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Adolescent nicotine exposure promotes adulthood opioid consumption that persists despite adverse consequences and increases the density of insular perineuronal nets

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Abstract

Adolescence marks a sensitive period for neurodevelopment wherein exposure to drugs of abuse may disrupt maturation and induce persistent changes in neurophysiology which may exacerbate the risk for developing substance use disorders in adulthood. Adolescent nicotine exposure (ANE) enhances motivation to obtain drugs of abuse, particularly opioids, and increases vulnerability for the development of opioid use disorder (OUD). Here, we characterized ANE effects on learning about the adverse consequences of opioid consumption in adulthood in the absence of further nicotine administration. First, we show that ANE engenders punishment resistant fentanyl self-administration in a heterogenous seeking–taking chain schedule of reinforcement at least at the tested dose of fentanyl (0.75 μg/kg). We found that ANE rats consumed significantly more fentanyl and contingent foot shock punishment was less efficacious in limiting fentanyl seeking in ANE rats, relative to nicotine-naïve controls. Next, we demonstrated that ANE limits learning about the deleterious consequences of acute opioid intoxication in adulthood. In a combined conditioned taste avoidance and place preference paradigm we found that ANE resulted in significant reductions in the strength of morphine-induced CTA, and a simultaneous enhancement of CPP at a higher dose that was less capable of driving reinforcement in naïve controls. Finally, we examined the expression of perineuronal nets (PNNs) within insular cortex (IC) and found ANE rats to have increased density of PNNs across the anterior IC and significantly more parvalbumin-labeled IC cells relative to naïve controls. Together, these data lay the framework

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Author Contributions

GCL designed the experiments. SCH, AM, MSP, and EAG conducted the studies. GCL and SCH analyzed the data and wrote the manuscript. All authors have approved the final manuscript.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

for a mechanistic explanation of the extreme comorbidity between nicotine use and development of OUDs.

Keywords

Pavlovian conditioning; Punishment; Fentanyl; Morphine; Self-administration; Compulsive

1. Introduction

Adolescence represents a sensitive period for brain development. Exposure to drugs and environmental hazards during adolescence may disrupt neural development and induce lasting changes in brain regions and neurocircuitry involved in reward-processing and impulse control [1,2]. These adaptations may further contribute to the vulnerability to developing substance use disorders later in adulthood [3,4]. For instance, initiation of smoking and nicotine use typically occurs in adolescence [5,6] and individuals who initiate nicotine use at an earlier age are substantially more likely to develop nicotine-dependence relative to those who initiate nicotine use later in life [7,8]. Evidence suggests that the adolescent brain may be more sensitive to the effects of nicotine relative to the adult brain, and nicotine exposure during adolescence results in myriad persistent neurobiological changes that promote addictive-like behaviors [9,10]. Adolescent nicotine exposure (ANE) in rodents enhances sensitivity to the reinforcing properties of nicotine and reduces sensitivity to the aversive properties of nicotine [11–13]. ANE also enhances the motivation to obtain other drugs of abuse. Specifically, nicotine exposure during early adolescence enhances conditioned place preference (CPP) for cocaine, morphine and amphetamine when tested in adulthood [14,15], augments self-administration of multiple classes of commonly abused drugs [16–19], and promotes tolerance to the analgesic properties of morphine [20]. Importantly, the enhancement of drug self-administration induced by ANE persists into adulthood despite cessation of nicotine administration during adolescence [16], indicating that ANE alone results in prolonged neurobehavioral changes that persist in perpetuity. As such, exposure to nicotine during developmental periods highly exacerbates the liability for development of polysubstance abuse.

Of particular interest is the highly prevalent comorbid use of nicotine and opioids [21,22]. Notably, habitual use of nicotine containing products among individuals seeking treatment for an opioid use disorder (OUD) is estimated to be at a rate between 74 and 97% [23–25]. Moreover, genomic and phenomic biomarkers associated with OUD and nicotine use disorder overlap significantly and these smoking-related biomarkers represent the highest risk-indicator for future development of OUD [26]. In support, nicotine use is associated with an increased likelihood of misusing prescribed opioids in humans [27–29] and significantly escalates opioid consumption in rodent models. Interestingly, this escalation of opioid consumption persists despite administration of contingent punishment [30]. Additionally, nicotine administration obfuscates learning about the adverse consequences of opioid intoxication [31,32] without interfering with the sensitivity to their overall interoceptive, presumably reinforcing, properties [33]. Currently, the precise neurobiological adaptations through which developmental nicotine exposure promotes

subsequent problematic drug use are not fully elucidated. Given the appreciable impact of the ongoing opioid epidemic, identifying neurobiological and behavioral mechanisms through which ANE promotes persistent problematic opioid consumption is a pressing health concern; particularly in light of recent significant increases in adolescent nicotine exposure due to the advent of electronic nicotine delivery systems (ENDS) or “vaping” [34, 35]. Moreover, it has been demonstrated that addressing the use of both nicotine and opioids is more efficacious for treatment of OUDs than treating opioid use alone [22].

One brain area that may represent a promising target area for identifying neurobiological adaptations induced by ANE is the insular cortex (IC). The IC is a neuroanatomically heterogeneous cortical structure comprised of functionally distinct subareas, broadly categorized as anterior and posterior [36]. The anterior IC (aIC) has predominant connections with frontal and limbic structures while the posterior IC (pIC) has more connections with motor and sensory areas [37]. Such specificity of projections implicates the IC as a critical area for the integration of sensory and motivational processes [38]. Recently, IC has gained significant traction as a neural substrate contributing to the development and expression of numerous behavioral phenotypes associated with substance use disorders [39,40]. Intriguingly, many of the behavioral phenotypes induced by nicotine exposure in response to opioids [30,31] have been shown to be critically modulated by insular neural activity. More specifically, neural activity within IC contributes to the expression of punishment-resistant drug consumption [41–43] and the acquisition and recall of aversive and reinforcing drug-associated contextual memories, including drug-associated conditioned taste avoidance [CTA; 32,44], CPP [32,45] and drug withdrawal-induced conditioned place avoidance [CPA; 46], and relapse to drug seeking following volitional abstinence [47,48], all for which direct associations between nicotine exposure and opioids have been demonstrated. Taken together, these previous findings implicate the IC as a relevant area for the mechanistic effects of nicotine on learning about the consequences of opioid consumption.

Here, we sought to expand our previous work conducted on acute nicotine exposure during adulthood [30,32] by exploring the effects of ANE on learning about the adverse consequences of opioid consumption when examined later in adulthood and in the absence of further nicotine administration. To this end, we conducted two sets of experiments. In one, we examined the degree to which ANE promotes the development of opioid self-administration phenotype in adulthood that persists despite adverse consequences through using a heterogenous seeking–taking chain schedule of reinforcement [49]. In this operant intravenous self-administration paradigm, two distinct responses are trained as “links” in a “chain” of behaviors that must be performed in sequence in order to obtain a drug reinforcer. Because the appetitive seeking behaviors do not directly result in reinforcer delivery until the taking behavior is performed, the chained schedule allows for the distinction between behaviors of appetitive drug seeking and consummatory drug taking, which offers enhanced face and ecological validity. These models have been used extensively to demonstrate the development and expression of habitual, punishment-resistant drug seeking [50–52]. In an additional experiment, we asked if ANE altered the acquisition of morphine-induced CTA and CPP in adulthood again in the absence of further nicotine administration. Finally, given the established role of IC neural adaptations in the acquisition and expression of these

behavioral phenotypes, we examined the effect of ANE on the expression of perineuronal nets (PNNs) across the anterior-posterior axis of IC. PNNs are extracellular matrix structures expressed in multiple areas of the cortex [53], including IC. The role played by PNNs in the brain is multifaceted, serving various functions, including protecting fast-spiking parvalbumin+ interneurons from oxidative stress [54], limiting synaptic plasticity, and maintaining established, or preventing new, synaptic connectivity [53,55,56], all of which have many implications for learning and memory. Exposure to commonly abused drugs influences the expression of PNNs in multiple brain regions, and manipulations of PNNs reciprocally alters drug self-administration and extinction learning [41,57–59]. In short, our objective was to characterize the persistent changes in learning about the aversive and reinforcing consequences of opioid consumption in adult rats that result from former ANE. Furthermore, we sought to identify potential biomarkers of IC plasticity that may be altered in adult rats following ANE, relative to naive controls, in a manner that may explain the alterations in learning about the deleterious consequences of opioid consumption.

