



Published in final edited form as:

Pharmacol Biochem Behav. 2023 March ; 224: 173535. doi:10.1016/j.pbb.2023.173535.

Opioid induces increased DNA damage in prefrontal cortex and nucleus accumbens

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Abstract

Opioid use disorder (OUD) is a chronic disease characterized by compulsive opioid taking and seeking, affecting millions of people worldwide. The high relapse rate is one of the biggest challenges in treating opioid addiction. However, the cellular and molecular mechanisms underlying relapse to opioid seeking are still unclear. Recent studies have shown that DNA damage and repair processes are implicated in a broad spectrum of neurodegenerative diseases as well as in substance use disorders. In the present study, we hypothesized that DNA damage is related to relapse to heroin seeking. To test our hypothesis, we aim to examine the overall DNA damage level in prefrontal cortex (PFC) and nucleus accumbens (NAc) after heroin exposure, as well as whether manipulating DNA damage levels can alter heroin seeking. First, we observed increased DNA damage in postmortem PFC and NAc tissues from OUD individuals compared to healthy controls. Next, we found significantly increased levels of DNA damage in the dorsomedial PFC (dmPFC) and NAc from mice that underwent heroin self-administration. Moreover, increased accumulation of DNA damage persisted after prolonged abstinence in mouse dmPFC, but not in NAc. This persistent DNA damage was ameliorated by the treatment of reactive oxygen species (ROS) scavenger *N*-acetylcysteine, along with attenuated heroin-seeking behavior. Furthermore, intra-PFC infusions of topotecan and etoposide during abstinence, which trigger DNA single-strand breaks and double-strand breaks respectively, potentiated heroin-seeking behavior. These findings provide direct evidence that OUD is associated with the accumulation of DNA damage in the brain (especially in the PFC), which may lead to opioid relapse.

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CRedit authorship contribution statement

YW: performed experiments, analyzed data, and drafted the manuscript. AS, GL, SY, and KH: performed experiments, and analyzed data. ZJW: designed experiments, supervised the project, and wrote the manuscript.

Declaration of competing interest

The authors declare no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.pbb.2023.173535>.

Keywords

Opioid use disorder; DNA damage; Prefrontal cortex; Nucleus accumbens; Heroin self-administration; Heroin seeking

1. Introduction

Substance use disorder is a chronic and relapsing neuropsychiatric disease characterized by compulsive drug seeking and use despite adverse consequences (Becker and Hu, 2008; Leshner, 1997). Opioid use disorder (OUD) is one of the common substance use disorders that leads to a large socioeconomic burden (Goldstein and Volkow, 2011). However, there are only limited medications for the treatment of OUD, including buprenorphine, methadone, and naltrexone, which are ineffective in promoting abstinence and preventing relapse. Studies have shown that persistent molecular and epigenetic changes during prolonged abstinence contribute to long-lasting cellular and behavioral maladaptation, which eventually leads to relapse vulnerability (Koob and Le Moal, 2001; Koob and Volkow, 2016). Therefore, it is critical to further understand the neurobiology of OUD for the development of novel therapeutics.

The maintenance of genomic integrity is the prerequisite for normal gene expression and cellular function. In the brain, the major endogenous resources for DNA damage include reactive oxidative stress resulting from metabolism and gene transcription (Beckman and Ames, 1997; Madabhushi et al., 2015). Cells in the brain have high metabolic and transcriptional demand, therefore are extremely vulnerable to DNA damage, which includes modified bases, DNA adducts, DNA strand crosslinks, and single- or double-strand breaks (Jackson and Bartek, 2009). These types of DNA damage can cause changes in chromatin structure and function, which will disrupt normal gene expression (Caldecott, 2008; Price and D'Andrea, 2013). Recent studies have highlighted the central role of DNA damage and repair in brain diseases, especially in neurodegenerative diseases (Lu et al., 2004; Madabhushi et al., 2014) and psychiatric disorders (Andreazza et al., 2007; Andreazza et al., 2008; Markkanen et al., 2016).

Moreover, both clinical and preclinical studies have shown that DNA damage is implicated in substance use disorders. It is well known that chronic alcohol abuse can induce extensive DNA damage and genomic instability (Kruman et al., 2012). Other drugs also induce DNA damage. For example, both acute and repeated cocaine administration increased DNA damage in several brain regions in female rats, including the prefrontal cortex (PFC) (de Souza et al., 2014). Moreover, nicotine induced a significant dose-dependent increase in DNA migration in parotid gland single-cells (Ginzkey et al., 2009). Importantly, an increase in the number of chromosome breaks is noted in blood samples from opioid users (Shafer et al., 1990); and a recent RNA sequencing study using postmortem brain tissue from OUD subjects and healthy controls showed that the overlapping differentially expressed genes induced by opioids in PFC and nucleus accumbens (NAc) are enriched in DNA damage and repair related pathways, such as regulation of DNA damage checkpoint (Seney et al., 2021). These studies strongly suggest that DNA damage is linked to substance use disorder.

However, direct evidence for the role of DNA damage in OUD is still needed. Therefore, the hypothesis of this study is that DNA damage is directly related to OUD. We aim to investigate the DNA damage levels in PFC and NAc after heroin exposure, and elucidate whether manipulating DNA damage levels can change heroin-seeking behavior.

Here we reported that DNA damage is increased in the postmortem PFC and NAc tissue from subjects with OUD. We also found that DNA damage is elevated in the dorsomedial PFC (dmPFC) and NAc of mice that underwent heroin self-administration (SA). Moreover, the increased DNA damage in dmPFC persists after prolonged abstinence. Administration of *N*-acetylcysteine, an antioxidant that reduces DNA damage (Malins et al., 2002), ameliorates the accumulation of DNA damage in dmPFC during prolonged abstinence and attenuates heroin seeking. Furthermore, microinjection of DNA breaks-inducing agent topotecan or etoposide in the dmPFC during abstinence potentiates heroin seeking. Our data directly link DNA damage in the brain to opioid addiction, which provides novel insight into the understanding of the neurobiology of OUD.

2. Methods

2.1. Animals

The current study used both male and female C57BL/6J mice (about half males and half females were used in the current study. The exact number for males and females used in each experiment was provided in figure legend). Animals were housed under the temperature and humidity controlled by animal care facility with modified 12 h light/dark cycle (lights on at 11:00 A.M and light off at 11:00 P.M.). All the procedures were approved by the Institutional Animal Care and Use Committee, University of Kansas. All animals were maintained according to the National Institutes of Health guidelines in Association for Assessment and Accreditation of Laboratory Animal Care accredited facilities.

2.2. Human postmortem tissues

Frozen human postmortem tissues (Brodmann's Area 9 and nucleus accumbens) from 7 OUD subjects and 6 age matched healthy controls were obtained from the NIH NeuroBioBank. Detailed information about these subjects is included in Suppl. Table 1. Upon arrival, tissue was stored in a -80°C freezer until used for comet assay.

2.3. Drug

Heroin hydrochloride, generously gifted from the NIDA drug supply program, was dissolved in sterile saline. Heroin solutions were prepared on a weekly basis (0.1 mg/mL). Pump durations were adjusted according to the animals' body weights on a daily basis to ensure delivery of the correct dose of drug for each animal. *N*-acetylcysteine (Tocris), topotecan (Tocris) and etoposide (EMD Millipore) were prepared by dissolving in sterile saline or DMSO stock, and then diluted with sterile saline before use.

