EFFECT OF ADOLESCENT COCAINE USE: CONTEXT-INDUCED DRUG SEEKING BEHAVIORS AND HIPPOCAMPAL NEUROPLASTICITY

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Psychology – Master of Arts

2024

ABSTRACT

The health and economic costs associated with substance use disorders (SUDs) are immense, with a rise in overdose deaths related to stimulant or stimulant and opiate use from 2016-2021 (NIDA). Drug use during adolescence increases the risk for development of SUDs, with the severity of diagnosis associated with worse outcomes later in life. Recent reports of increased stimulant use among 15–23-year-olds and the number of overdose deaths associated with cocaine use highlight the need to understand both the behavioral and underlying molecular changes associated with craving and relapse in this age group.

We developed an abbreviated cocaine self-administration (Coc-SA) procedure which allowed us to examine relapse during adolescence and found that the magnitude of contextual cocaine-seeking is the same for adolescent and adult cocaine-exposed rats after 1 day of abstinence. However, adolescent rats had significantly higher seeking after 15 days of abstinence (incubation of craving). The current proposal aims to examine whether changes in plasticity, specifically activation of NMDA and AMPA receptor subunits, occur during abstinence periods that precede relapse. Adolescent and adult rats received Coc-SA in a distinct context (2h sessions, 2x/day, minimum 10 sessions), followed by extinction training (EXT) in a second distinct context (2h session, 2x/day, minimum 8 session). Adolescent and adult rats were split into three separate Relapse Test groups: No Test (30min in homecage), Test in the EXT context (EXT), Test in the Cocaine-paired context after 1 day (T1), or 15 days (T15) of abstinence. Tissue samples from the dorsal hippocampus were collected 30min after Relapse Tests.

We found increased cocaine-seeking behavior in adolescent and adult rats after 15 days of abstinence, however we did not observe Age or Relapse Test-dependent changes in NMDA or AMPA subunit activation associated with craving and relapse.

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INTRODUCTION

Substance Use Disorders & Age

Substance use disorders (SUD) are a chronic and multifaceted disease characterized by life-long risk to engage in cycles of drug use, withdrawal and craving that precipitates a return to use (JB & Saunders, 2017). The transition from recreational use to dependence is defined by several criteria including increased time spent to obtain and use drug, larger amount of drug consumed and greater impact on normal day-to-day and social activities. The number of criteria met dictates the severity of SUD diagnosis, ranging from mild to severe- two to three vs six or more (Volkow & Blanco, 2023). The health and economic costs associated with SUDs are immense, with a rise in overdose deaths related to stimulant or stimulant and opiate use from 2016-2021 (NIDA). Patients diagnosed with SUDs often report their first experience with drug use during adolescence (Volkow et al., 2021). Drug use during adolescence increases the risk for development of SUDs, with the severity of diagnosis (mild, moderate, severe) associated with worse outcomes later in life (McCabe et al., 2022; Volkow & Wargo, 2022). Recent reports of increased stimulant use among 15–23-year-olds, and the number of overdose deaths associated with cocaine or dual cocaine use (2016- 2021), highlights the need to understand both the behavioral and underlying molecular changes associated with craving and relapse in this age group (McCabe et al., 2022; Ramo et al., 2012; Ramo & Brown, 2008).

Chronic Relapse as a challenge to treat SUDs

Intense craving and relapse is often triggered by exposure to the cues and environments present during drug use (Ehrman et al., 1992; Foltin et al., 2000), with craving persisting for up to a year after abstinence, a phenomenon known as incubation of craving (Gawin & Kleber, 1986; Grimm et al., 2001; Lu et al., 2004; Tran-Nguyen et al., 1998). Rates of relapse for adults diagnosed with SUDs are up to 84% or higher depending on the class of drug (Volkow & Blanco, 2023). Behavioral interventions such as cognitive behavioral therapy and voucher-

based programs can reduce craving during early abstinence, but they are not effective in preventing relapse with extended follow-up periods (Volkow & Blanco, 2023). Adolescent drug use patterns consist of shorter periods of use followed by long periods of abstinence, with relapse rates similar to those of adults. Surprisingly, much less is known about the adolescent craving and abstinence period (Acri et al., 2012; Silvers et al., 2019; Squeglia et al., 2019) despite ongoing development of brain regions important for flexible decision making, learning and memory, motivation and reward. It is therefore critical to understand the underlying drug-induced changes in adolescent brain plasticity to develop improved behavioral and pharmacological therapies that minimize future risk to develop SUDs (Silvers et al., 2019; Winters et al., 2014).

