age, baseline thermal nociception was assessed across a range of temperatures with a warm-water tail-withdrawal assay. Anxiety-like behavior was assessed in an elevated plus-maze (EPM) and activity was measured in an open field arena. Starting at 23 weeks of age, baseline thermal nociception was re-assessed, nociception was determined after acute heroin or naloxone injection, and anxiety-like behavior was redetermined in the EPM. Adolescent heroin inhalation altered baseline thermal nociception in female rats at 12 weeks of age and in both female and male rats at ~23 weeks. Heroin-treated animals exhibited an increase in anxiety-like behavior when tested in the elevated plus-maze, showed blunted heroin-induced analgesia, but exhibited no effect on naloxone-induced hyperalgesia. The present study demonstrates for the first time that heroin vapor inhalation during adolescence produces behavioral and physiological consequences in rats that persist well into adulthood.

INTRODUCTION

The US opioid crisis, which is associated with high rates of non-medical use of both prescription and illicit opioids, has been marked by escalating rates of opioid-associated overdose and increasing prevalence of addictive use disorders [1-4]. Treatment admissions of adolescents and young adults for heroin use is second only to admissions for cannabis in the US [5]. At the same time, there has been an alarming rise in the use of Electronic Nicotine Delivery Systems (ENDS; e-cigarettes), especially within adolescent populations. Adolescents who administer nicotine using ENDS devices are more likely to also use them to vape cannabis [6], and, among college students, ENDS devices are increasingly being used for the administration of substances other than nicotine [7]. ENDS devices can be readily adapted to deliver a range of psychoactive drugs [8], and reports are emerging on their use to administer substances such as opioids, cathinones, and amphetamines [9,10]. The popularity of ENDS for administering non-nicotine substances has reached a level that recommends rethinking the terminology; in truth, Electronic Drug Delivery Systems (EDDS) is a more accurate term.

Adolescence represents a critical developmental period associated with increased risk-taking and impulsive behavior [11]. Experimentation with drugs and alcohol is heightened during this period, and adolescents are therefore at an increased risk of developing substance abuse disorders [12,13]. In animal models, chronic drug exposure during adolescence can result in long-lasting effects on nociception and drug reinforcement. For example, mice that self-administered oxycodone during adolescence exhibit a decrease oxycodone anti-nociception in adulthood [14] and tolerance to morphine anti-nociception is accelerated in adult rats that were chronically treated with morphine during adolescence [15]. Morphine conditioned place preference (CPP) is enhanced in adult mice treated with oxycodone [16] and in adult rats treated with morphine during adolescence [17]. Similarly, oxycodone self-

administration in adolescence potentiates oxycodone CPP in adult mice [14]. While the lasting effects of opioid treatment during adolescence have been studied in animals, to date they have mainly been studied using parenteral injections, or implanted delivery systems, as the route of administration. Therefore, the route-specific effects of inhaled opioids within a developmental context, if any, are presently unknown.

Inhalation is a common route of administration among opioid users [18] that dates back to Mediterranean antiquity [19]. The inhalation of opioids can be achieved by heating the drug over a surface such as aluminum foil and inhaling the resulting vapors, a method often referred to as ‘chasing the dragon’ [20,21]. EDDS technology can be adapted to deliver active doses of many psychoactive drugs to rats by inhalation, including opioids. Non-contingent vapor delivery produces anti-nociception after inhalation of heroin, THC, or the combination [22-26], decreases body temperature after nicotine, THC or cannabidiol inhalation [22,23,26-28], increases in locomotor activity after exposure to nicotine [28], methamphetamine [29,30], and heroin [25,26], and decreases activity following THC [26]. Additionally, EDDS delivery of nicotine vapor produces CPP in adult and adolescent rats [31]. *In utero* exposure to vaporized THC produces effects on body weight in adolescence [32], and a recent study shows evidence of the self-administration of THC by vapor inhalation [33]. Recent studies also present compelling evidence for the self-administration of nicotine by rats [34,35] and mice [36,37]. Similarly, mice and rats will make responses to obtain doses of fentanyl vapor [38,39], and rats will make responses to obtain doses of the potent opioid sufentanil in vapor form in an extended daily access paradigm [40] and heroin vapor in a short-access paradigm [24]. Importantly, signs of dependence were observed in both the mouse and rat opioid vapor self-administration studies described.

The purpose of this study was to assess the long-term effects of repeated adolescent heroin vapor exposure on thermal nociception, injected opioid anti-nociception, and on anxiety-like behavior in adulthood. Following repeated heroin vapor exposure during adolescence and upon reaching adulthood, rats were tested for differences in nociceptive sensitivity using a warm water tail-withdrawal assay. Anxiety-like behavior was assessed using an elevated plus-maze (EPM) and open field tests. Finally, anti-nociceptive effects of acute heroin injection and hyperalgesic effects of injection with the opioid receptor antagonist naloxone were determined. This is the first study to assess the long-term effects of adolescent opioid exposure using inhalation as the route of administration.