2.4. Comet assay

Comet assay was performed based on previous publications (Olive and Banath, 2006) with modifications. Frozen human postmortem PFC and NAc tissues (from OUD subjects and

healthy control, Fig. 1) or dmPFC and NAc tissue punches (from mice underwent heroin or saline self-administration [see below for methods] followed by 1-day or 14-day forced abstinence, Fig. 2 and Fig. 3) were homogenized on ice in 0.5 mL cold PBS using a glass homogenizer. After homogenization, cells were collected through a 40 μ m cell strainer and diluted to 1×10^5 cells/mL with cold PBS. Diluted tissue cells or Alkaline Control Cells (R&D Systems, Catalog # 4256-010-CC, CometAssay Alkaline Control Cells consist of a healthy population (CC0) and cells treated with increasing doses of Etoposide in populations CC1 (low damage), CC2 (intermediate damage), and CC3 (high damage)) were mixed with 37 °C molten LMAgarose (Trevigen, 4250-050-02) at a ratio of 1:10 (v/v), then 50 μ L of the mixture was immediately pipetted onto each well of a CometSlide (Trevigen, 4250-050-03). CometSlides were then placed flat at 4 °C in the dark for 30 min, and then immersed in pre-chilled lysis solution (Trevigen, 4250-050-01) with 10 % dimethyl sulfoxide (DMSO, Chem-Cruz, sc-202581) overnight at 4 °C in the dark. After overnight lysis, slides were immersed in freshly prepared cold alkaline unwinding solution (300 mM NaOH, 1 mM EDTA) for 1 h in dark at 4 °C. Slides were then subjected to electrophoresis in 850 mL cold alkaline electrophoresis solution (200 mM NaOH, 1 mM EDTA) using CometAssay[®] Electrophoresis System II (Trevigen, 4250-050-ES) for 21 V 30 min at 4 °C. After electrophoresis, slides were washed with cold neutralization buffer (0.4 M Tris-HCl, pH 7.4) for 15 min twice at 4 °C in the dark, and then subjected into 70 % ethanol at room temperature (RT) for 30 min. After washing, slides were dried at 37 °C for 30 min to bring cells in a single plane to facilitate observation and stained with 100 μ L diluted SYBR Gold stock (Invitrogen, S11494) for 30 min at RT in dark. After 30 min, slides were rinsed briefly in ddH₂O and allowed to completely dry at 37 °C. Slides were imaged using Leica DM6-B Microscope (Figs. 1A and D, 2A and D) and Nikon Ti2 Microscope (Figs. 2G and J, 3A and D) with a 10 \times objective. Comet images were analyzed using ImageJ plugin OpenComet with default settings. DNA damage was quantified as the % tail DNA (tail DNA content as a percentage of comet DNA content), tail moment (tail length \times %tail DNA), and tail DNA (sum of pixel intensities inside the tail). 1–2 images were taken for each slide from non-overlapping regions.

2.5. Self-administration test chambers

The experimental chambers have been described elsewhere (Gancarz et al., 2015; Singh et al., 2022; Wang et al., 2017; Wang et al., 2016) with modifications. Briefly, 16 standard Med Associates Inc. (St. Albans, VT) chambers containing two nose-poke holes each with infrared monitoring were used. Two stimulus lights were mounted within each nose-poke hole, with a house light in the center back wall of the test chamber. All chambers were housed in sound-attenuating boxes and controlled through a Med Associates interface.

2.6. Jugular catheterization surgery

Mice were anesthetized with 100 mg/kg ketamine and 5 mg/kg xylazine and then implanted with chronic indwelling jugular catheters as previously described (Wang et al., 2017; Wang et al., 2016) with modifications. The catheter was inserted into the right jugular vein and sutured up in place. The catheter was threaded subcutaneously over the shoulder blade and was connected to the harness (Instech). Following surgery, catheters were flushed daily with 0.05 mL of heparinized saline to preserve catheter patency. At the beginning of behavioral

testing, each animal received an i.v. infusion of ketamine hydrochloride (1 mg/mL in 0.05 mL), and the behavioral response was observed to verify catheter patency. Loss of muscle tone and righting reflexes served as behavioral indicators of patency.

2.7. Self-administration

All SA experiments occurred in the standard SA test chambers described above. Before drug SA, all mice underwent 3 days of water training (one session per day, with 12–16 h water restriction prior to training). During water training, the mice were trained to make nose poke responses to get water delivery directly in the active hole (~20 μ L). The fixed ratio (FR) for water training increased from fixed-ratio 1 (FR1) to FR3 across the 3 days. Mice were given 20 min water access after water training session. One day after water training, the mice began heroin or saline SA as previously described (Gancarz et al., 2015; Martin et al., 2018; Singh et al., 2022; Wang et al., 2017; Wang et al., 2016) with modifications. Mice were subjected to daily 3 h SA training, during which responses to the active alternative snout-poke hole resulted in i.v. injections of heroin (0.05 mg/kg/infusion) or saline (as control) according to a FR1 schedule of reinforcement, which was increased daily to FR3 and maintained at FR3 for the remaining SA protocol. Infusions were accompanied by a 5 s illumination of the stimulus light inside the active snout-poke hole followed by a 10 s time-out period, during which time the house light was extinguished. Responses to the inactive hole resulted in no programmed consequences. The following criteria for acquisition of operant responding were adopted from publications (Martin-Garcia et al., 2011; Soria et al., 2008; Wilkerson et al., 2017): mice will be included if they maintain stable responding with 1) <30 % deviation from the mean of the total number of infusions earned in the last three consecutive sessions, 2) at least 65 % responding on the reinforced nose-poke, and 3) a minimum of 5 reinforcers per session.

Following heroin or saline SA, one batch of animals were subjected to 1 day or 14 days of forced abstinence, then dmPFC and NAc tissues were collected for comet assay (Fig. 2). Other batches of animals were subjected to 14 days of abstinence and received treatment with *N*-acetylcysteine (Figs. 3 and 4), topotecan (Fig. 5) or etoposide (Fig. 5) for the study of DNA damage level (comet assay) or drug-seeking behavior (drug-seeking test).

2.8. Drug-seeking test

After saline or heroin acquisition, the mice underwent forced abstinence for 14 days (a time period showed high extinction responding for heroin (Shalev et al., 2001)), which mimics the real-life situation in which environmental cues precipitate relapse behavior following an extended period of abstinence. Then mice were placed back in the same chambers (context) for a 1 h context- and cue-induced drug-seeking test (thereafter referred to drug-seeking test). During this test, the active responses produced discrete cues previously paired with drug delivery, but the drug (saline or heroin) was not available. Therefore, this active response-produced event during drug-seeking test was termed as attempted infusions.

2.9. Locomotor activity

Mice locomotor activities were tested for 15 min in an open field transparent plastic apparatus (70 \times 50 \times 35 cm). At the beginning of the test, a mouse was placed gently

in a corner square with its head facing the corner. The activity was monitored automatically through a video surveillance system (iSpy, Western Australia, Australia). The total number of lines crossed cover in 15 min was recorded.

2.10. Systemic drug administration

After the last session of saline or heroin SA, mice were counterbalanced based on the averaged SA performance for the last 4 sessions and assigned to receive intraperitoneal injections of *N*-acetylcysteine (100 mg/kg, once per day (Reichel et al., 2011)) or vehicle (sterile saline) for 13 consecutive days, which yielded four groups of animals: saline-*N*-acetylcysteine, saline-vehicle, heroin-*N*-acetylcysteine and heroin-vehicle. 24 h after the last injection (i.e., on the 14th day of abstinence), one batch of the four-group animals were sacrificed for dmPFC and NAc tissue collection for comet assay (Fig. 3); another batch of the four-group animals was subjected a to drug-seeking test.