2. Materials and Methods

2.1. Animals and Housing

Subjects consisted of 138 adolescent male and female Wistar rats (Envigo; Indianapolis, IN). Rats were individually housed in polycarbonate cages in a temperature and humidity-controlled facility and maintained on a 12:12h reverse light cycle. Rats arrived at the facility on PND 27 and, following 24-hour acclimation to the facility, all animals were handled for three days until the experiment began. All procedures were approved by the University at Buffalo Institutional Animal Care and Use Committee and were carried out in accordance with all relevant guidelines and regulations, including applicable Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.

2.2. Chemical Reagents

Nicotine tartrate (NIDA DSP) was dissolved in sterile saline at a concentration of 0.4 mg/ml (freebase) and the pH was adjusted to ~7.4 with dilute NaOH. All nicotine injections were administered subcutaneously (s.c.) at a dose of 0.4 mg/kg (volume of 1 ml/kg). Fentanyl hydrochloride (Cayman Chemical; Ann Arbor, MI) was dissolved in 0.9% sterile saline at a concentration of 4 μg/ml, and intravenous infusion of fentanyl was delivered at a dose of 0.75 μg/kg/infusion. Morphine sulfate (Spectrum Chemicals; Gardena, CA) was dissolved in 0.9% sterile saline to concentrations of 5.0 and 10 mg/ml. Saccharin sodium (Sigma-Aldrich; St Louis, MO) was dissolved in tap water at a concentration of 0.1% (w/v).

2.3. Behavioral Procedures

2.3.1. Operant Self-Administration Procedures

Adolescent Nicotine Exposure.: 20 adolescent male (n=10) and female (n=10) Wistar rats (Envigo; Indianapolis, IN) were used in the seeking-taking procedures. Rats were weight matched and assigned to receive injections of either nicotine or saline. During a 10-day period of adolescence, consisting of PND 34–43, rats were weighed and given twice daily injections of nicotine (0.4 mg/kg) or saline (0.1 ml/kg) s.c., during the dark phase, spaced six

hours apart on each of the 10 consecutive days. Following each injection, rats were placed in operant chambers for 1 h to expose the rats to the future operant self-administration context [60]. During each 1-h exposure session, the house light was illuminated and ventilation fans were activated, though no levers were presented and no cue lights were illuminated. Fig. 1 presents a timeline for each of the procedures performed across each of the experiments.

Surgical procedures.: Upon reaching adulthood (~PND 70), all rats underwent jugular catheterization in which chronically indwelling catheters were surgically implanted as described previously [32]. Briefly, rats were anesthetized with isoflurane (1–3%) inhalation and a 15-cm catheter line (C30PU-RJV1402, Instech) was implanted into the right external jugular vein. The opposite end of the catheter was connected to an externalized vascular access button (VABR1B/22, Instech) positioned just posterior to the scapular region on the dorsal side of the rat. Catheters were flushed with 0.1 ml of heparinized saline and enrofloxacin (Baytril, Bayer HealthCare LLC; Shawnee Mission, KS) once daily for 10 days to prevent infection. Following the post-operative recovery period, rats were flushed before and after self-administration sessions with 0.1 ml of heparinized saline. Catheter patency was verified at the conclusion of the study by administering 0.1 ml of ketamine (10 mg/ml, IV) and confirming the expression of ataxia.

Acquisition of Intravenous Fentanyl Self-Administration.: During acquisition training, rats were placed in operant conditioning chambers in sound-attenuating cabinets (Med Associates; St Albans, VT). The operant chambers were equipped with two retractable levers, a red house-light, a ventilation fan, and two stimulus lights located above each lever. During this phase, only one lever was made available and was designated as the “taking” lever (right or left levers were assigned in a semi-random counterbalanced fashion across both groups). Rats were trained in six daily 2-hour sessions on a fixed ratio-1 (FR-1) schedule of reinforcement such that one press of the taking-lever resulted in retraction of the lever along with illumination of the corresponding cue light and delivery of an intravenous infusion of 0.75 μg/kg fentanyl. The cue light remained illuminated during the ~3-s infusion period.

Training of the Seeking-Taking Chain Schedule.: Following six days of acquisition of the taking response, the training phase for seeking-taking chain began. Here, the opposite lever was designated as the “seeking” lever. Rats were trained such that pressing the seeking-lever provided access to the formerly trained taking-lever. During this phase, at the beginning of each trial the seeking-lever inserted into the cage and a variable interval (VI) timer was initiated. Once the VI had elapsed, the next press on the seeking-lever completed the first link of the chain and resulted in retraction of the seeking-lever and insertion of the taking-lever. The second link of the chain was completed once the rat pressed the taking-lever, resulting in retraction of the lever, illumination of the cue light, and delivery of fentanyl. Upon completion of the two-link chain cycle, there was a 10-s timeout period during which the house light turned off and no levers were available. Following the 10-s timeout, the “seeking” lever was inserted again thus initiating the next cycle. The average duration of the VI was gradually increased across five training sessions (2, 10, 30, 60, and 120 s) up to a terminal VI of 120-s.

Punishment of Fentanyl Seeking.: Following training of the two-link seeking-taking cycle, all rats were given an additional five days of responding on the VI-120-s chain which was designated as the "unpunished" baseline phase. Following these 5 days of baseline seeking-taking, a foot shock punishment was introduced wherein on ~50% of the cycles, completion of the first link of the chain resulted in delivery of a 0.5-s foot shock instead of gaining access to the "taking" lever. The remaining ~50% of the cycles resulted in presentation of the taking lever and the opportunity to deliver an infusion of fentanyl exactly as described above. The parameters of the punished sessions remained identical to the baseline sessions in terms of the VI-120 seeking timer and the 10-s timeout period. Once the 10-s timeout period was over, the "seeking" lever was made available and the next cycle was initiated. There were five total punished sessions and the intensity of the foot shock was systematically escalated daily over three days in the following order: 0.18, 0.24, & 0.3 mA and 0.3 mA was used for the remaining two sessions (three days total). These methods were adapted from previously published studies [51,61].

2.3.2. Pavlovian Conditioning Procedures

Adolescent Nicotine Exposure.: 118 adolescent male (n=60) and female (n=58) Wistar rats were used in the Pavlovian conditioning procedures. Rats were weight matched and assigned to receive injections of either nicotine or saline (vehicle). During a 10-day period of adolescence, PND 34–43, rats were weighed daily and given injections of nicotine (0.4mg/kg) or saline (0.1ml/kg) s.c., according to assignment, two times daily, six hours apart for 10 consecutive days and returned to their home cages.

Conditioned Taste Avoidance and Conditioned Place Preference Procedure.: Following the nicotine exposure period, all rats were left undisturbed in their home cage until maturing to PND 75. Subsequently, all rats were randomly assigned in a weight-balanced fashion to be conditioned with one of two doses of morphine (5.0 & 10 mg/kg) or saline for unconditioned controls. Combined CTA/CPP procedures largely mirrored those of our previous report [32]. Briefly, rats were maintained on a water schedule in which fluid was available for a 30 min period each day. Following acclimatization to this schedule (~5 days), rats were placed in the CPP apparatus and baseline preferences for the two contexts were assessed. The next day, all rats were given access to water for 30 min and then immediately injected with saline and transferred to the CPP apparatus equipped with the more preferred context for 30 min. The next day, rats were given access to 0.1% (w/v) saccharin for 30 min and then immediately injected with their assigned dose of morphine (0.0, 5.0, or 10 mg/kg) and transferred to the CPP apparatus equipped with the least preferred context for 30 min. This pattern was repeated 4 times for a total of 8 conditioning days. On the 9th day, the preference for the two contexts in the CPP apparatus was again assessed.