METHODS

Subjects

Adolescent female (N=24) and male (N=24) Wistar rats were obtained from Charles River Laboratories (Charles River) in two sex-balanced cohorts. Two cohorts of female (N=12 per cohort) and male (N=12 per cohort) rats, designated Cohort 1 (C1) and Cohort 2 (C2), arrived on Post-natal Day (PND) 29 and were habituated to the vivarium through PND 35. The vivarium was kept on a 12:12 hour light-dark cycle and behavior studies were conducted during the rats' dark period. Food and water were provided ad libitum in the home cage. Body weights were recorded weekly, beginning at around 5 weeks of age (PND 36) and continuing through the end of the study. Weight data are reported in the **SUPPLEMENTAL MATERIALS (Figure S1**). Experimental procedures were conducted in accordance with protocols approved by the IACUC of the University of California, San Diego and consistent with recommendations in the NIH Guide [41].

Drugs

Heroin (diamorphine HCl) was administered by vapor inhalation and subcutaneous injection. Inhalation/vapor doses are varied in this model, and are therefore described, by altering the concentration in the propylene glycol (PG) vehicle, the puff schedule and/or the duration of inhalation sessions in this approach [23,28,29]. Vapor drug treatment methods are fully described in the **SUPPLEMENTAL MATERIALS**. The heroin was dissolved in the PG to achieve target concentrations and then loaded in the EDDS tanks in a volume of ~ 0.5 ml per session. Fresh solutions were used for each session. Heroin and naloxone HCl were dissolved in physiological saline for subcutaneous (s.c.) or intraperitoneal (i.p.) injection, respectively. Heroin was provided by the U.S. National Institute on Drug Abuse and naloxone (Tocris Bioscience) was purchased from Fisher Scientific.

Thermal nociception

Baseline nociception

The first set of nociception assays (Time-1) were performed at 12 weeks of age over two days, with two temperatures assessed per test day (48ºC and 52ºC on one day and 46ºC and 50ºC on the other) and one day in between test days (See **SUPPLEMENTAL MATERIALS)**. A range of water temperatures was used to ensure any between-groups differences were not obscured by ceiling or floor effects. A second set of tail-withdrawal measurements (Time-2) were performed at 23 weeks of age in C2 rats and 29 weeks of age in C1 rats. On each test day, tail-withdrawal measurements for each of the two temperatures

were separated by 120 minutes and a counterbalanced order was used for the two designated test temperatures.

Heroin and naloxone challenge

The anti-nociceptive effects of acute heroin injection were assessed by warm water tail-withdrawal assay (See **SUPPLEMENTAL MATERIALS)** in rats at 26 (C2) and 31 (C1) weeks of age . Rats were administered heroin (0.5 mg/kg, s.c.) 30 minutes prior to the tail-withdrawal measurement. Rats were then injected with heroin (1.0 mg/kg, s.c.) 120 minutes after the first injection and tail-withdrawal latencies were once again measured after a 30-minute interval. Baseline tail-withdrawal latencies from the Time-2 re-assessments were used for baseline comparisons. Water temperature for both assessments was set to 52°C and a cutoff of 15 sec was used.

At 28 (C2) or 34 (C1) weeks of age, rats were administered naloxone (0.3 mg/kg, i.p.), followed by naloxone (1.0 mg/kg, i.p.) 120 minutes after the first injection. Rats underwent tail-withdrawal testing in a warm water bath set to 48°C 15 minutes after each antagonist injection. Baseline tail-withdrawal latencies for this experiment were measured immediately before the first naloxone injection. The cooler temperature was selected to provide parametric range to observe any anticipated hyperalgesic effects of naloxone injection.

Anxiety-like behavior

The effects of adolescent heroin inhalation on the expression anxiety-like behavior were assessed using an elevated plus-maze (EPM) test and by assessing behavior in an open field

arena (see **SUPPLEMENTAL MATERIALS**). Elevated plus-maze testing was conducted on PND 93 in C1 rats and PND 226 in C2 rats. Test sessions were conducted in a dimly lit room and animals' activity was recorded for 5 minutes. The apparatus had two opposing open arms and two opposing closed arms perpendicular to the open arms. Arms measured 50 cm in length and 10 cm in width. The walls of the closed arms were 40 cm in height. The apparatus had four legs, each positioned towards the distal portion of an arm, which suspended it 50 cm above the floor. Behavior was recorded and measured using AnyMaze software (Stoelting, Wood Dale, IL). The apparatus was cleaned with an ethanol solution prior to the testing of each subject.

Data Analysis

Data collected from cohort 1 and 2 animals during baseline nociception measurements, EPM testing, and from the heroin and naloxone challenges were combined for analysis. Data were analyzed by Analysis of Variance (ANOVA) with repeated measures factors for Time in weight data and open field data, for Temperature in tail-withdrawal experiments where multiple temperatures were assessed, and for Treatment Condition in the heroin and naloxone challenge experiments. Mixed-effects analysis was used when there were missing data. Significant effects were followed by post-hoc analysis using the Sidak (two-level factors) or Tukey (multi-level factors) methods. A criterion of $p < 0.05$ was used to infer significant effects. Analyses were performed using Prism 9 for Windows (GraphPad Software, Inc, San Diego, CA).