2.11. Cannula implantation

After heroin SA, mice were subjected to 14 days of forced abstinence. During this time, mice received cannula implantation. Mice were deeply anesthetized with 100 mg/kg ketamine as well as 5 mg/kg xylazine and placed on a stereotaxic apparatus (RWD instruments, CA, USA). Body temperature was maintained with a heating pad throughout the surgery. Bilateral holes were made in the skull for the implantation of the double guide cannula (with a dummy) directed on top of the dmPFC (AP: +2.0, ML: \pm 0.3, DV: -1.7) (sFig. 6E). The guide cannula was fixed with dental cement. Animals were allowed to recover from surgery for 7 days.

2.12. Intra-PFC drugs infusion

On the last 3 days of abstinence from heroin SA, the cannula cap was removed from the animal's head, and a microinjector with 0.5 mm projection beyond the guide cannula tip aiming at dmPFC (AP: +2.0, ML: \pm 0.3, DV: -2.2) was used for microinjection. Animals were counterbalanced based on the averaged SA performance for the last 4 sessions and assigned to receive intra-PFC infusion of topotecan (1 μ mol/L) (Uckun et al., 1995) or vehicle (1 μ L/hemisphere) for testing the effect of increased DNA single-strand breaks on drug-seeking behavior. Similarly, another batch of animals received intra-PFC infusion of etoposide (1 μ mol/L) (Laurie et al., 2005) or vehicle (1 μ L/hemisphere) for testing the effect of increased DNA double-strand breaks on drug-seeking behavior. An infusion pump (KD Scientific, MA, USA) with a flow rate of 50 nL/min was used for microinjections. The microinjector was kept in place for two extra minutes at the end of the microinjection to allow diffusion. Drug-seeking test was performed 24 h after the last infusion.

2.13. Statistical analysis

Multi-factor ANOVA was performed on the number of infusions and nose-poke responses during acquisition for heroin SA followed by Tukey's post hoc comparisons. Other comparisons of dependent variables with 4 groups (2 factors) were analyzed with two-way ANOVA followed by Tukey's post hoc comparisons. Comparisons between two groups were analyzed with unpaired student *t*-test. All data were analyzed using SPSS software (IBM

Corp., Armonk, NY, USA) or Graphpad (GraphPad Software, San Diego, CA), and were represented as the mean \pm SEM, with $p < 0.05$ indicating significance. The number of animals in each group was provided in figure legends. Statistical details were provided in Suppl. Table 2.

3. Results

3.1. DNA damage is increased in postmortem PFC and NAc tissue from OUD subjects

To determine whether prolonged opioid exposure alters the DNA integrity in the brain, we performed comet assay with the PFC tissue (Brodmann's area 9) and NAc tissue from OUD individuals vs age-matched control subjects (sTable 1). We used alkaline denaturing method which allows the detection of the migration of global DNA lesions resulted from single and double strand breaks, alkali-labile sites and DNA cross link (Fairbairn et al., 1995). Among the different parameters for comet assay, we used comet moment, which is the product of the amount of DNA in the tail and the mean distance of migration in the tail. This is considered as the most informative feature of the comet image (Olive et al., 1990). In addition, we included the measurement of %tail DNA, which is also an indicator for DNA integrity (Kumaravel and Jha, 2006). Using these measurements, we first validated our experimental settings by running control cells with or without DNA damage. We found that tail moment ($F_{3, 187} = 38.35, p < 0.0001$, one-way ANOVA), %tail DNA ($F_{3, 187} = 31.88, p < 0.0001$, one-way ANOVA) and tail DNA ($F_{3, 187} = 24.03, p < 0.0001$, one-way ANOVA) analysis of damaged CC1, CC2 and CC3 cells were all increased compared to undamaged CC0 cells, and these increases were proportional to the damage levels of the cells (sFig. 1).

Using this validated method, we started to examine the changes in DNA damage in postmortem brain tissues from OUD subjects and healthy controls. We found that the tail moment was significantly increased in the postmortem PFC tissue (Fig. 1A–B, $p < 0.01$, t -test) as well as in the postmortem NAc tissue (Fig. 1D–E, $p < 0.05$, t -test) from OUD subjects compared to controls ($n = 24$ – 33 slides from 6 to 7 subjects). Similarly, %tail DNA was also increased in the OUD postmortem PFC (Fig. 1C, $p < 0.01$, t -test). Although we noticed a significantly increased tail DNA in the NAc tissue (sFig. 2B, $p < 0.05$, t -test) from OUD subjects compared to controls, there is no significant increase of %tail DNA (Fig. 1F, $p = 0.3234$, t -test). These data suggest that opioid exposure induces increased DNA damage in postmortem human brain.

3.2. DNA damage is increased in the dmPFC and NAc from mice that underwent heroin self-administration

Next, we sought to determine the temporal profile of opioid-induced changes in DNA damage. To do so, we used a mouse heroin SA model. Using multi-factor repeated ANOVA analysis, we found a main effect of drug (heroin/saline) ($F_{1, 21}(\text{drug}) = 77.478, p < 0.001$) and a significant interaction between heroin and saline for the infusion numbers (sFig. 3A), suggesting that mice self-administered significantly more heroin than saline. Moreover, the total active responses were increased over the course of the 10 training sessions (sFig. 3B, $F_{1, 21}(\text{drug}) = 75.494, p < 0.001$), indicating the increased behavioral responding for heroin.

Additionally, there were no changes in the total inactive responses (sFig. 3C, $F_{1, 21}(\text{drug}) = 3.404$, $p = 0.079$). These data suggest that these mice acquired heroin SA.

To examine whether DNA damage is altered following heroin SA, mice were counterbalanced based on their behavioral performance (i.e., number of infusions earned during the last 4 sessions of SA) and assigned to 1-day or 14-day abstinence group. After abstinence, brain tissue punches containing dmPFC and NAc were collected for comet assay studies. We found that after 1 day of abstinence (1DA), the tail moment (Fig. 2A–B, $p < 0.0001$, t -test) and %tail DNA (Fig. 2C, $p < 0.0001$, t -test) were obviously increased in the dmPFC tissue from mice underwent heroin SA compared to saline controls. Moreover, compared to saline controls, there was a significant increase in tail moment (Fig. 2D–E, $p < 0.0001$, t -test) and %tail DNA (Fig. 2F, $p < 0.0001$, t -test) in the NAc from animals with a history of heroin SA followed by 1-day abstinence. Additionally, we found that the tail moment (Fig. 2G–H, $p < 0.0001$, t -test) and %tail DNA (Fig. 2I, $p < 0.0001$, t -test) was increased in the dmPFC of mice that underwent 14-day abstinence (14DA) following heroin SA. In contrast, no changes in tail moment (Fig. 2J–K, $p = 0.2051$, t -test) and %tail DNA (Fig. 2L, $p = 0.0953$, t -test) were found in the NAc from animals with a history of heroin SA followed by 14-day abstinence. Collectively, these data suggest that DNA damage in dmPFC and NAc is increased after heroin exposure, and the damage is persistent in dmPFC but not in NAc after prolonged abstinence from heroin SA.