Immunohistochemistry.: At ~PND 100, a subset of rats (male n=25, female n=17) were euthanized with a sodium pentobarbital solution (Fatal Plus 150 mg/kg, Vortech) and subsequently perfused transcardially with saline followed by buffered 4% paraformaldehyde (PFA). Rats were chosen from the Pavlovian conditioning procedures to ensure that all rats had an equal amount of exposure to opioids and only differed in whether they had received nicotine or were naïve. Brains were removed, blocked at the insular cortex, and post-fixed

in 4% PFA for an additional 24 hours. Fixed brain blocks were sectioned on a freezing microtome (Hacker Instruments) at 40 μm. Free-floating sections were subjected to a series of washes with phosphate-buffered saline (PBS) prior to blocking with a glycoprotein-free blocking solution (SP-5040, Vector Laboratories). Following blocking, sections were incubated in biotinylated wisteria-floribunda agglutinin (WFA) primary antibody (1:1000; B-1355–2, Vector Laboratories) for 2 hours at room temperature and then overnight at 4° C. The next day, tissue sections were again washed with PBS prior to incubation in Dylight-488 streptavidin (1:200; SA-5488–1, Vector Laboratories) secondary antibody for 2 hours at room temperature. The following day, a subset of these tissue sections was again washed in PBS and blocked with normal goat serum (Vector Laboratories) prior to incubation in a parvalbumin primary antibody (1:1000; 195–011, Synaptic Systems) for 2 hours at room temperature and overnight at 4° C. The next day, sections were washed in PBS and incubated in Alexa Fluor 555 (1:200; A-31570, Thermo-Fisher) secondary antibody for 2 hours at room temperature. Following immunohistochemistry, tissue sections were then mounted on glass slides and cover slipped with VectaShield anti-fade mounting medium (H-1000, Vector Laboratories).

Immunohistochemical Image Analyses.: Analyses were performed on 4 sections per animal representing the insular cortex at 4 different anterior/posterior levels (~+2.2, +1.7, +1.0, & −0.3, relative to bregma). Images of the right hemisphere of the insular cortex were taken at 10x on an epifluorescent microscope (Nikon Eclipse 80i) and stitched together; all acquisition parameters were held constant for every section for every rat. Quantification of WFA fluorescent density and quantification of parvalbumin+ cell counts were performed with ImageJ (National Institutes of Health) software as described previously [62] by an experimenter blinded to treatment conditions. Briefly, for WFA fluorescent intensity, the background intensity was measured for an area of the insula in which no PNNs were present. This value was then subtracted from measurements of fluorescence intensity of PNNs within the insular cortex. For counting of parvalbumin+ cells, manual cell counts were obtained in ImageJ following application of thresholding to normalize background staining.

2.4. Data Analyses

Operant Self-Administration Procedures.—All data were analyzed with Statistica 12 software package. For the acquisition phase, total earned infusions were analyzed for each session of the six-session acquisition phase using a mixed factors analyses of variance (ANOVA) with Nicotine exposure condition (exposed or naïve) and Sex (male or female) as between-subject factors and Session as a within-subjects factor. For the seeking-taking phase of the experiment, we compared responses/average VI in min on the “seeking” lever and total number of cycles completed during the five baseline sessions and the five punished sessions using repeated measures ANOVAs with Nicotine condition and Sex as between-subjects factors and Session and Punishment context as within-subjects factors.

For the punishment sessions, suppression scores were calculated to express the degree to which rats decreased responding as a function of the foot shock punishment. This score was represented as a percent change from baseline as a function of prior punishment from each subsequent punished session and was calculated for each animal by taking the difference

in number of cycles completed at baseline (last unpunished session before foot shock was introduced) from the number of cycles completed during that punished session and then dividing that difference by the number of cycles completed at baseline and multiplied by 100. The suppression scores were analyzed using a repeated measures ANOVA with Nicotine condition and Sex as between-subjects factors and Punishment and Session as the within-subjects factors.

Finally, punishment efficacy was calculated for the final four punishment sessions by dividing the inverse of the punishment suppression score by the total number of shocks earned from all of the preceding sessions. This score represents the degree of punishment-induced suppression of behavior as a function of the total number of foot shocks earned prior to that session. The punishment efficacy score shows how "efficacious" the punishment was at suppressing behavior once foot shock was introduced. Since the punishment efficacy for each session was calculated based on foot shocks earned in the preceding sessions, the first day of punishment could not be included in this analysis. The punishment efficacy scores were analyzed using a repeated-measures ANOVA with Nicotine condition and Sex as between-subjects factors and Punishment Session as the within-subjects factor.

All significant interaction effects were further analyzed using Bonferroni corrected post-hoc tests when appropriate.

Pavlovian Conditioning.—For CTA experiments, change in consumption of the CS+ (saccharin) was analyzed with a mixed factors ANOVA where Sex, Nicotine exposure (exposed or naïve), and morphine Dose (0.0, 5.0, 10 mg/kg) served as between-subjects factors and conditioning Day was the within-subjects factor. For CPP experiments, change in preference for the drug-paired context was analyzed with a mixed factors ANOVA where Sex, Drug, and morphine Dose were between-subjects factors.

PNN fluorescent intensity analyses were conducted with a mixed factors ANOVA where Sex and Drug were between-subjects factors and the anterior-posterior Area of the insular cortex was the within-subjects factor.

Similarly, PV+ cell counts were analyzed with a mixed factors ANOVA where Sex and Drug were between-subjects factors and anterior-posterior Area of the insula was the within-subjects factor.

All significant interaction effects were further analyzed using Bonferroni corrected post-hoc tests when appropriate.

3. Results

3.1. Operant Conditioning

Adolescent nicotine exposure enhances intravenous fentanyl self-administration.—Nicotine exposure during adolescence resulted in significantly higher fentanyl intake across the 6-day acquisition phase, relative to naïve controls, (Fig. 2). Analysis of earned infusions in a three-factor ANOVA (Nicotine X Sex X Session) revealed that both groups, regardless of nicotine or saline condition, escalated their responding for

fentanyl across days of IVSA acquisition (main effect of Session; $F_{(5,80)} = 9.99$, $P < 0.001$). Treatment with nicotine during adolescence resulted in an overall higher number of fentanyl infusions relative to saline controls (main effect of Drug; $F_{(1,16)} = 10.08$, $P < 0.01$). There were no significant main or interactive effects of Sex.

Adolescent nicotine exposure augments both seeking and taking of fentanyl.—Adolescent nicotine exposure, relative to saline, produced significantly higher seeking responses and this difference was even more pronounced following introduction of contingent punishment (Fig. 3A). A four-factor ANOVA (Nicotine X Sex X Punishment X Session) conducted on seeking responses across both unpunished and punished sessions revealed that ANE rats made significantly more seeking responses than naïve controls, regardless of punishment or session (main effect of Nicotine; $F_{(1,16)} = 12.67$, $p < 0.01$). Seeking responses made by rats exposed to nicotine solely during adolescence, relative to naïve controls, were less impacted following administration of punishment (Drug X Punishment interaction; $F_{(1,16)} = 12.36$, $p < 0.01$). There were no significant main or interactive effects of Sex.