RESULTS

Thermal nociception

Initial analysis of thermal nociception on PND 85 and PND 87 (12 weeks of age; **Figure 1**) across four temperatures confirmed a main effect of Temperature ($F(3, 126) = 222.4$, $p < 0.0001$), an interaction of Sex with Adolescent Drug ($F(1, 42) = 8.639$, $p < 0.01$), and a three- way interaction of Temperature, Sex, and Adolescent Drug ($F(3, 126) = 3.197$,

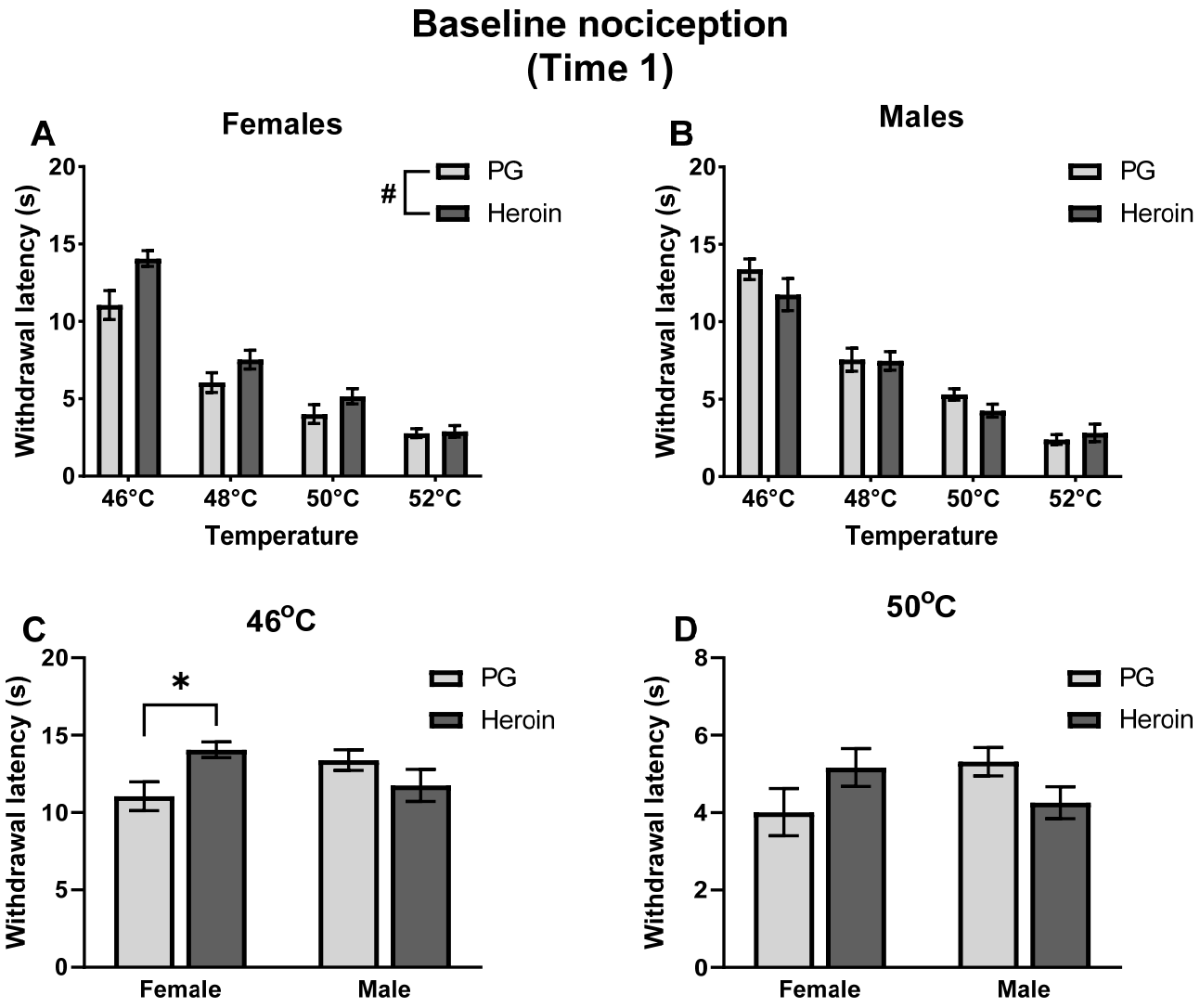

***Figure 1**. Mean (±SEM; N=12 per group) tail withdrawal latencies at 12 weeks of age (PND 85-87). Latencies for female (A) and male (B) rats, treated with PG or Heroin during adolescence, across four temperatures (46°C, 48°C, 50°C, and 52°C). Withdrawal latencies for female and male animals, treated with PG or heroin during adolescence, at 46°C (C) and 50°C (D). A significant difference between adolescent drug groups (within sex) collapsed across temperature with #; a difference between adolescent drug groups (within sex) for a given temperature with *.*