3.3. Antioxidant treatment during abstinence ameliorates DNA damage in dmPFC and attenuates heroin seeking

Persistent molecular changes during abstinence contribute to relapse vulnerability (Koob and Le Moal, 2001; Koob and Volkow, 2016). DNA damage can directly affect gene expression (Lu et al., 2004). Therefore, we next asked if alleviating the DNA damage burden during abstinence (following heroin SA) can affect drug-seeking behavior. It is well-recognized that the major source of DNA damage in the brain is free radicals from metabolic processes (Dizdaroglu et al., 2002; Halliwell, 1992). *N*-acetylcysteine is a powerful antioxidant prodrug to L-cysteine and can replenish intracellular level of antioxidant glutathione (Atkuri et al., 2007). Therefore, mice were first subjected to heroin or saline SA, then were counterbalanced based on their last 4 days of SA performance and assigned to receive intraperitoneal injections of *N*-acetylcysteine-(100 mg/kg, once per day for 13 days (Zhou and Kalivas, 2008)) or vehicle (sterile saline, sFig. 4A–C). Then we tested if persistent DNA damage in dmPFC will be alleviated. We found that there was a significant main effect of drug (heroin/saline, $F_{1, 207}(\text{drug}) = 158.1$, $p < 0.0001$) and treatment (*N*-acetylcysteine/vehicle, $F_{1, 207}(\text{treatment}) = 28.22$, $p < 0.0001$), and a significant interaction between drug and treatment ($F_{1, 207}(\text{interaction}) = 18.42$, $p < 0.0001$) for tail moment in the dmPFC (Fig. 3A–B). Similar pattern was found in %tail DNA in the dmPFC (Fig. 3C): there was a significant main effect of drug ($F_{1, 207}(\text{drug}) = 216.5$, $p < 0.0001$) and treatment ($F_{1, 207}(\text{treatment}) = 7.447$, $p < 0.01$), and a significant interaction between drug and treatment ($F_{1, 207}(\text{interaction}) = 4.48$, $p < 0.05$). Post hoc analysis revealed that mice received *N*-acetylcysteine treatment during abstinence following heroin SA showed significantly lower tail moment ($p < 0.0001$) and %tail DNA ($p < 0.01$) in the dmPFC compared to vehicle controls (Fig. 3A–C). Moreover, *N*-acetylcysteine treatment in mice underwent saline SA did not change the tail

moment and %tail DNA in the dmPFC (Fig. 3A–C). In comparison, there was no significant drug or treatment effect in tail moment and % tail DNA in the NAc (Fig. 3D–F), which is consistent with our previous finding showing there is no increase of DNA damage in the NAc after 14-day abstinence following heroin SA (Fig. 2J–L).

To test if *N*-acetylcysteine treatment can attenuate heroin seeking, mice were subjected to saline or heroin SA and received *N*-acetylcysteine or vehicle treatment during abstinence as described above. Two-way ANOVA analysis revealed that there was no significant difference in infusion numbers (Fig. 4A, $F_{1, 30}(\text{treatment}) = 0.739, p = 0.397$) as well as in total active and inactive responses (sFig. 5) between mice treated with *N*-acetylcysteine and vehicle during saline or heroin acquisition, suggesting there was no pre-existing bias in saline or heroin intake. On the contrary, we found a significant treatment effect (*N*-acetylcysteine/vehicle, $F_{1, 30}(\text{treatment}) = 4.382, p < 0.05$) and a significant interaction between heroin and saline ($F_{1, 30}(\text{drug}) = 8.867, p < 0.01$) for the total active responses during drug-seeking test (Fig. 4B). Post hoc analysis showed that mice with a history of heroin SA and treated with *N*-acetylcysteine during abstinence had significantly lower total active responses (Fig. 4B) and attempted infusions (Fig. 4C) compared to vehicle controls (mice underwent heroin SA and received vehicle treatment). In addition, *N*-acetylcysteine did not change the total active responses in mice underwent saline SA (Fig. 4B). Importantly, there was no change in neither total inactive responses (Fig. 4D) nor locomotion activity (Fig. 4E) across all groups, suggesting that the changes in behavioral responses during drug-seeking test were not attributed to altered nose-poking behavior or baseline locomotion activity.

3.4. Intra-PFC infusion of topotecan and etoposide during abstinence potentiates heroin seeking

As *N*-acetylcysteine not only affects DNA damage but also influences many other cellular processes such as synaptic function (da Costa et al., 2017; Kathuria et al., 2019; Zhou and Kalivas, 2008), we next asked whether direct manipulation of DNA damage (e.g., DNA single strand or double strand breaks) in the dmPFC during abstinence can affect heroin-seeking behavior. To do so, we chose the agents topotecan (Uckun et al., 1995) and etoposide (Hande, 1998; Laurie et al., 2005), which can induce DNA single-strand breaks and double-strand breaks through inhibition of topoisomerase I and II, respectively. Mice were subjected to 10 days of heroin SA. During abstinence, mice were counterbalanced based on heroin acquisition performance during the last 4 sessions and assigned to receive topotecan or vehicle infusion into dmPFC at 1 $\mu\text{mol/L}$ (Uckun et al., 1995) once per day for 3 days starting from the 11th day of heroin abstinence. Drug-seeking test was performed on the 14th day of heroin abstinence (Fig. 5A). We found that there was no pre-existing difference in infusion numbers (Fig. 5A, $F_{1, 130}(\text{treatment}) = 0.6763, p = 0.4124$) as well as in total active and inactive responses (sFig. 6A–B) between topotecan and vehicle group during heroin acquisition. On the contrary, *t*-test showed that mice with a history of heroin SA and treated with topotecan had significantly higher total active responses (Fig. 5B, $p < 0.05$) and attempted infusions (Fig. 5C, $p < 0.05$) compared to vehicle controls. In addition, topotecan treatment did not alter the total inactive responses (Fig. 5D) and locomotion activity (Fig. 5E).

Similarly, etoposide or vehicle was infused into dmPFC at 1 $\mu\text{mol/L}$ for 3 days starting from the 11th day of heroin abstinence. Drug-seeking test was performed on the 14th day of heroin abstinence (Fig. 5F). We found that there was no significant difference in infusion numbers (Fig. 5F, $F_{1, 120}(\text{treatment}) = 0.6187$, $p = 0.4331$) as well as in total active and inactive responses (sFig. 6C–D) during heroin acquisition between mice treated with etoposide and vehicle. On the contrary, t -test showed that mice with a history of heroin SA and treated with etoposide had significantly higher attempted infusions (Fig. 5H, $p < 0.05$) and a trend toward increase in total active responses (Fig. 5G, $p = 0.0583$) compared to vehicle controls. In addition, etoposide treatment did not alter the total inactive responses (Fig. 5I) and locomotion activity (Fig. 5J), suggesting that the changes in behavioral responses during drug-seeking test were not credited to changes in movement.

4. Discussion

In the current study, we showed that opioid induces increased DNA damage in dmPFC and NAc, two of the key brain regions involved in drug addiction. Moreover, the increased DNA damage in dmPFC persisted during prolonged abstinence. Importantly, antioxidant *N*-acetylcysteine treatment during abstinence significantly reduced DNA damage in dmPFC and attenuated heroin-seeking behavior. Furthermore, directly introducing DNA breaks in dmPFC during abstinence potentiated heroin-seeking behavior. Together these findings provide direct evidence for the role of DNA damage in opioid addiction.

In this study, we used comet assay, also known as single cell gel electrophoresis (SCGE) assay, for DNA damage detection. It is a rapid, sensitive, and relatively simple method for detecting DNA damage in individual cells. Cells with different treatments were embedded in agarose microgels on microscope slides. Lysis solution was applied to the slides to lyse the cells and remove cellular proteins (McKelvey-Martin et al., 1993). The DNA was then allowed to unwind under alkaline condition for electrophoresis which allows the detection of the migration of global DNA lesions resulted from single and double strand breaks, alkali-labile sites and DNA cross link (Fairbairn et al., 1995). The broken DNA fragments or damaged DNA migrate away from the nucleus and form a comet-like appearance after staining with a DNA-specific fluorescent dye with the head being the intact DNA and the tail being damaged DNA fragments. Tail moment and %tail DNA were used as the major measurements in the current study to analyze DNA damage. As mentioned in Methods, the tail moment is defined as the product of the tail length and the fraction of total DNA in the tail (tail moment = tail length \times % of DNA in the tail) (Mozaffarieh et al., 2008), considering both the migration of the genetic material as well as the relative amount of DNA in the tail (Hellman et al., 1995). Our data suggested increased accumulation of DNA damage in the postmortem PFC tissues from human OUD subjects compared to healthy controls, with elevated tail moment and %tail DNA. Although the increase of %tail DNA (% of DNA in the tail) in the postmortem NAc tissue did not reach significance (Fig. 1F), we observed increased tail moment in the postmortem NAc from OUD brains compared to controls (Fig. 1E), suggesting a greater degree of the DNA migration (Møller et al., 2014). Taken together, our study indicates that opioids induce DNA damage in the PFC and NAc.