Similarly, nicotine exposure during adolescence significantly increased the number of seeking-taking cycles completed and this difference was even more evident following administration of punishment (Fig. 3B). Cycles completed (sum of earned fentanyl infusions and earned shocks) were calculated for each IVSA session and analyzed with a four-factor ANOVA (Nicotine X Sex X Punishment X Session). This analysis revealed that nicotine-exposure during adolescence, relative to naïve controls, resulted in a significantly higher number of completed cycles of the 2-link chain sequence regardless of punishment (Main effect of Nicotine; $F_{(1,16)} = 12.06$, $p < 0.01$). Nicotine-exposed rats were significantly less impacted by introduction of punishment relative to naïve controls (Drug X Punishment interaction; $F_{(1,16)} = 5.13$, $p < 0.05$). A significant Sex X Session interaction was observed ($F_{(4,64)} = 2.51$, $p < 0.05$) driven by the finding that males and females differed in cycles completed on the third unpunished session, though not significantly, and significant sex differences did not occur on any other day of the procedure, suggesting this was likely a spurious effect. Importantly, we found no interactive effects between Sex and Nicotine history or Sex and Punishment, demonstrating that the effects of ANE on opioid motivation did not differ between sexes.

Adult rats exposed to nicotine during adolescence are resistant to punishment of fentanyl consumption.—Rats treated with nicotine during adolescence suppressed responding for fentanyl in the presence of punishment significantly less relative to rats treated with saline during adolescence (Fig. 4). A three-factor ANOVA (Nicotine X Sex X Session) conducted on punishment suppression scores calculated from cycles completed across punished sessions revealed that all rats, regardless of nicotine or saline condition, somewhat reduced responding in the presence of punishment across sessions (main effect of Session; $F_{(4,64)} = 9.02$, $p < 0.001$). However, nicotine-exposed rats suppressed the number of cycles completed following introduction of punishment significantly less than naïve controls (main effect of Nicotine; $F_{(1,16)} = 7.16$, $p < 0.05$). We also found a main effect of Sex (main effect of Sex; $F_{(1,16)} = 4.52$, $p < 0.05$), driven by the

finding that males, regardless of nicotine or saline condition, produced higher suppression scores relative to females, indicating that males suppressed responding to a greater degree than female rats. Importantly, we observed no interactions between Sex and Nicotine, showing that the effects of ANE were similar across sexes.

Next, we calculated a punishment efficacy score in order to determine the degree to which the number of punishers earned were efficacious in suppressing responding. We found that nicotine-exposed rats were significantly less responsive to foot shock punishment despite receiving more total punishers (Fig. 5). A three-factor ANOVA (Drug X Sex X Session) conducted on punishment efficacy scores calculated for cycles completed across punished sessions revealed a main effect of Nicotine ($F_{(1,16)} = 8.52$, $p < 0.05$). We also found a main effect of Sex ($F_{(1,16)} = 4.75$, $p < 0.05$), driven by the finding that females, regardless of nicotine exposure, generally had lower punishment efficacy scores relative to males, again indicating that punishment was more efficacious in reducing responding in male rats relative to female rats.

3.2. Pavlovian Conditioning

Adolescent nicotine exposure alters learning about the interoceptive properties of morphine.—Exposure to nicotine solely during adolescence significantly reduced the strength of morphine conditioned taste avoidance across multiple doses when measured in adulthood (Fig. 6A). A mixed factors ANOVA revealed a significant Nicotine x Dose x conditioning Day interaction ($F_{(4,212)} = 4.17$, $P < 0.01$). Post-hoc analyses demonstrated that naïve controls, relative to nicotine-exposed rats, suppressed saccharin intake more on the final conditioning day with 5.0 mg/kg morphine but this difference failed to survive Bonferroni correction ($P = 0.0058$ vs a significance level set at 0.0055). There were no main or interactive effects of Sex on any measure.

Similarly, exposure to nicotine solely during adolescence increased the reinforcing properties of higher doses of morphine as assessed in the conditioned place preference procedure (Fig. 6B). A factorial ANOVA revealed a significant Drug x Dose interaction ($F_{(2,106)} = 3.31$, $P < 0.05$). Post-hoc analyses revealed that ANE and control rats did not differ in conditioning with 0.0 or 5.0 mg/kg morphine while nicotine-exposed rats displayed a significantly stronger CPP when conditioned with 10 mg/kg morphine ($P < 0.0055$). There was also a significant Sex x Dose interaction ($F_{(2,106)} = 5.33$, $P < 0.01$) driven by the finding that male rats, regardless of nicotine history, had significantly lower CPP scores to 5.0 mg/kg morphine relative to female rats. Yet again, there were no interactive effects of Sex on nicotine Drug history.

Exposure to nicotine during adolescence increases the density of insular perineuronal nets in adulthood.—Fluorescent density analyses revealed that there was more intense labelling of perineuronal nets within the insular cortex of adult rats exposed to nicotine during adolescence, compared to unexposed controls (Fig. 7A). A mixed factors ANOVA revealed a significant main effect of Nicotine ($F_{(1,38)} = 4.55$, $P < 0.05$). Because there were no main or interactive effects of Sex on these measures, we ran this analysis with Sex removed as a factor. These analyses revealed a significant Nicotine x Area interaction

($F_{(3,120)} = 2.63$, $P = 0.05$). Posthoc analyses on this interaction revealed that the differences between ANE and naïve control rats in PNN density were greatest in the anterior areas of the IC with much lower differences at the posterior level though no group difference at any individual level survived post-hoc correction.

Similarly, adolescent nicotine-exposed rats had significantly more parvalbumin-labelled cells in the insula than did nicotine-naive control rats (Fig. 7B). A mixed factors ANOVA revealed a significant main effect of Drug ($F_{(1,18)} = 4.67$, $P < 0.05$). Again, there were no main or interactive effects of Sex on these measures

4. Discussion

In the present study, we demonstrate that nicotine administered exclusively during adolescence induces a multifaceted behavioral state that markedly increases the liability for problematic opioid use later in adulthood. Specifically, we found that ANE resulted in adult rats that displayed significantly higher rates of appetitive and consummatory responding for intravenous fentanyl and enhanced punishment-resistance (Fig. 3A & B), such that ANE rats display increased seeking and taking of fentanyl that was significantly less impacted by contingent administration of foot shock punishment (Fig. 4 & 5). Likewise, we demonstrated that ANE alters learning about the aversive and reinforcing properties of morphine such that ANE appears to reduce responding to the adverse properties of morphine as demonstrated by a significant reduction in the strength of morphine-induced CTA (Fig. 6A), and a simultaneous enhancement of morphine CPP, particularly at the highest dose (10 mg/kg; Fig. 6B), relative to nicotine-naïve controls. Additionally, we found concomitant changes to the extracellular matrix within the IC of rats following ANE in a manner that could likely mechanistically explain the reduced capacity for learning about the deleterious consequences of opioid consumption. More specifically, we observed that ANE resulted in increased intensity of PNN fluorescent staining within the IC relative to unexposed control rats (Fig. 7A). This effect was specifically prevalent at the anterior and mid regions of the IC, but diminished within a posterior area. Likewise, we found that ANE rats had significantly more parvalbumin-labeled neurons within IC relative to unexposed control rats (Fig. 7B). Again, this effect was most pronounced at the anterior-most areas of the IC.