Preclinical Models to Study Craving and Relapse

Preclinical models of rodent cocaine self-administration are widely used to understand the mechanisms that contribute to craving and relapse, with volitional intake. In general, behavioral procedures consist of three phases: 1) self-administration training, 2) extinction training and/or an abstinence period and 3) reinstatement testing. During self-administration, rats press one of two levers- one to obtain cocaine (active) or another that does not result in cocaine (inactive). Self-administration training occurs in the presence of background contextual stimuli (that do not change with cocaine infusions). Rats then progress to extinction training or an abstinence period. During extinction training, rats are placed in a second, distinct context in which lever responses result in no programmed consequences. During reinstatement (Relapse Test), rats are placed back into the previous cocaine-paired context. Lever responses result in no reward during this phase and therefore serve as an index of cocaine-seeking behavior. During abstinence, rats remain in their homecage until reinstatement testing. An increase in lever responses after extinction is referred to as reinstatement of cocaine -seeking (or context-induced cocaine-seeking) behavior (Feltenstein et al., 2021). A larger increase in seeking

behavior resulting from longer periods of abstinence, or a time-dependent increase in seeking, is termed incubation of cocaine-seeking (or incubation of craving) in rats. As with humans, cocaine or stress priming, cocaine-paired cues, and contexts can trigger cocaine-seeking, with cocaine-paired cues more likely to elicit incubation of cocaine-seeking behavior (Perry et al., 2014; Fuchs et al., 2009).

Adolescent model of craving and relapse

The brief length of adolescence in rats and the extended training time needed for operant self-administration studies are hurdles in in studying the effect of cocaine use on adolescent craving and relapse (Piekarski et al., 2017). Existing literature shows that adolescent rats exposed to cocaine have equal or lower cue reinstatement and incubation of craving when compared to adult rats. (Anker & Carroll, 2010; Li et al., 2018; Madsen et al., 2017; Zbukvic et al., 2016). Regarding cocaine and stress-priming, adolescent cocaine-exposed rats demonstrate higher cocaine-seeking behaviors compared to adults (Anker & Carroll, 2010; Wong & Marinelli, 2016). Furthermore, there is evidence that adolescent rats show higher reactivity to a cocaine-paired environment, when tested with conditioned-place preference and habit-based procedures (Brenhouse & Andersen, 2008).

We developed a procedure to study the impact of adolescent cocaine use on subsequent context-induced relapse. In our abbreviated cocaine self-administration (ABRV Coc-SA) procedure, rats undergo two Coc-SA sessions/day in a unique context, followed by two extinction (EXT) sessions/day in a second distinct context. Rats undergo 1 day or 15 days of abstinence, followed by reinstatement tests (Relapse Test) in the EXT or Cocaine-paired context (Cho et al., 2020; Olekanma et al., 2023).

As summarized in **Figure 1**, we found that adolescent and adult rats have equal intake of cocaine and similar lever responses during Coc-SA and EXT phases.

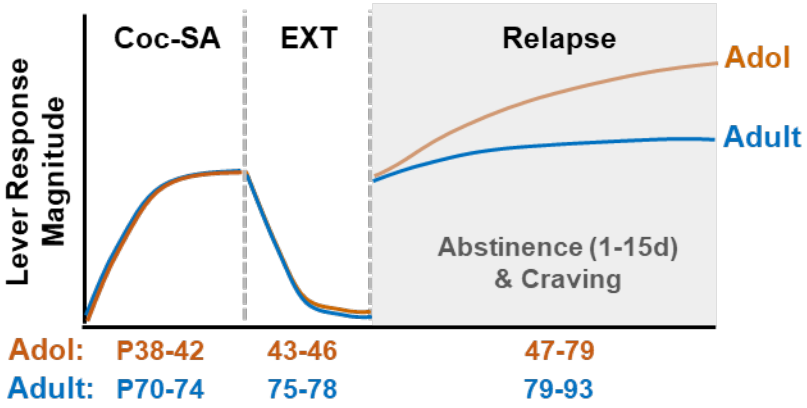

Figure 1. Summary of previous behavioral findings between adolescent (adol) and adult male rats during abbreviated cocaine self-administration (ABRV-Coc-SA), extinction (EXT) and reinstatement tests (Relapse Tests) in a previous cocaine-paired context after 1 or 15 days of abstinence. Adol and adult rats displayed similar magnitude of active lever responses during Coc-SA, EXT, and Relapse Tests after 1 day of abstinence. Incubation of craving, higher responses after 15 days of abstinence, was only observed in adol rats.

After 1 day of abstinence, both age groups have high cocaine-seeking in a previous Cocaine-paired context, compared to EXT. After 15 days of abstinence, only adolescent rats show higher cocaine-seeking behavior compared to 1 abstinence day. These findings demonstrate that adolescent cocaine-exposed rats display context-induced incubation of craving, compared to their adult counterparts and that the ABRV Coc-SA procedure allows for testing during adolescence and young adulthood to reveal latent behavioral effects of incubation.

Neurobiological Mechanisms Underlying Craving and Relapse

Rodent models of Coc-SA have been used to determine brain regions, circuits and molecular mechanisms associated with craving and relapse (Bossert et al., 2013). Key regions of the mesocorticolimbic pathway including the hippocampus, basolateral amygdala and prefrontal cortex, promote contextual cocaine-seeking behavior (Lasseter et al., 2010; Ramirez et al., 2009). As summarized in **Figure 2**, temporal inactivation of the dorsal and ventral subregions of

the hippocampus (DH, VH) reduced contextual cocaine-seeking behavior in adult rats (Fuchs et al., 2007; Lasseter et al., 2010). Within the DH, plasticity at NMDA receptors (which has been associated with enhanced learning and memory) also promote seeking behavior. For example, impaired signaling at NMDA receptors, via AP-5 infusion into the DH, reduced cocaine-seeking behavior. Furthermore, reduced Src family kinase signaling, via PP-2 infusion into the DH, impaired cocaine-seeking and reduced downstream phosphorylation of the NR2b subunit of the NMDA receptor (pGluN2b) (Wells et al., 2016a; Xie et al., 2013a). There is also evidence that activation of AMPA receptors also contribute to cocaine-seeking and incubation of craving in adult rats, although this has mostly been studies in the nucleus accumbens (NAc) (Pickens et al., 2011).