$p < 0.05$). Follow-up post-hoc analysis of Temperature confirmed significant differences between all temperatures examined. Follow-up two-way ANOVAs were then performed at each level of Sex and Temperature to further parse effects. Within the females, a significant effect of Adolescent Drug ($F(1, 22) = 12.08$, $p < 0.01$; **Figure 1A**) was confirmed; females treated with heroin as adolescents exhibited longer latencies to withdraw compared with PG-treated females. A significant effect of Temperature ($F(3, 66) = 104.5$, $p < 0.0001$; **Figure 1A**) was additionally confirmed and the follow-up analysis confirmed significant differences between all temperatures. Within male animals, a significant effect of Temperature ($F(3, 60) = 121.7$, $p < 0.0001$; **Figure 1B**) was also confirmed and the post-hoc analysis further confirmed significant differences between all temperatures examined in male rats. No main effect of Adolescent Drug was confirmed for the male groups. When Adolescent Drug and Sex were considered at each level of Temperature, interactions of Adolescent Drug by Sex were confirmed at 46ºC ($F(1,42) = 8.474$, $p<0.01$; **Figure 1C**) and at 50ºC ($F(1,42) = 5.164$, $p<0.05$; **Figure 1D**). Post-hoc analysis of the interaction at 46ºC confirmed a difference between females treated with PG and those treated with heroin during adolescence. The post-hoc analysis did not confirm any differences between groups at 50ºC, however. The 15 second cutoff duration for warm-water tail submersion during testing was only reached by 3 PG female, 6 PG male, 8 heroin female and 2 heroin male rats in the 46°C condition.

The three-way analysis of tail-withdrawal latencies at the Time 2 measurement confirmed significant effects of Temperature ($F(2, 84) = 126.9$, $p < 0.0001$; **Figure 2A**) and Adolescent Drug ($F(1, 42) = 6.757$, $p < 0.05$; **Figure 2A**) but not of Sex (**Figures 2B and 2C**); no significant interactions of factors were confirmed. That is, heroin-treated animals exhibited significantly shorter latencies compared with PG-treated animals, collapsed across Temperature or Sex. The post-hoc analysis of the marginal means for Temperature confirmed

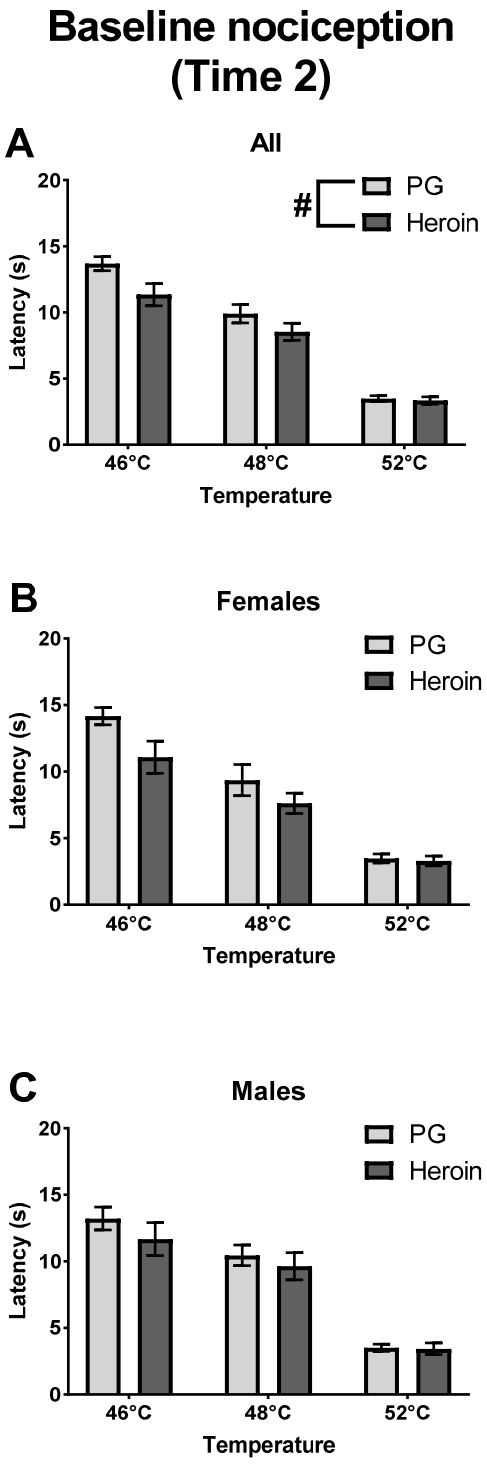

Figure 2. *Mean (±SEM; N=12 per group) tail-withdrawal latencies for female and male rats at the Time-2 measurement. Withdrawal latencies collapsed across sex (A) and separated into female (B) and male (C) groups. A significant difference between groups (collapsed across sex and temperature) is indicated with #.*

that latencies at 52°C were significantly lower compared with those at 46°C or 48°C and that latencies at 48°C were significantly lower compared with those at 46°C.