These data expand upon our previous findings that acute nicotine administration prior to IVSA sessions substantially increases consumption of opioids (remifentanil and morphine) in rats [32], and this escalation in consumption persists despite administration of two different forms of punishment: contingent foot shock and histamine adulteration [30]. Specifically, the present data establish that nicotine exposure, occurring solely during adolescence, markedly facilitates the development of a habitual-like opioid consumption phenotype in adulthood, an effect similar to the pharmacological actions of acutely administered nicotine. Furthermore, we have previously demonstrated that this enhancement in punishment resistance is unique to opioids, as nicotine-mediated augmentation of responding for a non-drug reinforcer was immediately lost upon the introduction of contingent foot shock punishment [30]. Here, we employed a heterogenous seeking-taking chained operant conditioning procedure to demonstrate enhanced punishment resistance of fentanyl seeking in ANE rats, compared to nicotine-naïve controls. ANE rats acquired

fentanyl IVSA in adulthood at the same rate as naïve controls yet consumed significantly more fentanyl during acquisition than the saline-treated controls (Fig. 2). Notably, we found no main or interactive effects of sex on responding for fentanyl during acquisition, demonstrating that ANE increased opioid consumption equally in both males and females. It has previously been reported that female rats acquire fentanyl IVSA more quickly than male rats [63]; however, under the experimental conditions employed here using Wistar rats, sex did not significantly influence responding for fentanyl and, importantly, did not differentially mediate the effect of ANE upon augmentation of fentanyl responding. This increase in fentanyl consumption persisted once the rats were transitioned into the seeking-taking portion of the experiment (Fig. 3A & B). Additionally, we found that ANE rats likewise demonstrated significantly increased rates of seeking responses (Fig. 3A) in line with their enhanced fentanyl consumption. We calculated a corrected seeking score to represent seeking responses during the operant session controlled for by each individual rat's average VI time in mins. We found that nicotine-treated rats produced significantly higher rates of seeking responses during the two-hour session relative to saline-treated controls and, interestingly, the seeking response in ANE rats escalated across the five unpunished days of responding while seeking remained relatively stable in nicotine-naïve rats.

The increases in both the seeking responses and cycles completed in the ANE group remained significantly higher than saline-treated controls despite the administration of punishment. To quantify these, suppression scores were calculated from the seeking-taking cycles completed during punished sessions to represent the percent change in responding for fentanyl that occurred on each session relative to each rat's own final day of unpunished responding (Baseline). ANE rats suppressed seeking-taking for fentanyl in the presence of punishment significantly less than control rats (Fig. 4). Furthermore, because ANE rats completed more cycles than naïve controls, they inherently earned significantly more foot shock punishers. Next, we further calculated the efficacy of punishment by expressing each rat's degree of suppression as a function of the total number of shocks that specific rat had earned previously. These punishment efficacy scores further revealed that punishment of fentanyl responding was less efficacious at deterring behavior in nicotine-exposed rats compared to those treated with saline (Fig. 5) such that ANE rats required significantly more punishers to suppress responding than naïve controls. It has been demonstrated by others that female rats respond differently than males to foot shock punishment [64,65], and female rats learn punishment avoidance faster than males, even while showing no difference in reinforcement-dependent associative learning [66]. In the present study, we did not observe a significant difference in fentanyl intake between sexes, but female rats were less impacted by contingent punishment of fentanyl seeking relative to male rats. Importantly, we again found that male and female rats were not differentially affected by ANE. Prior research demonstrates that a portion of rats will eventually develop punishment-resistant drug seeking, but only after a period of protracted drug self-administration [51,52]. For this study, we operationalized a rat as being punishment sensitive as suppressing their total number of cycles completed by greater than 40% on the final two days of punishment conditioning. Our finding that just a brief period of nicotine exposure during adolescence promotes habitual opioid responding in 80% of adult rats, relative to a rate of 40% in naïve controls, despite short-access fentanyl self-administration suggests that ANE produces

profound alterations in neural mechanisms and behavioral phenotypes that significantly impair responding to the adverse consequences of opioids in adulthood.

In agreement, using a combined CTA/CPP procedure we found that ANE rats, relative to naive controls, exhibited a significant reduction in the strength of morphine-associated CTA and a significant enhancement for CPP at the 10 mg/kg dose of morphine when measured in adulthood (Fig. 6A & B). There were no differences between ANE and controls rats in the unconditioned control group in either the CTA or CPP domain. Additionally, we found that male rats expressed somewhat lower CPP scores relative to female rats, ultimately driving a main effect of Sex. This is consistent with previous studies demonstrating females show CPP at higher doses of morphine that are not found to be reinforcing to males in CPP measures [67] and demonstrating that females detect morphine at lower doses than males [68]. Again, these sex differences did not interact with ANE status, further indicating that ANE does not alter opioid-directed responding differently in males and females. These findings from the Pavlovian conditioning procedures strongly support that adolescent nicotine exposure may in some manner serve to disrupt learning about the adverse consequences of opioids. More specifically, the largest effects between ANE and naïve controls were in the CTA paradigm. Additionally, ANE only affected morphine-induced CPP at the highest dose tested (10 mg/kg). In naïve controls, peak CPP conditioning occurred at 5 mg/kg and the CPP score began to diminish at the 10 mg/kg dose, consistent with previous studies [32,69], presumably due to the malaise-inducing or sedative properties of morphine. This assertion that ANE specifically impacts the ability to learn about the adverse consequences of opioid intoxication would be consistent with our prior findings that rats treated both with acute nicotine, and prior ANE (Fig. 4 & 5), persist in opioid-seeking despite punishment [30]. Further, similar ANE procedures have been shown to interfere with the acquisition of ethanol-induced CTA [70]. Additionally, we have shown previously that nicotine delivered directly to the IC is sufficient to interfere with acquisition of morphine-induced CTA and to enhance morphine CPP, again at doses >10 mg/kg, relative to vehicle [32]. The IC is a brain area that is critically involved in the ability to learn about the stimulus effects of drug of abuse, especially their aversive effects and consequences [38, 42,44,45,71–74]. As such, it follows that ANE may produce profound insular dysfunction that interferes with the ability to process the adverse consequences of drugs of abuse in a manner that promotes the development of punishment-resistant drug consumption.

In an attempt to identify potential insular mechanisms that contribute to the observed learning and behavioral deficits engendered by ANE, we stained and quantified the density of expression of insular PNNs. PNNs are specialized components of the extracellular matrix that are most often found surrounding parvalbumin+ neurons of the cortex [75]. These structures are formed at the closing of the critical period and their expression is associated with maturation of the cortex and the transition from adolescence into adulthood [76]. It is thought that cortical PNN expression is associated with reductions in cortical plasticity [53,77–79] at least partially due to the ability for PNNs to increase the firing rate of fast-spiking parvalbumin+ interneurons and to interfere with the formation of new synaptic connections [80]. Increased expression of PNNs has been associated with chronic exposure to a multitude of commonly abused drugs [58,62,81]. As such, it follows that abnormal PNN expression may fundamentally underlie many of the maladaptive learning and behavioral

consequences of protracted drug use. Such an interpretation is supported by multiple studies that demonstrate that enzymatic degradation of PNNs supports drug-extinction based learning [81–83] and that removal of PNNs, solely in IC, limited compulsive-like ethanol drinking [41]. The IC has been shown to be critically involved in mediating the interoceptive signals relevant to intoxication and is critically involved in CTA, CPP, and contextual-associated punishment of drug seeking [42–45,48,84,85]. As such, IC PNNs may critically contribute to persistent maladaptive drug-associated behaviors. Here, we demonstrate that rats that received nicotine solely during adolescence had significantly higher density of fluorescent PNN staining in adulthood relative to saline-treated controls. This effect appeared most pronounced within the anterior and mid sections of IC with very little difference observed between ANE and control rats in the posterior section. In general, the density of PNN staining appeared to decrease across the posterior-most sections of IC. Likewise, we found that ANE was associated with significantly more PV+ neurons in the IC relative to naïve controls. PNNs have been shown to protect parvalbumin+ neurons from oxidative stress [54,86] and so the increased expression of these labelled neurons may indicate increased survivability of parvalbumin+ neurons into adulthood. Interestingly, a recent study similarly demonstrated enhanced PNN expression and increased parvalbumin+ neurons in the mPFC following chronic cocaine exposure and subsequent abstinence [81]. It is currently unknown how nicotine exposure during adolescence might upregulate PNN expression, but it is known that deposition of PNNs appears to be activity dependent [87] and that normal maturation of cortical parvalbumin+ interneurons is dependent on the activity of α7 nicotinic receptors [88]. As such, repeated activation of α7 nicotinic receptors during development may contribute to a persistent increase in cortical PNN and parvalbumin+ neuronal expression, and this increase may fundamentally underlie the deficits in aversive learning surrounding opioids observed following ANE.