Much less is known about the role of the DH and plasticity at NMDA and AMPA receptors in contextual cocaine-seeking in adolescent rats. It is appreciated that elements of the mesocorticolimbic system undergo dramatic changes during the adolescent period- for example, the DH and inputs to this region from the basolateral amygdala and prefrontal cortex, undergo extensive pruning during early adolescence (Bessières et al., 2019; Klune et al., 2021; Travaglia et al., 2016).

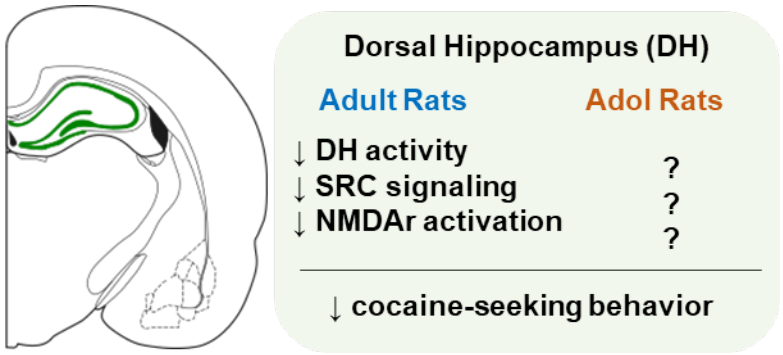

Figure 2. Summary of previous studies examining the role of the dorsal hippocampus (DH) in cocaine-seeking behavior. Temporal inactivation of the DH, reduced NMDA receptor or Src family kinase signaling, reduces contextual cocaine-seeking. Studies in adolescent (adol) cocaine-exposed rats are lacking.

Furthermore, plasticity and baseline expression of NMDA and AMPA receptors undergo dynamic changes during adolescence. During the peri-adolescence to adult transition, levels of DH GluN2b decline, GluA1 and GluA2 increase; whereas GluN2a and phosphorylated extracellular signal-regulated kinase (ERK) signaling, a downstream target of NMDA receptors, remain stable (Bessières et al., 2019; Jia et al., 2018). Taken together, cocaine-exposure during adolescence could impact plasticity at NMDA and AMPA receptors and result in long-lasting impacts on future cocaine-seeking behavior.

Current Study Aims

Our published data show that adolescent cocaine-exposed rats have higher context-induced incubation of craving, compared to adult counterparts. The current study aims to examine whether changes in plasticity, specifically activation of NMDA and AMPA receptor subunits, occur during drug-free abstinence periods that precede relapse. We predict that adolescent cocaine-exposed rats will display 1) higher activation at NMDA receptor subunits (GluN2b and GluN2a) when tested in a previous cocaine-paired context after 15 days of abstinence, compared to 1 day of abstinence. 2) higher activation at NMDA receptors subunits when tested in the cocaine-paired context, vs an EXT context, after 1 day of abstinence, 3) higher activation at NMDA receptors at 1d and 15d of abstinence when compared to adult cocaine-exposed rats. We predict that no changes will be observed in activation of AMPA receptor subunits based on age or Relapse Test conditions.

METHODS

Animals: Adult and Adolescent male Sprague Dawley rats (n = 41; Envigo Labs) were used for the current study. Rats were approximately postnatal day 25- P25 (84 gm, n = 22) or P65 (241 gm, n = 19) on arrival and were pair-housed until the start of behavioral training. The rat housing area was under a 12-hr reversed light cycle and all behavioral testing occurred during the dark cycle. All rats were handled approximately 6-7 days prior to surgical procedures. Following surgeries, rats were housed individually for the entire experiment. All rats were maintained on a limited diet consisting of approximately 15g for adolescents or 18g for adults, starting two days before self-administration training (Carroll, 1985; Cho et al., 2020; DePoy et al., 2016; Olekanma et al., 2023). All protocols utilized were approved by the institutional animal care and use committee (IACUC) at Michigan State University and followed the National Research Council's Guide for the Care and Use of Laboratory Rats.

Surgery: Rats received meloxidyl (orally, 0.9 mg/kg) two days prior to the start of surgeries. Catheters were assembled in house, 10cm length for adults and 9.7cm for adolescents, as previously described (Charpentier et al., 2023; Olekanma et al., 2023; Wong et al., 2013; Zbukvic et al., 2016). Rats were anesthetized via intraperitoneal injection (I.P.) with a combination of Xylazine + Ketamine (80-100 mg/kg + 5-10 mg/kg, respectively; Covetrus). Catheters were fed subcutaneously above the shoulder blades, and tubing implanted into the right jugular vein (Bal et al., 2019; Cho et al., 2020; Fuchs et al., 2005). Following surgery, rats were administered meloxidyl, and topical gentamycin. Catheters were flushed daily with 0.1mL of Cefazolin (0.1 mg/mL) dissolved in 70-unit heparinized saline (70 U Hep; Covetrus), followed by 0.1 mL of 10 U Hep, as previously described. Propofol was used to periodically assess catheter patency. Rats that failed the propofol test (loss of movement not present) were removed from the study.