Heroin and naloxone challenge

Injection of heroin (0.5, 1.0 mg/kg, s.c.) slowed tail withdrawal latencies in the nociception test. The 3-way ANOVA confirmed significant effects of Treatment Condition ($F(2, 84) = 191.4$, $p<0.0001$), Sex ($F(1, 42) = 6.719$, $p<0.05$), and Adolescent Drug ($F(1, 42) = 16.07$, $p<0.001$) on withdrawal latency. There was also a significant effect of the interaction of Adolescent Drug with Treatment Condition ($F(2, 84) = 6.535$, $p<0.01$; **Figure 3**) confirmed. Animals treated with heroin during adolescence, collapsed across sex, exhibited significantly shorter latencies to withdraw the tail from 52°C water compared with PG-treated animals (**Figure 3A**), and each Treatment Condition significantly differed from

each other condition, when collapsed across Sex and Adolescent Drug. Female rats exhibited significantly shorter withdrawal latencies compared with male rats, collapsed across Adolescent Drug and Treatment Condition (**Figure 3B**). The interaction was followed up by collapsing across Sex and performing a 2-way ANOVA. The effects of Adolescent Drug (F (1, 44) = 14.74, p<0.001), Treatment Condition (F (2, 88) = 179.8, p<0.0001), and the interaction of Adolescent Drug and Treatment Condition (F (2, 88) = 6.360, p<0.01) were again confirmed. The post-hoc analysis confirmed significantly slower tail-withdrawal latencies for animals treated with heroin during adolescence, compared with PG-treated animals, within each of the 0.5 mg/kg and the 1.0 mg/kg dosing conditions (**Figure 3A**).

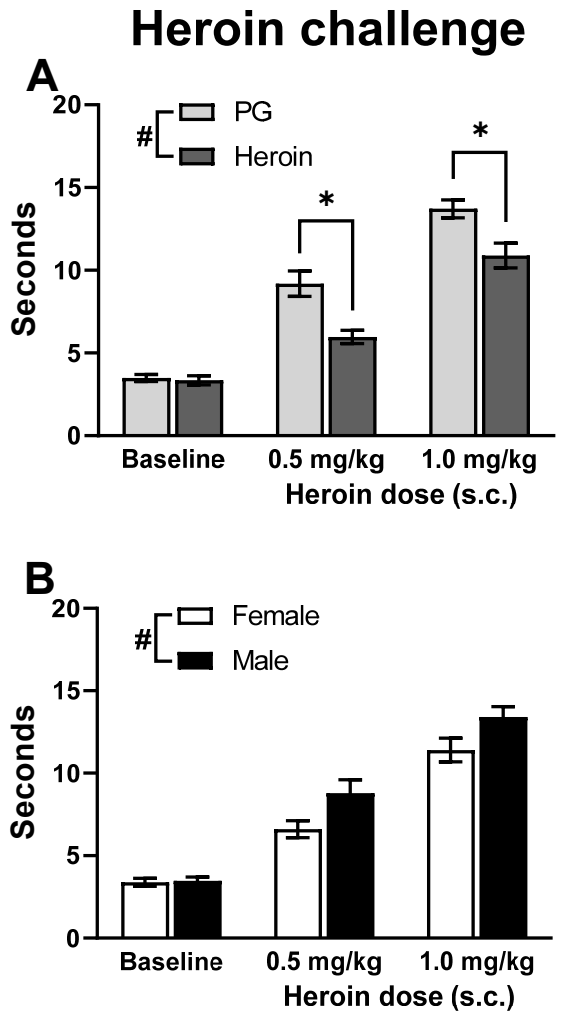

***Figure 3**. Mean (±SEM; N=24 per group) tail-withdrawal latency at 52°C under baseline or Heroin challenge (0.5, 1.0 mg/kg, s.c.) conditions collapsed across Sex (A) and Adolescent Drug (B), or at 48°C under baseline or naloxone challenge (0.3, 1.0 mg/kg, i.p.) conditions for Adolescent Drug. A significant difference between adolescent drug groups (within treatment condition is indicated with *; a difference between sexes with #; a difference between treatment conditions with §.*

A three-way analysis of latencies for tail-withdrawal from a 48° C water bath following naloxone injections confirmed a significant effect of Treatment Condition (F (2, 84) = 7.168, p<0.01; **Figure 4**). The post-hoc analysis confirmed a significant decrease in