One factor accounting for increased DNA damage could be increased DNA damage resources. DNA is under constant attacks from both exogenous and endogenous sources. Exogenous DNA damage results from exposure to environmental, physical, and chemical agents, such as UV and ionizing radiation. Our current study focused on the endogenous DNA damage. One endogenous source for DNA damage in the brain is gene transcription. For example, neuronal activity changes involve the changes in transcription of immediate early genes. During this process, DNA double strand breaks are formed in order to facilitate the start of gene transcription (Madabhushi et al., 2015). Another major endogenous source for DNA damage in the brain is reactive oxygen species (ROS). The brain accounts for about 20 % of the body's total oxygen consumption (Raichle and Gusnard, 2002). ROS resulted from electron leaking out of the respiratory chain during oxidative phosphorylation in the mitochondria can damage macromolecules in cells, including DNA (Beckman and Ames, 1997). *N*-acetylcysteine is a powerful scavenger of free radicals by directly interacting with ROS such as OH• and H₂O₂ (Aruoma et al., 1989; Weyemi et al., 2018). The highly reactive hydroxyl radical even counts for 60 % of DNA damage induced by ionizing radiation (Vignard et al., 2013). In addition, *N*-acetylcysteine can also increase the intracellular level of glutathione (GSH), which is the key component for one of the endogenous antioxidant defense mechanisms glutathione peroxidase (Yu, 1994). Studies have shown that *N*-acetylcysteine can ameliorate DNA damage in peripheral tissue (Malins et al., 2002; Zafarullah et al., 2003). For example, 14 days supplement of *N*-acetylcysteine significantly reduced the mutagenic 8-hydroxyguanine in DNA from the hind leg of the BALB/c mouse (Malins et al., 2002). Our studies showed that systemic *N*-acetylcysteine treatment can alleviate heroin-induced persistent DNA damage in the brain (especially in dmPFC, Fig. 3). The amelioration of DNA damage can potentially recover DNA function (i.e., gene transcription) and recover cellular function, which eventually attenuates heroin seeking (Fig. 4).

It is noteworthy that other studies have shown that *N*-acetylcysteine treatment can attenuate drug-seeking behaviors (Baker et al., 2003; Madayag et al., 2007; Zhou and Kalivas, 2008). For example, Zhou et al. reported that *N*-acetylcysteine administration during extinction training reduced extinction-responding and inhibited cue- and heroin-induced reinstatement (Zhou and Kalivas, 2008). Baker et al. showed that systemic administration of *N*-acetylcysteine prevented the cocaine-primed reinstatement of drug seeking (Baker et al., 2003). Although these studies showed that *N*-acetylcysteine treatment can attenuate drug seeking by restoring the in vivo cystine/glutamate exchange and recovering the extracellular glutamate level in the NAc, it is possible that amelioration of DNA damage in the PFC and recovery of glutamate level in the NAc together contribute to the attenuation of drug seeking induced by *N*-acetylcysteine.

One limitation of the current study is that *N*-acetylcysteine does not merely exert its effect on DNA damage. The compound has a broad effect on the cellular function (Madayag et al., 2007; Rice, 2011; Zafarullah et al., 2003). Therefore, we cannot rule out the possibility that other molecular mechanisms are involved in *N*-acetylcysteine-induced changes in heroin-seeking behaviors. We did not correlate drug-seeking behavior with DNA damage by sacrificing the mice for comet assay after drug-seeking test. This is because drug-seeking test can induce neuronal activity increase in PFC and NAc (Singh et al., 2022), and neuronal

activity can directly induce DNA breaks (Madabhushi et al., 2015). Interestingly, topotecan (Uckun et al., 1995) and etoposide (Hande, 1998) can induce DNA damage (mainly breaks) through inhibition of topoisomerase I and II respectively, which is independent of oxidative stress. Since our studies provide direct evidence showing that opioid induces increased DNA damage in dmPFC and NAc, and that topotecan and etoposide infusion in dmPFC during abstinence can potentiate heroin seeking (Fig. 5), we argue that DNA damage is directly linked to the vulnerability for heroin relapse. However, we do not know what the genomic locations of heroin-induced DNA damage and how manipulations with topotecan and etoposide affect these heroin-induced DNA damage sites and subsequently gene expression, especially when treatment of topotecan (Mabb et al., 2014; Pearson et al., 2016) and etoposide (Madabhushi et al., 2015) can alter a board number of gene expression in cortical neurons. Therefore, future studies using techniques with genome-wide single-nucleotide-resolution are needed to map out drug-induced DNA damage sites as well as to examine whether direct manipulation of these DNA damage sites could affect gene expression and consequently drug seeking.

Another factor that contributes to increased DNA damage may be limited DNA repair. In the brain, single base DNA lesion (e.g., oxidation and alkylation) can be repaired through base excision repair (Krokan and Bjoras, 2013), DNA double helix distortion can be repaired through nucleotide excision repair (Marteijn et al., 2014), and DNA breaks can be mended by single strand break repair (Caldecott, 2008) and double strand break repair (Lieber, 2010). Interestingly, studies have shown that different brain regions have different capacity to repair DNA damage. For example, one of the most common DNA adducts 8-Hydroxy-2'-deoxyguanosine (oxo-8-dG) shows higher levels in regions like midbrain, caudate putamen and hippocampus compared to cerebellum, cortex and pons and medulla, suggesting the regional capacity to repair oxo-8-dG is different (Cardozo-Pelaez et al., 2000). Intriguingly, our data showed that during prolonged abstinence from heroin SA, DNA damage was ameliorated in NAc but not in dmPFC, implying that heroin abstinence alters the original DNA damage repair capacity especially in dmPFC. It is still unclear that whether the DNA damage in dmPFC observed after 14-day abstinence were originally occurred during early abstinence period (i.e., 1 day abstinence) or newly accumulated during prolong abstinence. This persistent or newly formed DNA damage in PFC may contribute to persistent epigenetic and transcriptional abnormality in PFC cells, which contribute to the maladaptation of PFC in addiction and lead to relapse (Goldstein and Volkow, 2011). Because of the brain region specificity of DNA damage repair, it is also interesting to explore whether other brain regions (e.g., ventromedial PFC, sometimes plays distinct roles in regulating drug seeking compared to dmPFC (Ball and Slane, 2012; Bossert et al., 2012; Capriles et al., 2003; Fuchs et al., 2005; McLaughlin and See, 2003; Peters et al., 2008)) have similar or different levels of DNA damage after opioid exposure.