Cocaine Self-Administration (Coc-SA) Training: Coc-SA training took place within operant chambers (29.5 x 24 x 28 cm; Med Associates Inc., St. Albans, NY) designed to represent two distinct contexts. As shown in **Figure 3**, Context A was comprised of a continuous white house light (0.4 fc brightness), intermittent tone (80 dB, 1 kHz; 2 sec on, 2 sec off), pine-scent freshener (Car Freshener Corp., Watertown, NY), and interweaved wire mesh flooring (26cm x 27cm). Context B was comprised of an intermittent illuminating white light over the inactive lever (1.2 fc brightness; 2 sec on, 2 sec off), continuous tone (75 dB, 2.5 kHz), vanilla scented freshener (Car Freshener Corp., Watertown, NY), and bar flooring with spaces and black acrylic plate bisecting the floor at a 45-degree angle (19 cm × 27 cm). Rats were assigned to start Coc-SA in Context A or B in a counterbalanced design (Cho et al., 2020; Fuchs et al., 2008; Khoo et al., 2017).

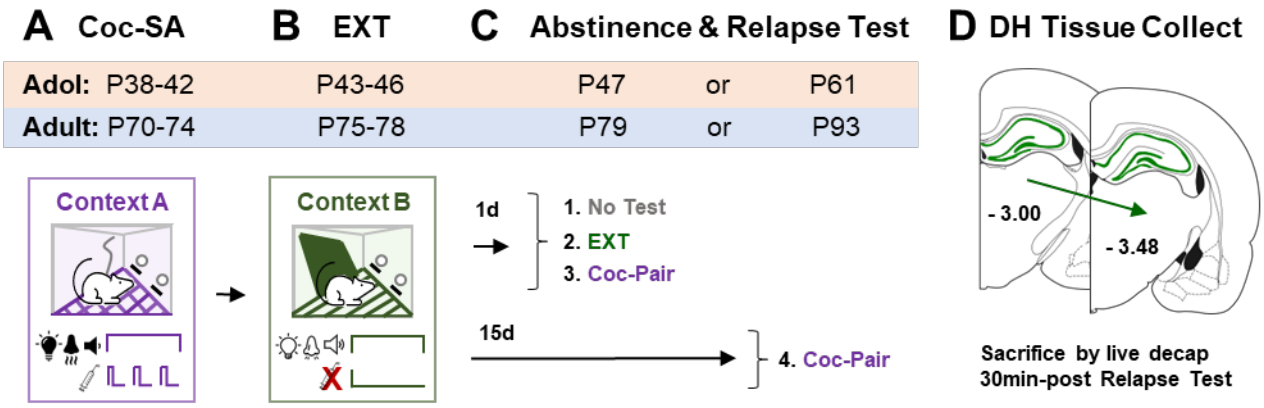

Figure 3: Experimental design to investigate context-induced incubation and neuroplasticity. **(A)** Cocaine self-administration (Coc-SA) occurred in a context with distinct background stimuli: Context A, 2h session, 2x/day, minimum of 10 sessions. **(B)** EXT was conducted in a second distinct context: Context B, 2h session, 2x/day, minimum of 8 sessions, no drug rewards. **(C)** After 1 day of abstinence, adol and adult rats were split into three separate Relapse Test groups: 1) No Test, 2) EXT context, 3) Cocaine-paired context (T1). After 15 days of abstinence adol and adult rats were tested in 4) Coc-paired context (T15). **(D)** Dorsal hippocampal (DH) tissue punches were collected from fresh frozen tissue obtained through live decapitation 30min-post Relapse Test.

Cocaine Hydrochloride (Cocaine-HCl; NIDA Drug Supply System, Research Triangle Park, NC) was prepared in 0.9% sterile saline. Responses on an active lever resulted in an IV infusion of 0.05 ml of Cocaine-HCl (0.5 mg/kg per each infusion) on a Fixed Ratio (FR1) schedule of reinforcement. Infusions were delivered via an infusion pump (Med Associates Inc; Model PHM-107) over 2 seconds, with a 20 second time out. Responses on an inactive lever resulted in no reward. Weight was recorded daily and cocaine concentration in syringes was adjusted for 50g increase in weight, as previously described (Anker & Carroll, 2010; Cho et al., 2020). Coc-SA consisted of 2 h sessions, 2x/day over 5 days, for a minimum of 10 sessions (criteria = 10 infusion minimum). Coc-SA stated at period in which adolescent rats were post-pubertal (McCutcheon & Marinelli, 2009) In between sessions, rats were returned to their home cages.