It follows that punishment resistant drug seeking may emerge from myriad neurobiological mechanisms that likely differ based on the behavioral history of the organism. For instance, punishment resistant drug seeking may emerge due to an augmented motivational state in which the drug reinforcer becomes so salient as to negate the consequences of punishment. Additionally, this punishment resistant phenotype may emerge due to cognitive deficits that manifest in an inability to learn the new punishment contingency. Of course, these are just a few of the potential underlying psychological mechanisms that may drive problematic drug use and they may be acting independently or in concert to produce the observed phenotype [89]. Therefore, one cannot assume that, for instance, neural adaptations that produce continued drug seeking despite adverse consequences in one experimental paradigm are the same as those produced by a different experimental manipulation despite nearly identical behavioral outcomes. As such, we are currently exploring experimental manipulations in an attempt to rescue the punishment insensitivity induced by ANE. For instance, removal of insular PNNs may improve the ability to learn about adverse contingencies surrounding continued drug seeking. Additionally, nicotine exposure may facilitate the development of loss of goal-directed opioid seeking through promoting dorsal striatal stimulus-response control of opioid consumption [90]. One potentially important limitation of our study was the focus on a single dose of fentanyl for the IVSA procedures. It is possible that ANE may alter behavioral responding to the pharmacological properties

of fentanyl, for instance increasing tolerance to its behavioral depressant effects, in a manner that may have influenced the present results. Previously, we have demonstrated that nicotine exposure failed to alter the locomotor response to multiple doses of morphine despite augmenting self-administration [32] which somewhat contradicts such a possible interpretation. Regardless, we are currently conducting IVSA procedures that seek to determine fentanyl self-administration as a function of altering infusion dose with the aim of determining whether nicotine exposure produces lateral or vertical shifts in the dose-response function.

These findings from the present study strongly support that ANE induces a protracted vulnerability to opioid addiction-like behavior in rats that is most commonly associated with extended experience with drug taking [51,52,61]. Likewise, ANE impairs learning about the adverse consequences of administration of moderately high doses of opioids in Pavlovian conditioning procedures. These effects may be due to neurobiological changes induced by nicotine during the sensitive developmental period of adolescence. Consistent with such an interpretation, we demonstrated that ANE rats displayed a significantly greater density of PNNs within the anterior insular cortex relative to nicotine-naïve controls. While we demonstrated a relationship between ANE and fluorescent density of PNNs within the IC, the present study presents correlative data between behavioral responses to opioids with changes to PNNs within the IC; therefore, it will be important for future studies to explicitly determine the behavioral consequences of changes in PNN expression within the IC and these are studies actively being conducted in our lab. Taken together, these findings support that adolescent nicotine exposure produced profound changes in behavioral responding to opioids and lay the groundwork for supporting alterations in insular plasticity in contributing to this behavioral phenotype.

Data availability

Data will be made available on request.

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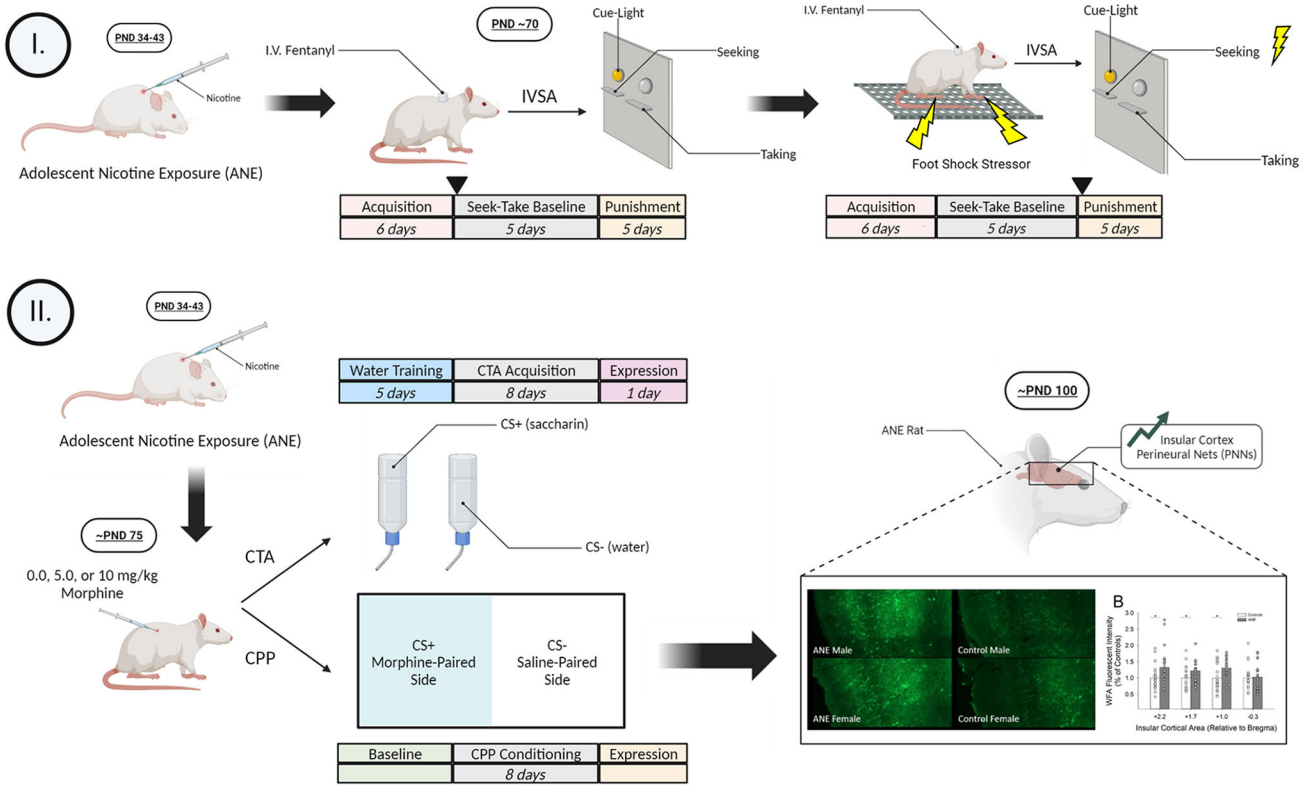

Fig. 1.

Graphical timeline for the various procedures performed in experiments I & II. Images created with Biorender.com

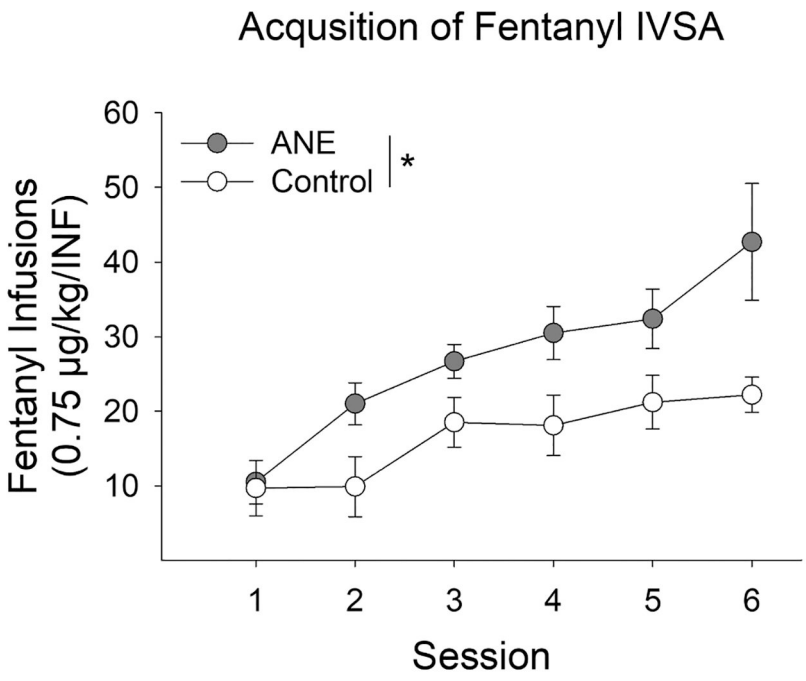

Fig. 2.