In conclusion, our study shows that DNA damage is increased in postmortem PFC and NAc tissue from OUD patients as well as in dmPFC and NAc from mice that underwent heroin SA. The ROS scavenger *N*-acetylcysteine attenuates the accumulation of DNA damage after prolonged abstinence in mouse dmPFC. Moreover, intra-PFC topotecan and etoposide infusions that induce DNA breaks during abstinence potentiate heroin-seeking behavior. Both increased DNA damage sources and reduced DNA repair capacity may contribute to

the increased DNA damage. We not only show that opioids directly induce DNA damage in the brain, but also prove that manipulations alleviating or exacerbating DNA damage in the dmPFC can attenuate or potentiate heroin seeking, respectively. This study provides a novel substrate for understanding the neurobiology of opioid addiction. Future studies are needed to elucidate the exact role of DNA damage and repair processes in drug addiction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Adam Smith, Arim Hong, Julia Doderer, Anasuya Subramanian, Tyler Sarovich, Jeeshitha Pulkuri and Rebecca Pavot for their technical support. We thank NIH NeuroBioBank for providing human tissues in this study. ZJW is supported by NIH grant DA050908 and DA056804, University of Kansas start-up funding. This project was also supported by an Institutional Development Award (IDeA) from the National Institute of General Medical Sciences of the National Institutes of Health under grant number P20 GM103418. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of General Medical Sciences or the National Institutes of Health.

Data availability

Data will be made available on request.

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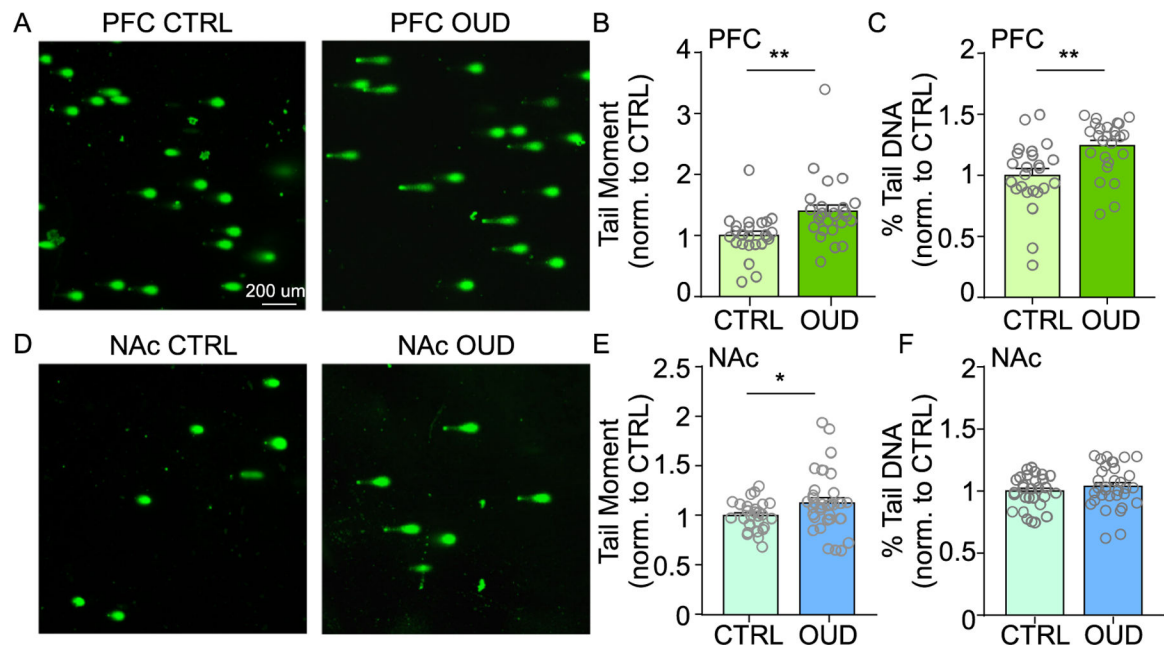


Fig. 1.

DNA damage was increased in postmortem PFC and NAc tissue from OUD subjects.

(A–C) Representative images (A) and quantification of comet assay (B–C) by measuring tail moment (B) and %tail DNA (C) using postmortem PFC homogenates from OUD and control subjects. (D–F) Representative images (D) and quantification of comet assay (E–F) by measuring Tail moment (E) and %Tail DNA (F) using postmortem NAc homogenates from OUD and control subjects. CTRL: healthy control, OUD: opioid use disorder, PFC: prefrontal cortex, NAc: nucleus accumbens. Comet assay was performed under alkaline conditions. Data are expressed as mean \pm SEM, $n = 24$ – 33 images from 6 to 7 subjects/group, * $p < 0.05$, ** $p < 0.01$.