Extinction Training (EXT) and Reinstatement (Relapse) Tests: EXT was comprised of 2h sessions, 2x/day over 4 days, for a minimum of 8 sessions (criteria = less than 25 responses on final 2 sessions). During EXT, no consequences resulted from active or inactive lever responses. After the last EXT session, rats were split into several groups. After 1 day of abstinence, adolescent and adult rats were split into three separate Relapse Test groups: 1) No Test (remained in homecage), 2) Test in the EXT context, 3) Test in the Cocaine-paired context (T1). After 15 days of abstinence adolescent and adult rats were tested in 4) Cocaine-paired context (T15). After the 30min Relapse Test, rats were sacrificed immediately by live decapitation, brains were extracted, and flash frozen in isopentane and stored at -80^{o}C to preserve phosphorylation. A 30min test length was used based on previous studies from our lab that found a trend for increased pGluN2b and pGluN2a at both 30min and 1h post Relapse Test. Previous studies that examined changes in neuroplasticity-linked proteins within the central amygdala and nucleus accumbens, also sacrificed rats 30min after Relapse Tests (Dong et al., 2017; Szumlinski et al., 2016; Wells et al., 2016a; Xie et al., 2013a).

Western Blot Analysis: Tissue punches from the hippocampus were collected on a cryostat using neuropunches from the dorsal (-2.28 to -3.40) and ventral (-4.56 to -5.50) hippocampus. Samples were sonicated in lysis buffer containing protease and phosphatase inhibitors and protein concentrations determined using DC protein assay (Bio-Rad).Equal amounts of protein (15 μg) were resolved on 4% - 15% Criterion TGX gradient gel (Bio-Rad) and transferred to Immobillon membranes at 100V for 1 h (Millipore) Upon successful transfer, membranes underwent blocking in 1X buffer for 1 h, followed by primary antibody incubation overnight at 4^0C. Primary antibody dilutions were based on our previous publications and dilution curves from the lab (Higginbotham et al., 2021). Membranes were rinsed the following day in TBS with 0.01% Tween 20 (TBST) and incubated with infrared secondary antibody for 1hr at room temperature, Membranes were rinsed again with TBST and imaged with LiCor Odyssey (CLx-1547) and quantified using LiCor Image Studio software. Phosphorylated proteins were normalized to total and calnexin protein, as previously described (Arguello et al., 2014; Wells et al., 2016; Xie et al., 2013).

Statistical Analysis: Analysis of Variance (ANOVA's) or independent t-tests were utilized to assess for preceding differences in Relapse Test groups (EXT, T1, T15) for: cocaine intake, mean lever responses during the final 3 sessions of Coc-SA, final session of EXT, and session to acquire Coc-SA and EXT. To assess behavioral differences during Coc-SA and EXT by Age, data from adult and adolescent *Relapse Test Groups* (EXT, T1, T15) pre-treatment were collapsed. Interaction effects for within-subject factors: *Coc-SA* or *EXT sessions*, or the between-subject factor of *Age* were assessed. For behavioral phases (Coc-SA, EXT, Relapse Test), no *Age* x *Context* x *Relapse Test Group* interaction effects were found. To assess behavioral differences at the Relapse Test, 2 x 3 ANOVAs for *Age* x *Test Group* (EXT, T1, T15) were conducted. Significant effects were analyzed with Tukey's post hoc test, with the alpha set at 0.05. Rats that lost catheter patency were not included in the analysis.

RESULTS

Adolescent and adult behavior during Coc-SA, EXT, and Relapse Tests: To examine pre-existing behavioral differences between adolescent and adult rats, lever responses for all phases before Relapse Test (Coc-SA and EXT) were combined by Age. Overall, there were no pre-existing differences by Age in the number of sessions to acquire Coc-SA, lever responses or cocaine intake during the last 3 sessions of Coc-SA (**Figure 4A**). The 2 x 10 ANOVAs for Coc-SA did not reveal significant *Coc-SA session* x *Age*, *Coc-SA intake* x *Age* interaction effects, or main effect of *Age*. A significant main effect of *Coc-SA session* (active: $F_{1,39}$ = 412.171, $p < 0.001$, inactive: $F_{1,39}$ = 11.445, $p < 0.001$) was observed.

During EXT, there were no pre-existing differences by Age in the number of sessions needed to meet EXT training criterion or lever responses on the last session of EXT (**Figure 4B**). The 2 x 8 ANOVAs of lever responses during EXT did not reveal significant *EXT session* x *Age* interaction, or *Age* main effects. A significant main effect of *EXT session* (active: $F_{1,39}$ = 36.784, $p < 0.001$, inactive: $F_{1,39}$ = 49.436, $p < 0.001$) was observed. Tukey's post-hoc comparisons revealed that both adolescent and adult rats decreased lever responses by the final EXT session (active and inactive: EXT S1> S8, $^{\#}p<0.01$)

To examine potential behavioral differences between adolescent and adult rats during the Relapse Test, lever responses for each Group (EXT, T1, T15) were assessed by Age. The 2 x 3 ANOVAs of active lever responses during Relapse Test did not reveal significant *Test Group* x *Age* interaction, or *Age* main effects. A significant main effect of *Test Group* (active: $F_{1,35}$ = 9.209, $p < 0.001$) was observed. Tukey's post-hoc comparisons revealed that adolescent and adults displayed cocaine-seeking behavior (**Figure 4C**). Adolescent and adult rats had higher active responses between T15 and EXT Groups ($^{*}p<0.01$), but no significant differences between T1 and EXT, or T1 and T15 Groups was observed. During the Relapse Test, no significant differences were observed for inactive lever responses.