latencies in animals following treatment with 0.3 or 1.0 mg/kg naloxone compared with baseline. Additionally, there was a significant effect of the interaction of Treatment Condition with Sex ($F(2, 84) = 3.423$, $p < 0.05$). Follow-up analysis of this interaction, by collapsing across Adolescent Drug, again confirmed significant effects of Treatment Condition ($F(2, 88) = 7.389$, $p < 0.01$) and of the interaction of Treatment Condition with Sex ($F(2,88) = 3.552$, $p < 0.5$) on withdrawal latencies. Post-hoc analysis of the interaction confirmed a significant difference between female and male rats at baseline which appeared to be driven by the C1 PG-exposed female group exhibiting faster withdrawal latencies than any other group at baseline (**SUPPLEMENTAL MATERIALS; Figure S2**). Latencies were also faster than those recorded for this group in the original evaluation at 48°C (**Figure 2B**), unlike other groups, potentially compromising any detection of hyperalgesic effects of naloxone. The reason for this is unknown. Additionally, the post-hoc analysis of the interaction of Treatment Condition with Sex confirmed a significant difference between each naloxone treatment condition and baseline in the male rats, but not the female rats. A full depiction of tail-withdrawal latencies by Group and Cohort can be found in the **SUPPLEMENTAL MATERIALS** (**Figure S2**); importantly, inclusion/exclusion of the C1 female groups has no differential impact on the interpretive outcome for this experiment.

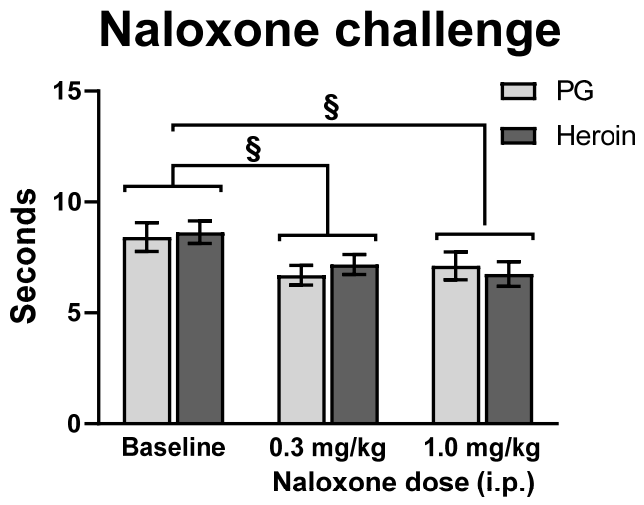

***Figure 4**. Mean (±SEM; N=24 per group) tail-withdrawal latency at 48°C under baseline or naloxone challenge (0.3, 1.0 mg/kg, i.p.) conditions for Adolescent Drug. A significant difference between treatment conditions is indicated with §.*

Anxiety-like behavior

Rats treated with heroin during adolescence spent significantly less time in the open arms of the elevated plus-maze compared with PG-treated animals, and males spent significantly less time in the open arms compared with female rats. The statistical analysis of time spent in the open arms of the elevated plus-maze confirmed significant effects of Adolescent Drug ($F(1, 41) = 5.632$, $p < 0.05$; **Figure 5A**) and of Sex ($F(1, 41) = 5.442$, $p < 0.05$; **Figure 5A**). Significant effects of Sex, but not Adolescent Drug, were also confirmed for mean speed (($F(1, 41) = 18.21$, $p < 0.0001$; **Figure 5B**), total distance traveled (($F(1, 41) = 18.52$, $p < 0.0001$; **Figure 5C**), and overall immobility **(**($F(1,41) = 49.56$, $p < 0.0001$; **Figure 5D)**. Female rats were significantly faster and traveled more distance compared with male rats, and male rats spent significantly more time immobile compared with female rats**.** No differences in open arm entries or time spent in the center were confirmed (not shown).

Separate 3-way analyses including Age/Cohort as a factor were performed for Time spent in the Open Arm zones, average Speed, total Distance traveled, number of Entries into the Open Arm zones, overall Immobility, and Time spent in the Center zone to assess the impact of conducting the elevated plus-maze test at different ages in the two cohorts (see S**UPPLEMENTAL MATERIALS**). A significant effect of Adolescent Drug was only confirmed for Time spent in the Open arms (see **SUPPLEMENTAL MATERIALS**; **Figure S3**). Testing the Cohorts at different ages did not have an impact on Adolescent Drug differences in the Open arms; there was no main effect of Age confirmed for Time spent in the Open Arms, nor any interaction of Age with Adolescent Drug or with Sex. However, there was a significant interaction of Age with Sex ($F(1, 37) = 19.23$, $p < 0.0001$). The post-hoc analysis confirmed that female rats tested at 32 weeks of age spent significantly *more* Time in the Open arms compared with 13-week old female rats whereas 32-week old male rats spent significantly *less*

Time in the Open arms compared with 13-week old male rats. Furthermore, 32-week old female rats spent significantly more Time in the Open arms compared with male rats at 32 weeks of age, but there were no significant differences between sexes at the 13-week assessment.

Open field behavior data are reported in the **SUPPLEMENTAL MATERIALS**.