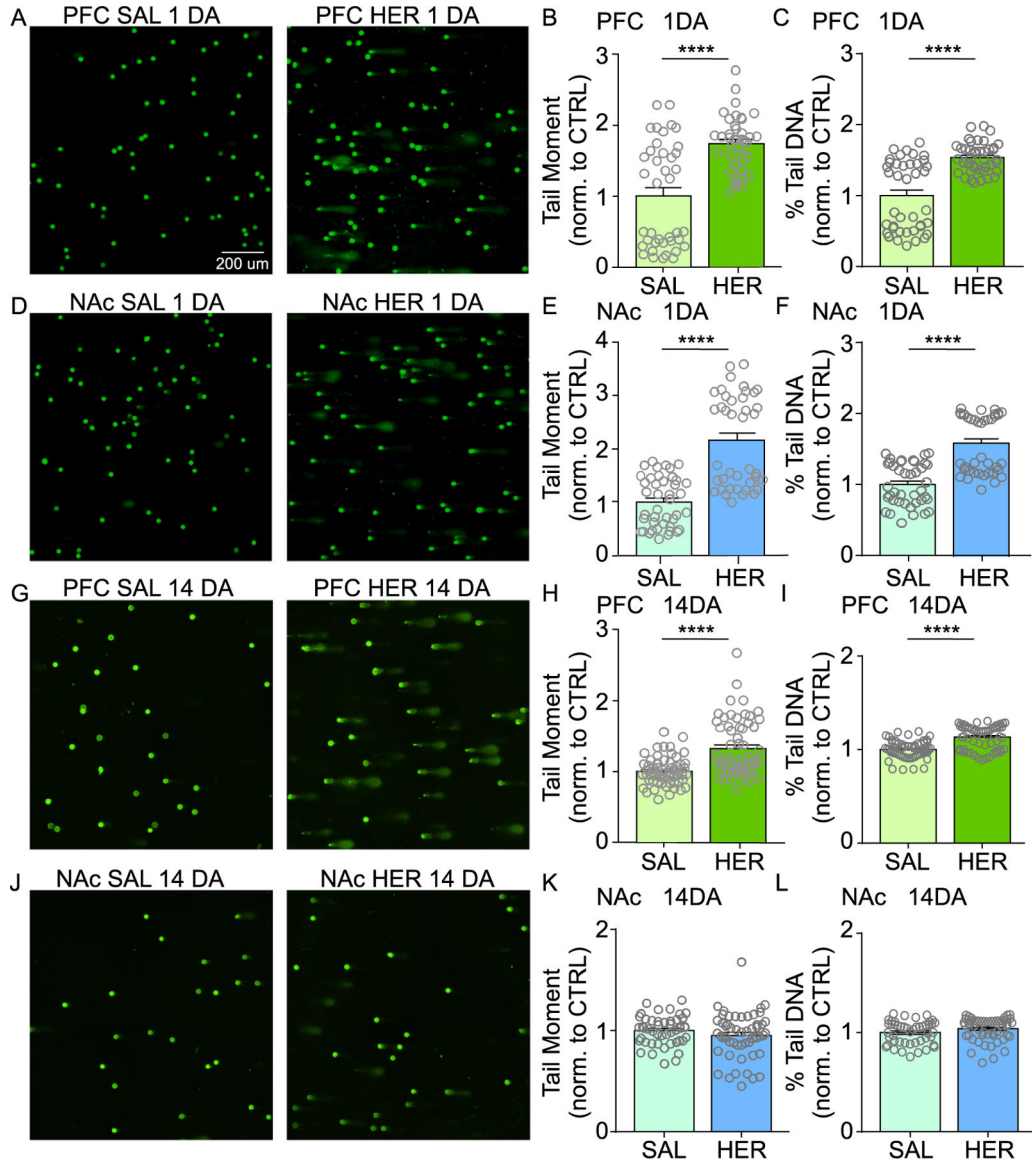


Fig. 2. DNA damage was increased in the dmPFC and NAc from C57BL/6J mice underwent heroin self-administration. (A–F) Representative images (A, D) and quantification of comet assay by measuring Tail moment (B, E) and %Tail DNA (C, F) using dmPFC (A–C) and NAc (D–F) tissues from mice underwent saline (SAL) or heroin (HER) SA followed by 1 day of abstinence (1 DA). $n = 40–41$ images from 3 mice/group (2 males, 1 female). (G–L) Representative images (G, J) and quantification of comet assay by measuring Tail moment (H, K) and %Tail DNA (I, L) using dmPFC (G–I) and NAc (J–L) tissues from mice underwent saline or heroin SA followed by 14 days of abstinence (14 DA). $n = 47–62$ images from 3 mice/group (2 males, 1 female). DA: day of abstinence, dmPFC: dorsomedial prefrontal cortex; NAc: nucleus accumbens. Comet assay was performed under alkaline conditions. Data are expressed as mean \pm SEM, **** $p < 0.0001$.

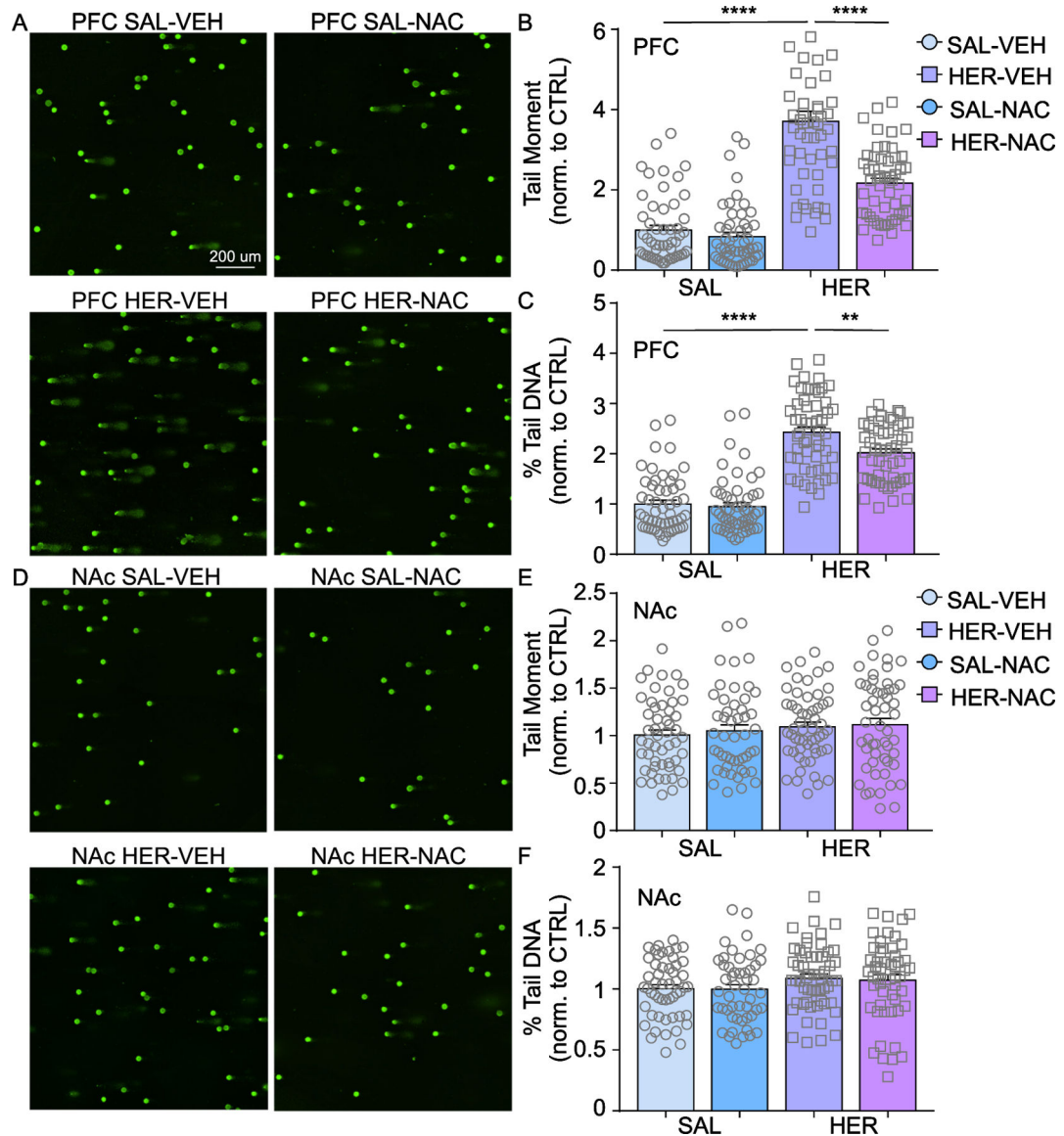


Fig. 3. Antioxidant treatment during abstinence ameliorated DNA damage in dmPFC from C57BL/6J mice. (A–C) Representative images (A) and quantification of comet assay by measuring tail moment (B) and % tail DNA (C) using dmPFC tissues from mice underwent saline or heroin SA and received vehicle (VEH) or *N*-acetylcysteine (NAC) treatment (100 mg/kg, i.p., once per day for 13 days) during abstinence. $n = 52$ – 53 images from 3 mice/group (1 male, 2 females). (D–F) Representative images (D) and quantification of comet assay by measuring tail moment (E) and % tail DNA (F) using NAc tissues from mice underwent saline or heroin SA and received vehicle (VEH) or *N*-acetylcysteine (NAC) treatment during abstinence. $n = 49$ – 55 images from 3 mice/group (1 male, 2 females). SAL-VEH: mice underwent saline SA and received vehicle i. p. injections. HER-VEH: mice underwent heroin SA and received vehicle i.p. injections. SAL-NAC: mice underwent saline SA and received *N*-acetylcysteine i.p. injections. HER-NAC: mice underwent heroin SA

and received *N*-acetylcysteine i.p. injections. Comet assay was performed under alkaline conditions. Data are expressed as mean \pm SEM, ** $p < 0.01$, **** $p < 0.0001$.