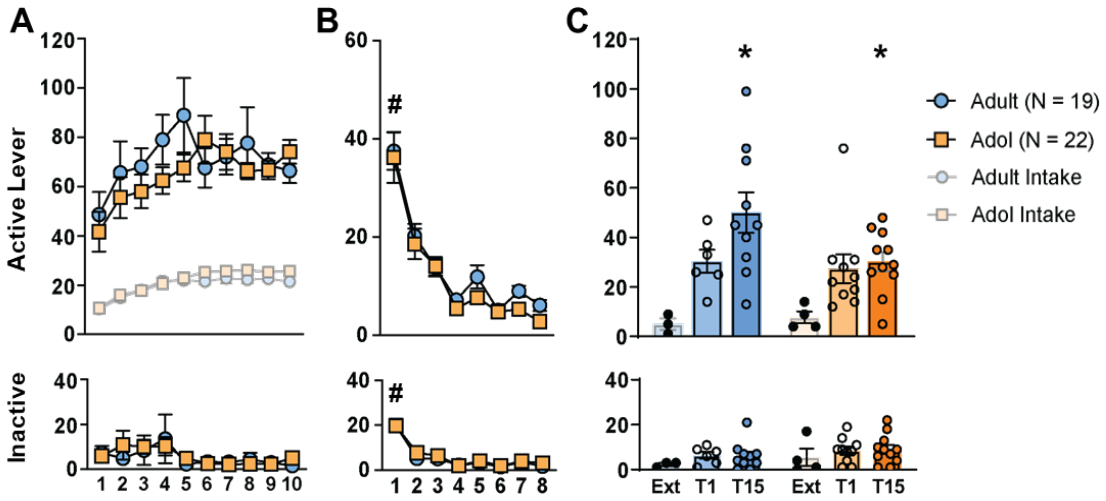

Figure 4. Schematic of Behavioral Experiments in adolescent and adult rats.
Mean ± SEM of active and inactive lever responses for adol and adult rats during abbreviated **(A)** Cocaine self-administration (Coc-SA, 2h sessions, 2x/day, minimum of 10 sessions), **(B)** Extinction training (EXT, 2h sessions, 2x/day, minimum of 8 sessions), and **(C)** reinstatement test (Relapse Test, 30min) that were conducted in the previous EXT or Coc paired context after 1 day of abstinence (Ext, T1) or after 15 days of abstinence (T15). Symbols indicate significant within-subject differences revealed by Tukey's test **(B)** EXT session 1>8, $^{\#}p<0.01$ **(C)** EXT session<T1, *p<0.01. Groups denoted by: Blue = adult (n=19), Orange = adolescent (n=22).

NMDA and AMPA receptor activation at Relapse Test

To examine for potential differences in NMDA and AMPA receptors activation associated with cocaine-seeking in adolescent and adult rats, tissue punches were collected after a 30min Relapse Test. An additional No Test Group was added for adolescent and adult rats. These test groups went through Coc-SA, EXT, and 1 day of abstinence, but remained in their homecage during tests. The levels of protein expression were examined by Age for each Relapse Test Group (No Test, EXT, T1, T15).

The A 2 x 4 ANOVAs of pGluN2b or pGluN2a levels during Relapse Test did not reveal significant *Relapse Test* x *Age* interaction or main effects (**Figures 5A, B**). Furthermore, no significant *Relapse Test* x *Age* interaction or main effects were observed for levels of pGluA1 or

pERK (**Figures 6A, B**). We also separately examined for potential differences in protein levels during Relapse Test for adolescent or adult rats. The 1 x 4 ANOVAs did not reveal any significant differences in levels of pGluN2b, pGluN2a, pGluA1 or pERK between the Relapse Test Groups.

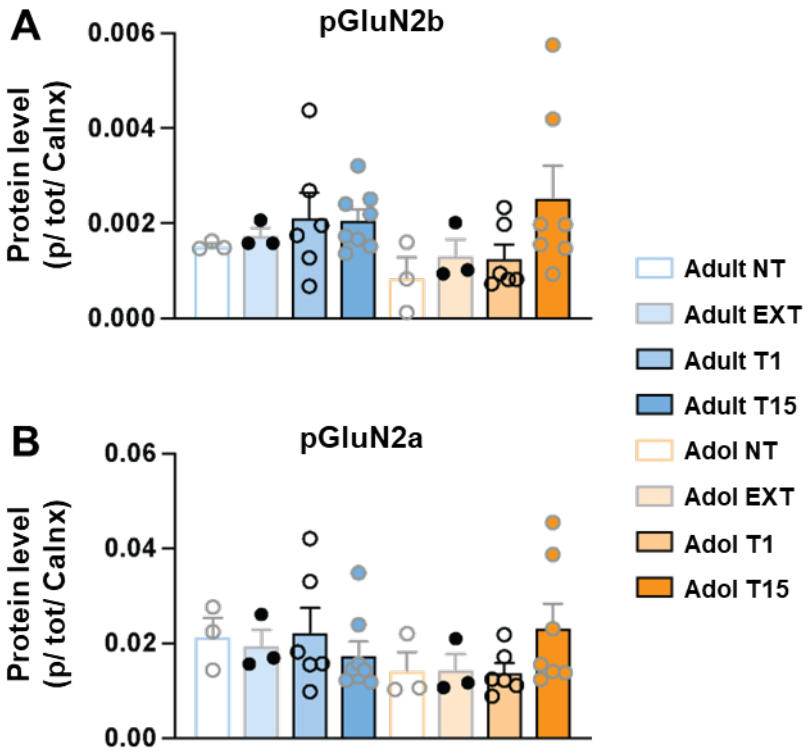

Figure 5. NMDA receptor subunit phosphorylation and Relapse Test. Protein levels of phosphorylated NMDA receptor subunits during Relapse Tests. **(A)** pGluN2b and **(B)** pGluN2a levels for Relapse Test Groups: No Test, extinction context after 1day abstinence (EXT), cocaine-paired context after 1 day (T1) or 15 days of abstinence (T15). No significant differences were noted between Ages or Test Groups.