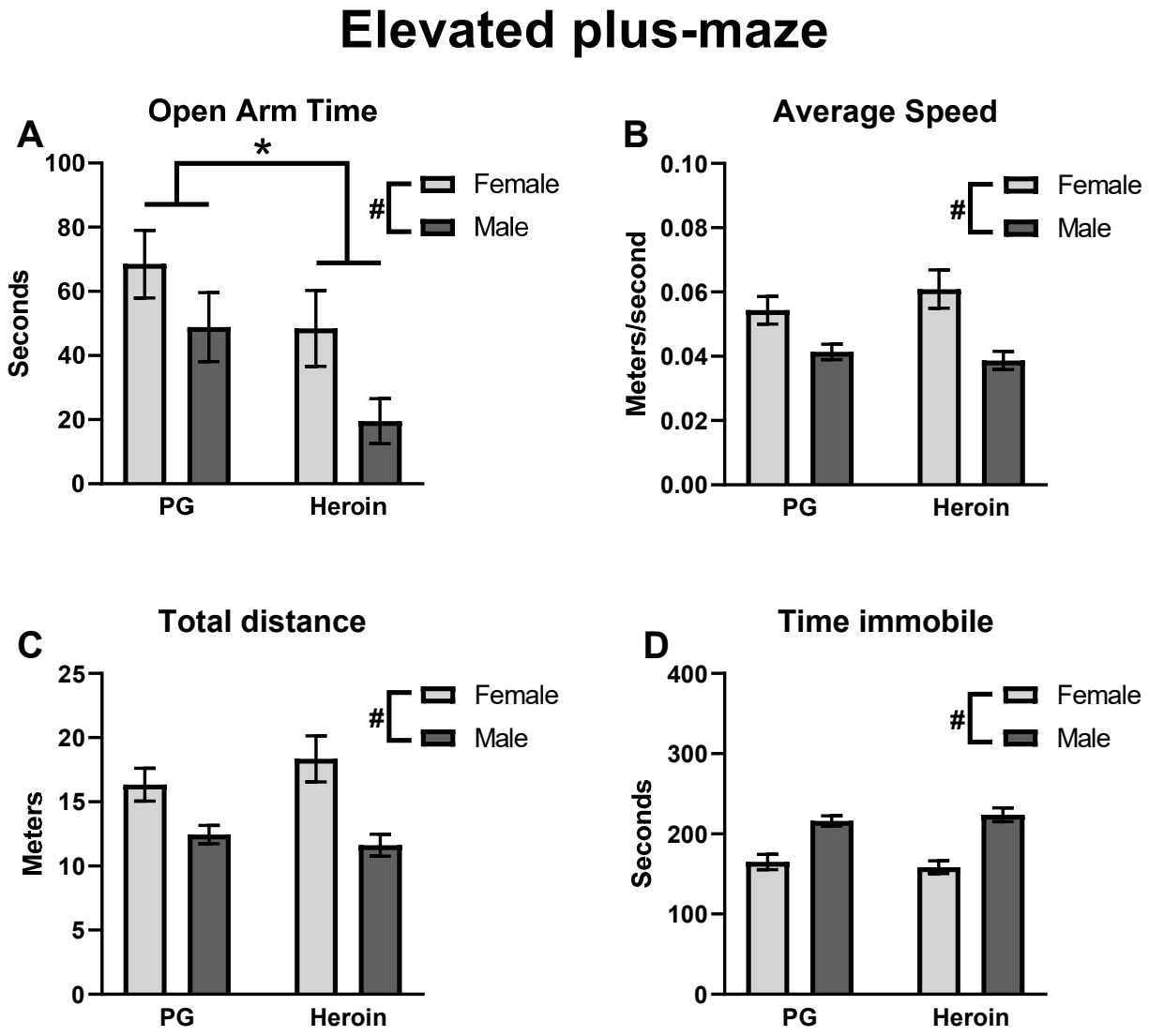

***Figure 5**. Mean (±SEM; N=12 per group) A) time spent in the Open Arms, B) speed of movement, C) distance traveled and C) time spent immobile in the elevated plus-maze. A significant difference between adolescent drug groups with *; a difference between sexes with #.*

DISCUSSION

Repeated exposure to heroin vapor during adolescence produced behavioral and physiological changes that lasted into adulthood in both female and male rats, in this study. The data confirmed adolescent heroin inhalation decreased sensitivity to thermal nociception in female rats upon reaching early adulthood, but increased sensitivity in both female and male rats when re-assessed at 23 and 29 weeks of age. This hyperalgesia was further confirmed by a reduction in anti-nociceptive efficacy of acute heroin injections in the groups exposed to heroin vapor. Long-term expression of anxiety-like behavior was also observed in the elevated plus-maze test in female and male rats exposed to heroin. This is the first study to report long-term hyperalgesia, decreased opioid anti-nociceptive efficacy, or anxiety-like behavior in adult animals exposed to opioids as adolescents where inhalation was used as the experimental route of drug administration. In humans, inhaled heroin produces more positively rated subjective effects compared with those produced by intravenous administration, despite lower C_{MAX} heroin levels [42]. While this may suggest that heroin inhalation is protective compared with intravenous administration, there are risks specific to route such as toxic leukoencephalopathy associated with “chasing the dragon” [18,43]. There are also pharmacokinetic differences between routes, such as an increase in the heroin metabolite morphine-6-glucuronide when heroin is inhaled [42]. The contributions of such differences to the lasting consequences of opioid exposure are presently not well understood, therefore the findings reported here provide information about how method of exposure can potentially impact outcome. Lasting, potentially life-long, increases in anxiety and a need for higher opioid doses for effective analgesia caused by adolescent opioid inhalation may be risk factors for developing later life opioid use disorders [44,45].