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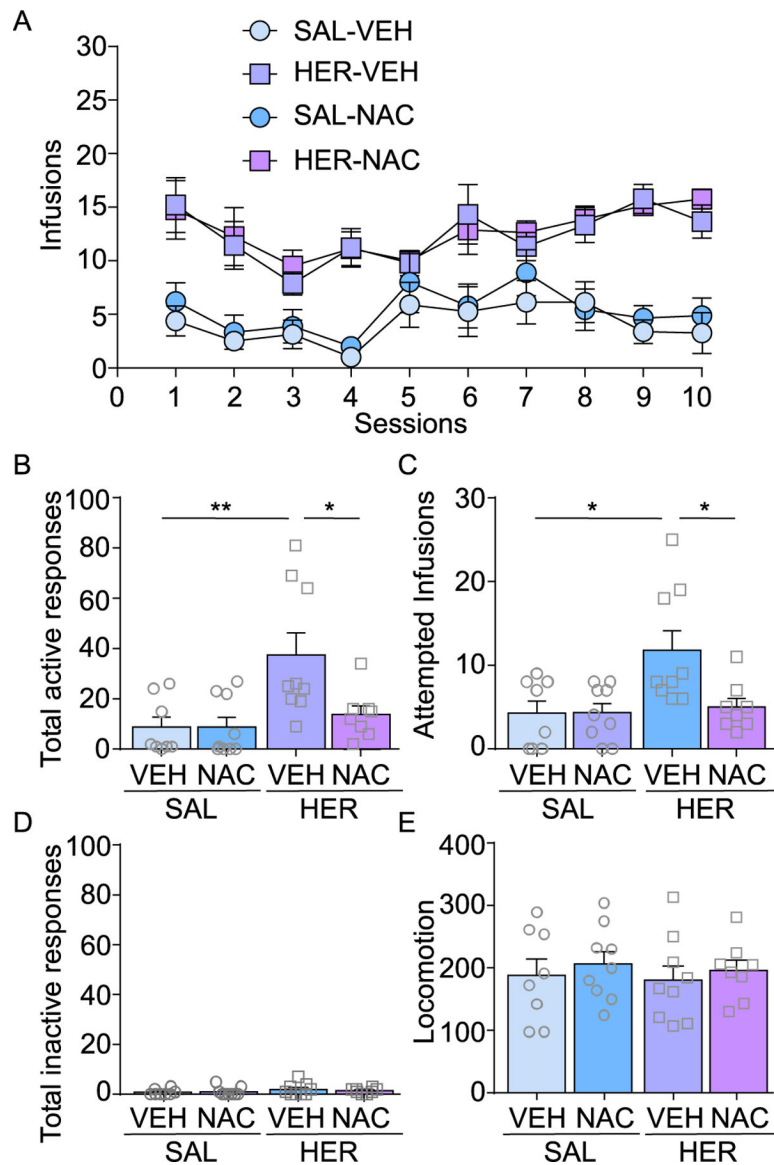
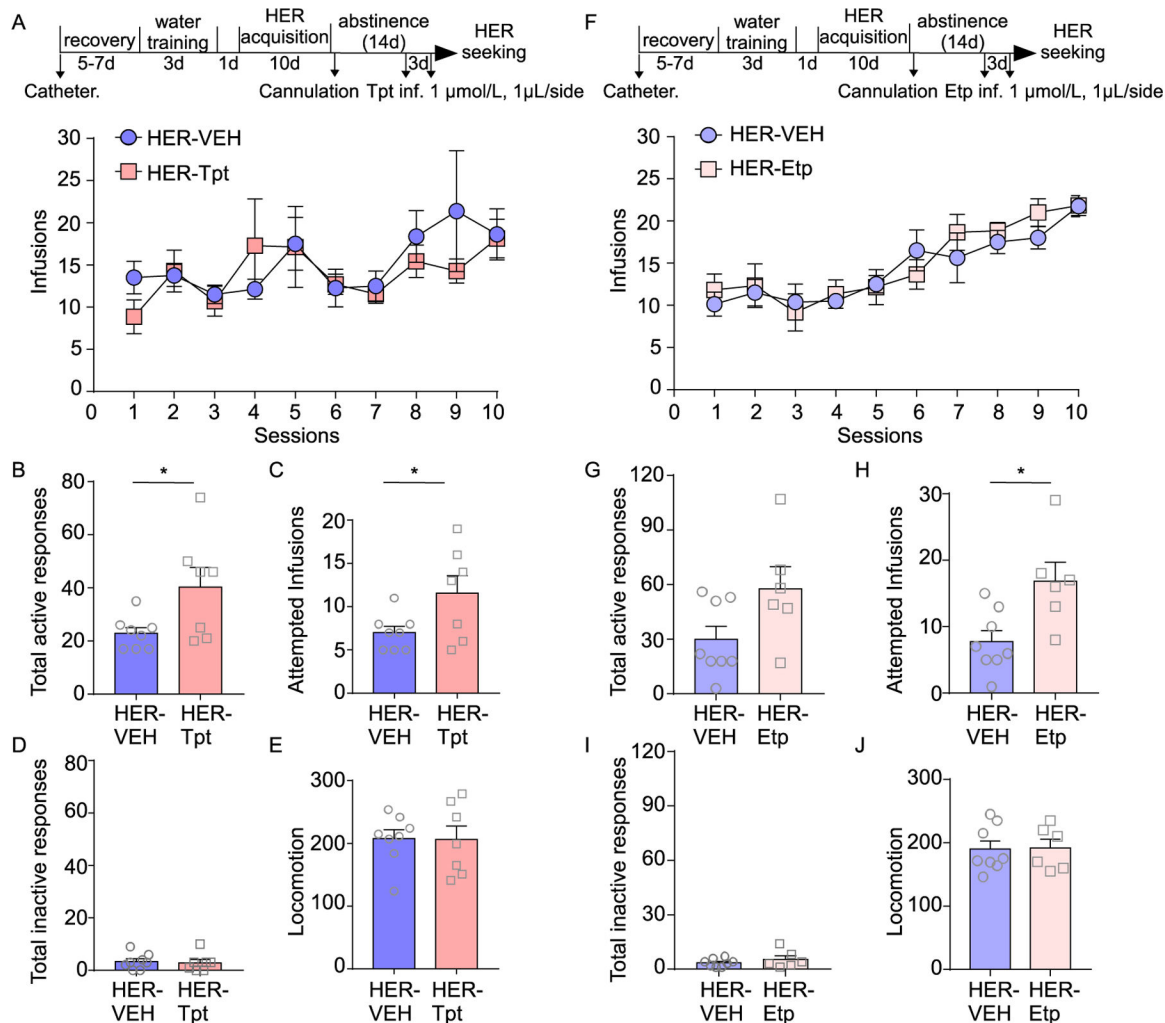


Fig. 4. Antioxidant treatment during abstinence attenuated heroin-seeking behavior in C57BL/6J mice. (A) Mean numbers of infusions per session during acquisition of SA for all groups including mice self-administered saline and received i.p. injection of vehicle (SAL-VEH) or *N*-acetylcysteine (100 mg/kg, SAL-NAC) and mice self-administered heroin and received i.p. injection of vehicle (HER-VEH) or *N*-acetylcysteine (HER-NAC). (B–D) Mean numbers of total active responses (B), attempted infusion (C) and total inactive responses (D) during drug-seeking test. (E) Mean numbers of line crossing during locomotion test. Data are expressed as mean \pm SEM, $n = 8$ – 9 /group (4–5 males, 4 females), * $p < 0.05$, ** $p < 0.01$.

**Fig. 5.**

Inducing DNA damage in dmPFC during heroin abstinence potentiated heroin seeking in C57BL/6J mice. (A) Timeline for the experimental design (upper). HER: Heroin, Tpt: Topotecan. Mean numbers of infusions per session during acquisition of heroin SA for mice received vehicle (HER-VEH) or topotecan (1 μmol/L, HER-Tpt) infusions in the dmPFC (1 μL/hemisphere) (lower). (B–D) Mean numbers of total active responses (B), attempted infusion (C) and total inactive responses (D) during heroin-seeking test. (E) Mean numbers of line crossing during locomotion test. $n = 7\text{--}8/\text{group}$ (3–4 males, 4 females). (F) Timeline for the experimental design (upper). HER: Heroin, Etp: etoposide. Mean numbers of infusions per session during heroin SA for mice received vehicle (HER-VEH) or etoposide (1 μmol/L, HER-Etp) in the dmPFC (1 μL/hemisphere) (lower). (G–I) Mean numbers of total active responses (G), attempted infusion (H) and total inactive responses (I) during heroin-seeking test. (J) Mean numbers of line crossing during locomotion test. $n = 6\text{--}8/\text{group}$ (3–4 males, 3–4 females). Data are expressed as mean \pm SEM, * $p < 0.05$.