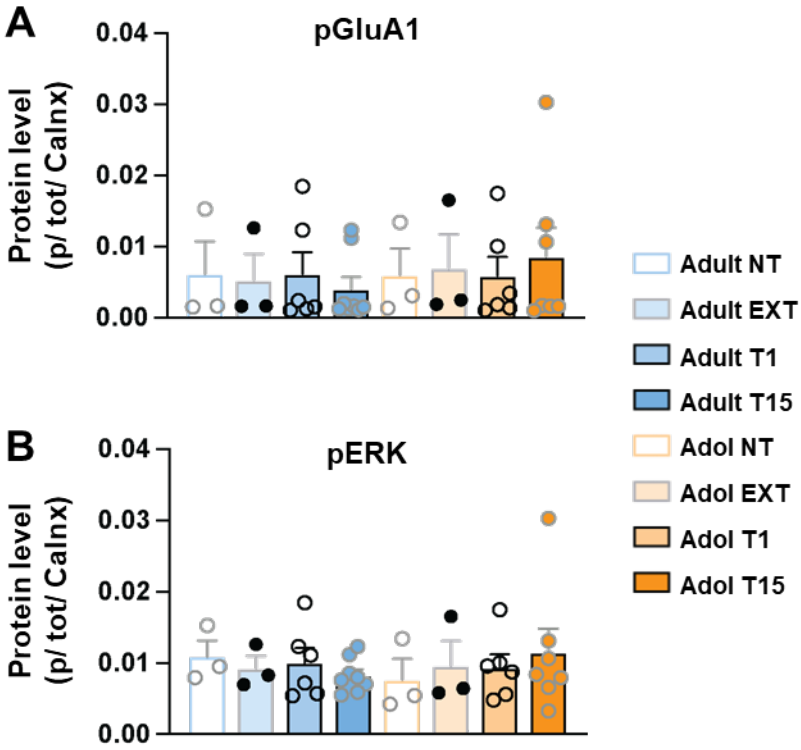

Figure 6. AMPA receptor subunit and ERK phosphorylation, and Relapse Test. Protein levels of phosphorylated AMPA receptor subunit or ERK protein during Relapse Tests. **(A)** pGluA1 and **(B)** pERK levels for Relapse Test Groups: No Test, extinction context after 1day abstinence (EXT), cocaine-paired context after 1 day (T1) or 15 days of abstinence (T15). No significant differences were noted between Ages or Test Groups.

DISCUSSION

Overall Behavioral Conclusions

The aim of the current study was to examine the underlying changes in dorsal hippocampal (DH) plasticity- specifically phosphorylation of NMDA and AMPA receptors that occur during drug-free abstinence periods that precede relapse. Our published data found that adolescent cocaine-exposed rats displayed higher context-induced incubation of craving after longer periods of abstinence, compared to adult counterparts. We therefore hypothesized that higher activation at NMDA and AMPA receptor subunits may contribute to these observed behavioral effects.

To test our hypothesis, we used our published abbreviated cocaine self-administration (ABRV Coc-SA) procedure in both adolescent and adult rats. We found no differences in Coc-SA behaviors- both adolescent and adult rats had similar cocaine intake, active and inactive lever responses, stable responses by the last three sessions of training, and both groups distinguished between the active and inactive levers. No age-dependent differences were observed during extinction (EXT) training. Both adolescent and adult cocaine-exposed rats extinguished responses by the end of this behavioral phase. The current data replicate previous findings from our group and others which showed no pre-existing, age-dependent differences in behavioral phases that preceded abstinence and Relapse Tests (Cho et al., 2020; Crombag & Shaham, 2002; Fuchs et al., 2005; Hamlin et al., 2008; Olekanma et al., 2023).

To examine changes in plasticity at NMDA and AMPA receptors during abstinence periods, we modified the experimental design after EXT training was complete. Adolescent and adult rats were divided into four separate Relapse Test groups. After 1 day of abstinence, rats received 1) No Test- remained in the homecage, 2) Test in EXT context, 3) Test in the Cocaine-paired context. After 15 days of abstinence, a separate group of adolescent and adult rats received 4) Test in the Cocaine-paired context. In the current study we found that both adolescent and adult rats had increased cocaine-seeking behavior after 15 days of abstinence,

when compared to the EXT context, which is in line with our previous results (Cho et al., 2020). However, we did not observe cocaine-seeking behavior after 1 day of abstinence for both age groups. Additionally, we did not find higher cocaine-seeking (or incubation of seeking) in adolescent cocaine-exposed rats. The absence of time-dependent differences in cocaine-seeking behavior in adolescent rats contrasts with our previous findings (Olekanma et al., 2023). The length of Relapse Tests likely contributed to these results. Our previous work examined cocaine-seeking behavior over a 2-hour period, with adolescent cocaine-exposed rats continuing to respond into the 2nd hour of testing. In the current study, both age groups received 30 min Relapse Tests based on previous literature that observed increased phosphorylation at NMDA and AMPA subunits at this shortened test length (Bessières et al., 2019; Travaglia et al., 2016). Unpublished data from our group also found trends for increased levels of DH pGluN2b and pGluN2a at both 30min and 1h test lengths (Cho, unpublished results).