Adolescent nicotine exposure significantly increases intravenous self-administration of fentanyl. Rats were trained in 2-h sessions for 6 days on an FR-1 schedule of reinforcement to press a retractable lever in standard operant chambers for IVSA of fentanyl (0.75 µg/kg/Inf). In general, rats that received nicotine during adolescence, relative to nicotine-naïve controls, earned significantly more IVSA fentanyl infusions across the 6 acquisition sessions. *s indicate significant group differences ($P < 0.05$).

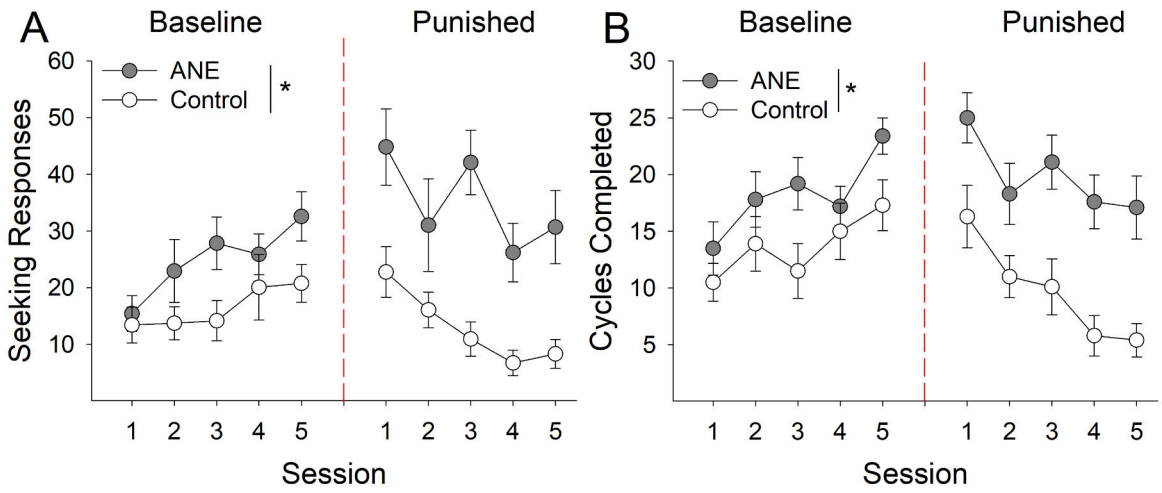

Fig. 3.

Adolescent nicotine exposure (ANE) significantly increases both seeking and taking of fentanyl and this difference was even more pronounced following introduction of contingent punishment. Rats were trained such that pressing a seeking-lever provided access to a taking lever in a heterogenous seeking-taking chain procedure. (A) ANE rats displayed significantly elevated seeking responses across both baseline and punished operant sessions. Moreover, seeking was significantly less impacted by implementation of foot shock punishment in ANE rats, relative to nicotine-naïve controls. Seeking responses across the operant session were corrected for each rat's average VI for that given session. (B) Likewise, the number of fentanyl consumption cycles was significantly elevated in ANE rats across both baseline and punished sessions and ANE rats, relative to nicotine-naïve controls, were less impacted by introduction of foot shock punishment. Cycles completed are the total number of earned fentanyl infusions and delivered foot shocks. *s indicate significant group differences ($P < 0.05$).

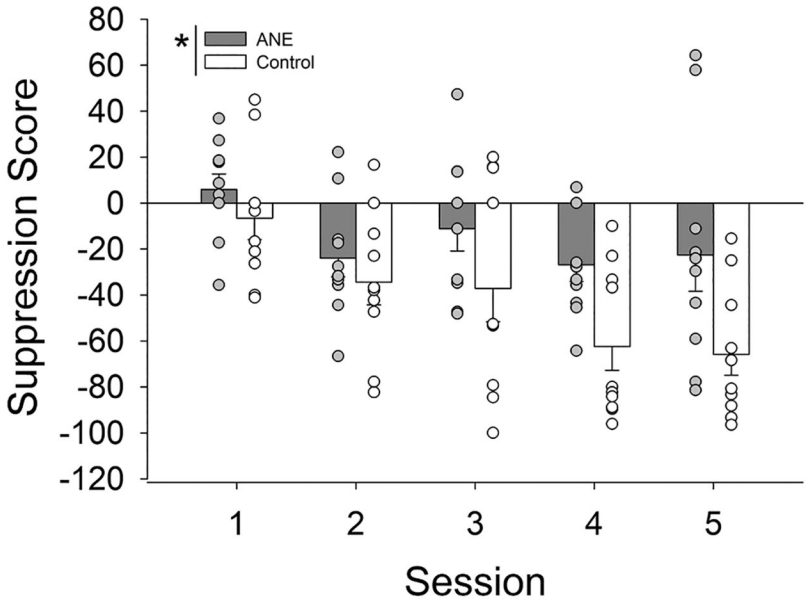

Fig. 4.

Adolescent nicotine exposure produces a habitual-like fentanyl seeking phenotype in adult rats that are significantly less likely to suppress fentanyl intake following implementation of punishment. To quantify the impact that implementation of foot shock punishment had on the number of fentanyl seeking cycles completed, suppression scores were calculated by taking the difference in number of responses at baseline from the number of responses on each punished session divided by the number of cycles completed at baseline multiplied by 100 to produce a percent change for each day of punishment. ANE rats suppressed responding to a significantly lesser degree relative to nicotine-naive controls, indicating that ANE rats were much more punishment-resistant in their responding for fentanyl.

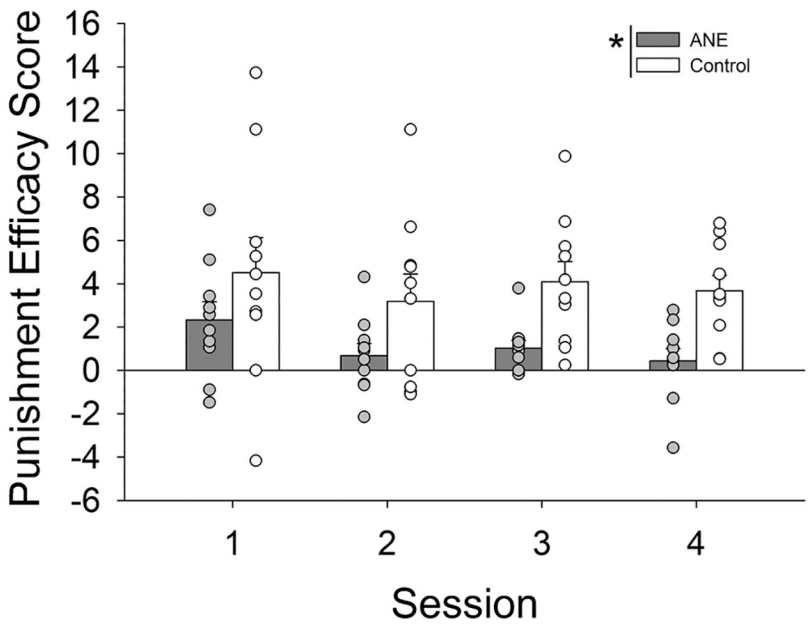

Fig. 5.

Adolescent exposure to nicotine significantly reduces the efficacy for foot shock punishment to suppress fentanyl consumption despite these rats earning significantly more punishers. Because ANE rats were less impacted by implementation of foot shock despite earning significantly more total foot shock punishers we calculated a punishment efficacy score by dividing the inverse of the punishment suppression score for each session by the total number of shocks earned from all of the preceding sessions. These scores revealed that ANE rats were significantly less responsive to delivery of foot shock and their escalated fentanyl consumption persisted despite this deterrent. *s indicate significant group differences ($P < 0.05$).