The present study found sex-specific effects of adolescent heroin exposure on baseline nociception at 12-weeks, but this shifted qualitatively by the time the second basal measurement was performed. An apparent h*ypo*algesia in the heroin-exposed female group changed to the *hyper*algesia present in the male group at both time points. Sex-specific analgesic effects of opioids have been documented in pre-clinical studies. For example, morphine produces greater acute anti-nociceptive effects and greater tolerance following chronic treatment in male rats [46]. Additionally, continuous opioid agonist infusion produces hyperalgesia faster and for a longer duration in female mice, but at higher doses persists equally and is prevented only in males by an NMDA antagonist [47]. The reasons for this interesting finding within the present study are not known and will require future studies to better understand the cause and implications of the qualitative shift in nociceptive sensitivity observed in heroin-exposed female rats from 12 weeks to 23/29 weeks of age. It may be the case, for example, that male rats would exhibit hypoalgesia if assessed at a timepoint closer to the adolescent heroin exposure.

Opioid tolerance and opioid-induced hypersensitivity to noxious stimuli are commonly reported adverse effects of repeated opioid treatment [48-54]. In the present study, animals exposed to heroin as adolescents displayed increased thermal sensitivity when assessed starting at 23 weeks of age, and a reduction of anti-nociceptive efficacy of acute heroin injections. It is not clear, however, if this was due to an apparent tolerance where increased N-Methyl-d-aspartate (NMDA) receptor mediated pronociceptive activity masked the anti-nociceptive effects of the injected heroin [55], or if this was due to persistent changes in opioid receptor function [54]. Interestingly, when injected with naloxone tail-withdrawal latencies were generally decreased, but there was no exacerbated response observed in the heroin-treated rats. Heightened endogenous mu-opioid anti-nociceptive activity, in response to heightened

pro-nociception [51], may therefore not necessarily be responsible for the behavioral observations. Determining the mechanisms responsible for the persistent nociceptive effects observed here is outside the scope of this study, however, and will require future studies to delineate the neurobiological substrates underlying the reported behaviors.

The relationship between substance abuse and anxiety disorders is a complex and cyclical one. The two often co-exist [56,57], anxiety disorders during adolescence increase the later risk of substance abuse disorders [58,59], and adolescent substance use increases the risk of anxiety disorders later in life [60,61]. Here we examined the effects of adolescent heroin vapor exposure on the expression of an anxiety-like behavioral phenotype in adult male and female rats, using an elevated plus-maze (EPM) approach. In contrast to a previous study reporting no alteration of anxiety-like behavior in an EPM test in adult rats repeatedly treated with morphine as adolescents [17], the present study found that adolescent heroin exposure decreased time spent in the open arms of the test apparatus; a decrease in time spent by an animal in the open areas of the EPM (or similar behavioral tests such as Elevated Zero Maze) is widely interpreted as an increase in anxiety-like behavior in rodents [62-64]. Importantly, the expression of anxiety-like behavior observed in this study extended from early to middle adulthood, long after the initial heroin exposure during adolescence. The impact of adolescent heroin was consistent across sex, despite the fact that male rats exhibited significantly higher overall immobility, lower average speed, and traveled less distance compared with female rats, irrespective of drug pre-treatment. Although no differences were confirmed in open field behavior between treatment groups, sex differences were also observed in the open field test in overall activity and in activity specific to the center region of the apparatus. These findings are in agreement with the well-documented sex-dependent differences in activity in rodents

[65,66] and serve as further validation of the EPM and Open Field methodology used in this study.

In summary, the findings of this study show that lasting behavioral and physiological changes are produced by repeated heroin vapor exposure during adolescence in rats. This included a demonstration that long-term changes in nociception persist deep into adulthood, a phenomenon that is not well-described in the literature. Additionally, these results suggest that sex, at least during early adulthood, may play a determinative role in the manner by which the nociceptive effects of repeated heroin exposure are expressed. The present study also shows that long-lasting anxiety-like effects are produced by repeated adolescent heroin exposure. Finally, these data show for the first time that heroin inhalation, a common route of administration for humans, is capable of inducing these persisting, possibly life-long, alterations in anxiety and nociception. This further validates use of EDDS technology for the study of the effects of inhaled opioid drugs in rodents. We conclude that adolescent heroin exposure may increase risk for later life development of opioid use disorder via reduced sensitivity to opioid analgesia and increased anxiety.

FUNDING AND DISCLOSURE

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AUTHOR CONTRIBUTIONS

AG and MAT conceptualized and designed the studies, conducted statistical analysis of data, and created figures. AG, KMC and ELH performed the research and conducted initial data analysis. AG wrote the initial draft of the paper. KMC, ELH, and MAT critically reviewed and / or revised draft version of the paper. All authors approved of the submitted version of the manuscript.

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