Overall NMDA and AMPA receptor plasticity Results

Previous unpublished work from our lab suggested that NMDA receptor activation (at GluN2b and GluN2a subunits) were higher in adolescent rats tested in a cocaine-paired context after 1d of abstinence, compared to those tested in an EXT context. Therefore, the current study extended these preliminary findings by adding several adult comparison groups: No Test, EXT test group, 1 day and 15 day abstinence groups (T1, T15). We also added additional adolescent rats to the T1 and added a new T15 group based on our contextual adolescent incubation of craving findings (Olekanma et al., 2023). Our primary goal was to determine whether plasticity markers associated with enhanced learning and memory would be increased during longer abstinence periods. We did not observe Age or Relapse Test dependent differences in phosphorylation at the NMDA GluN2b and GluN2a subunits or at the AMPA GluA1 subunit. Therefore, we also probed for changes downstream of NMDA and AMPA subunit activation, but also did not observe changes in ERK or CREB phosphorylation.

One possibility for the non-significant results of NMDA and AMPA receptor activation could be related to our behavioral Relapse Test results. As mentioned in the above paragraph, while cocaine-seeking was observed after 15 days of abstinence in adolescent and adult rats, it was absent after 1 day of abstinence and no time-dependent differences in incubation were observed in adolescent rats (EXT < T1 < T15). Given that lever responses remained high into the 2nd hour of the Relapse Test, it is possible that larger increases in pGluN2b might be observed if DH tissue was collected after 2 hours of testing. However, from our previous pilot data (Cho, unpublished) we did not observe higher pGluN2b levels in adolescent cocaine-exposed rats after a 2 hour vs 30min or 1 hour Relapse Test. From the current cohort of rats, we also took punches from the prelimbic cortex and did observe time-dependent increases in pGluN2b levels which suggest that the 30min test length was sufficient to observe changes in phosphorylation. These data are in line with evidence that within the prefrontal cortex and nucleus accumbens, activation of NMDA and AMPA receptors promote cue-induced incubation of craving rats (Dong et al., 2017; Szumlinski et al., 2016; Wolf, 2016). Future experiments may focus on Age and abstinence-related changes at NMDA and AMPA receptors in the prefrontal cortex or nucleus accumbens,

Another possibility for our observations of minimal change in NMDA receptor activation after abstinence, is that all adolescent and adult rats received cocaine self-administration training. It is possible cocaine masked our ability to detect changes in phosphorylation at NMDA and AMPA receptor subunits. Based on existing literature it is known that changes in NMDA and AMPA receptor activation can occur with acute cocaine or in cocaine-experienced rats, when compared to relative saline controls (Pickens et al., 2011). To assess this potential caveat and test for potential cocaine-related changes in neuroplasticity, additional groups of adolescent and adult rats that underwent saline self-administration would be needed.

Last, another possibility is that NMDA subunit activation plays a more important role in strengthening cocaine-associated memories, via reconsolidation mechanisms. A trend for increased pGluN2b between adolescent No Test and T15 groups was observed. From previous literature, increased levels of pGluN2b, pGluN2a and pERK are present in the basolateral amygdala and DH of adult cocaine exposed rats but are a result of 15min of re-exposure to a previous cocaine-paired context (Wells et al., 2013). Studies from the same group showed that there was no increased in pERK between No Test and Coc-pair T1 groups, however when activation of pERK was inhibited context-induced cocaine-seeking was reduced (Wells et al., 2016). Therefore, future experiments that functionally impair signaling through NMDA receptor subunits in adolescent cocaine-exposed rats, may reveal age-dependent differences in reduced craving.

CONCLUSIONS & FUTURE DIRECTIONS

In conclusion, we found increased cocaine-seeking behavior in adolescent and adult rats after 15 days of abstinence, however we did not observe Age or Relapse Test-dependent changes in NMDA or AMPA subunit activation associated with craving and relapse. Future directions could take advantage of novel methods to reduce activation at NMDA receptors. In particular, future studies could utilize viral methods to circuit-specifically inhibit NMDA activation (Whyte et al., 2021; Li et al., 2021). It is also known that sex-differences in craving and cocaine-seeking behavior exist, with different levels of cocaine-seeking observed between estrus and non-estrus cycling females (Nicolas et al., 2019; Kantak et al., 2007). We have preliminary data to suggest that adolescent female and male rats display similar levels of lever responding behavior during Coc-SA, EXT and Relapse Test after 1day of abstinence, however we have yet to examine for sex-dependent differences after 15days of abstinence.

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