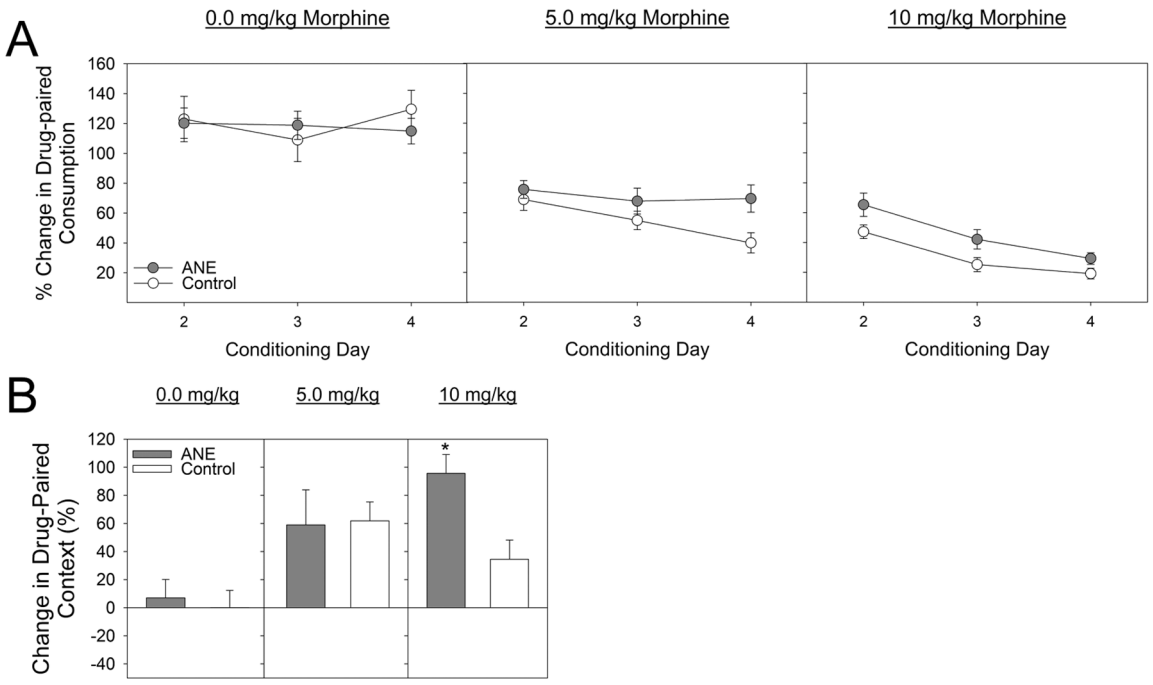

Fig. 6.

Adolescent nicotine exposure significantly impairs learning about the adverse effects of acute opiate intoxication in Pavlovian conditioning procedures. (A) ANE significantly reduced the strength of morphine conditioned taste avoidance across multiple doses of morphine relative to nicotine-naïve controls. Controls, relative to ANE rats, suppressed saccharin intake significantly more across conditioning with two doses of morphine (5.0 & 10 mg/kg). There were no differences in the unconditioned consumption of saccharin between ANE and control rats.(B) ANE increased the reinforcing properties of the higher dose of morphine in the conditioned place preference procedure. ANE and control rats did not differ in conditioning with 0.0, or 5.0 mg/kg morphine while ANE rats displayed a significantly stronger CPP when conditioned with 10 mg/kg morphine, a dose that was slightly less effective in conditioning a place preference in controls, relative to the 5.0 mg/kg dose. *s indicate significant group differences ($P < 0.05$).

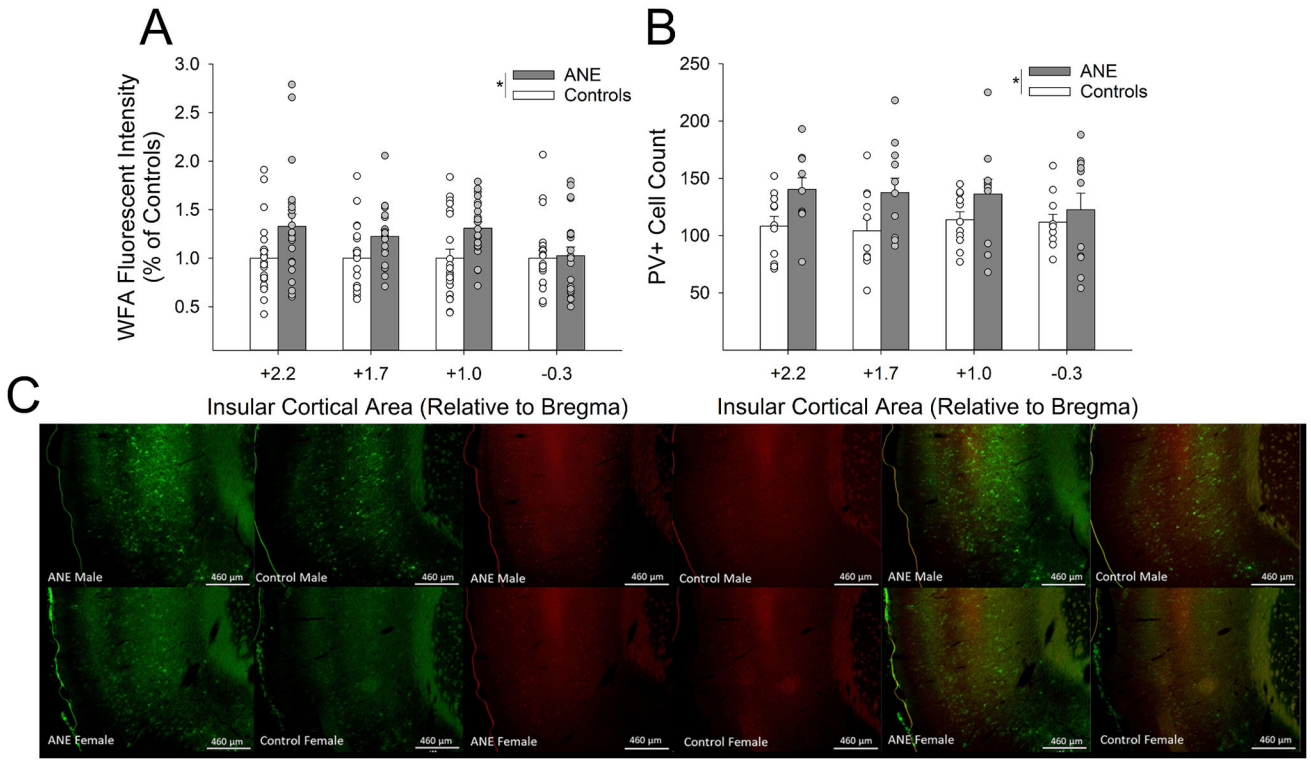

Fig. 7.

ANE augments the density of insular cortical perineuronal nets (PNNs) expressed in adulthood compared to saline controls and increases the number of parvalbumin-labelled (PV) cells. (A) ANE, relative to controls, significantly enhanced the density of PNNs as measured by WFA fluorescent staining intensity across multiple areas of the insula. Visual inspection of the data reveals that ANE PNN density, relative to saline controls, was higher at the anterior and mid regions of the IC (~+2.2, +1.7, & +1.0, relative to bregma) but not at the posterior section (−0.3) but these differences did not survive posthoc correction. (B) Likewise, there were significantly more PV+ labelled neurons across IC in adulthood in ANE rats relative to saline controls. Similar to PNN analyses, these differences were more pronounced in the anterior areas of IC but individual group differences failed to survive posthoc correction. (C) Representative images of PNNs (green) and PV+ cells (red) in male and female ANE and nicotine-naïve controls. (B) *s indicate significant group differences ($P < 0.05$).