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Extinction and reinstatement to cocaine-associated cues in male and female juvenile rats and the role of D1 dopamine receptor

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ABSTRACT

Extinction of behaviors in response to drug-associated cues and prevention of reinstatement are integral for addiction treatment, and can reverse or ameliorate the harmful consequences of drug use. The mechanisms controlling extinction and reinstatement involve prefrontal cortical dopamine receptors, which change in expression and activity during the juvenile and adolescent transitions until they mature in adulthood. Little is known about the role that PFC D1 dopamine receptors play in extinction of drug-paired associations early in life. We used extinction of place preferences for cocaine in juvenile male and female rats following genetic, cell-specific overexpression of D1 on glutamatergic cells in the PFC. All subjects needed to demonstrate cocaine preferences for inclusion in the extinction studies. Here, male juveniles with a preference to 10 mg/kg cocaine took longer to extinguish preferences compared to both male adults and female juveniles. Female juveniles extinguished more rapidly than male juveniles at 20 mg/kg cocaine. Overexpression of D1 in juvenile males significantly facilitated extinction relative to juvenile male controls, whereas D1 prolonged expression of extinction in adults overexpressing D1 and adolescents who naturally have elevated D1 expression. These data suggest that an immature D1 profile in juveniles prevented the learning of new associations, and D1 overexpression may provide sufficient activity to facilitate extinction learning. D1 overexpression reduced reinstatement to a priming dose of cocaine in juvenile males. Together, these data show D1 expression may re-program motivational circuitry to facilitate extinction learning during juvenility that is normally unavailable to juveniles and that sex differences exist.

1. Introduction

Drug-associated cues play an important role in relapse to drugs of abuse (Childress et al., 1999; Maas et al., 1998). In order to reduce the likelihood of relapse, a number of rehabilitation approaches focus on extinguishing drug-associated cues. Extinction of these associations requires that new associations are formed between the conditioned stimulus (CS) and cues that signal the *absence* of the unconditioned stimulus drug (US). Studies of extinction processes in rats have utilized operant paradigms and place conditioning (reviewed in Moorman et al. (2014)). In the operant version of extinction, the animal responds on an operanda (bar press or nose poke) and only the CS is delivered without the US. After repeated trials, responding diminishes over time. To assess vulnerability to relapse, a prime of a lower dose of drug is administered in the presence of the CS or no CS and the animal rapidly re-attains pre-extinction levels of responding. This is known as reinstatement and is a useful measure of motivation to use drugs. Under the simplest of conditions (stable responding on a schedule of reinforcement, extinction, reinstatement) the whole process after surgery and recovery (7 days itself) takes minimally 35 days. This protracted period of time is further complicated with additional stressful effects of single housing of subjects to prevent cage mates from chewing on catheters. Social isolation paradigms of short (Leussis and Andersen, 2008) or longer duration (Hall, 1998) affect the underlying reward systems we aim to study and do so in a

Abbreviations: CK, CamKIIα; CS, conditioned stimulus; GFP, green fluorescent protein; ilPFC, infralimbic prefrontal cortex; mPFC, medial prefrontal cortex; plPFC, prelimbic prefrontal cortex; US, unconditioned stimulus.

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developmentally dependent manner. Place conditioning provides an alternative to study these same issues of conditioning, extinction, and reinstatement with caveats, but also parallel results to self-administration paradigms (Aguilar et al., 2009; Tzschentke, 2007).

Place conditioning with extinction/reinstatement of drug-associated contexts requires less time (~15 days) and no single-subject housing, making it well-suited for developmental assessments within relatively short periods of time before the animals mature too much. For this reason, the few days (four in our paradigm) required for place conditioning is sufficient to provide a useful indicator of preferences or aversions to drug-associated environments (Cabib et al., 1996). The days until the subject reaches extinction criterion, which is set to less than 54% of the time originally spent on the preferred side, has lasted less than 12 days in most of our animals when animals are allowed to freely roam the chambers including both the drug-paired and drug-unpaired contexts (Brenhouse and Andersen, 2008).

Resistance to extinction may elevate risk of relapse significantly, whereas faster extinction would facilitate a reduction in relapse. Consistent with elevated addiction risk in teens (O'Brien and Anthony, 2005), adolescent rats take 75% longer than adults to extinguish conditioned associations when the environment that previously was associated with cocaine no longer predicts its availability (Brenhouse and Andersen, 2008). Reinstatement is also higher in adolescents at a lower, but not higher, dose of cocaine (10 and 20 mg/kg, respectively). Less is known about the pre-adolescent development of drug-cue extinction and reinstatement processes. Here, we investigate whether low rates of addiction in children (O'Brien and Anthony, 2005) may be partially facilitated by rapid extinction and lower reinstatement by examining these processes in juvenile rats (19 days of age).

Sex differences in extinction/reinstatement during development have also received little attention. Female rats show stronger place preferences for cocaine-associated environments than male rats in adulthood and adolescence (Brenhouse et al., 2009; Kuhn et al., 2001; Russo et al., 2003; Zakharova et al., 2009). Extinction of these cocaine place preferences is protracted in adult female mice compared with males (Hilderbrand and Lasek, 2014), whereas no sex difference in the degree of extinction is observed between male and female adult rats (Bobzean et al., 2010). Differences in the time course of extinction were not examined in this latter study leaving sex differences in the rate of extinction uncharacterized. Interestingly, reinstatement of preferences in rats following extinction was higher in females than males. Sex differences in a number of behaviors often emerge during adolescence (Sato et al., 2008). Place conditioning studies in juvenile females are highly limited (Freund et al., 2014). Previously, we found no sex differences in preferences for cocaine between sham-lesioned male and female juvenile rats. Here, we investigated the time course for extinction of conditioned place preferences to two doses of cocaine in intact male and female rats.

The protracted development of the medial prefrontal cortex (mPFC) is likely to play a role in age differences in extinction (Brenhouse and Andersen, 2008). The mPFC and its connections to the amygdala and the accumbens are involved in the original and the new extinction memory (Ciccocioppo et al., 2001; Sotres-Bayon and Quirk, 2010; Zavala et al., 2003). Lesions to the prelimbic PFC (plPFC) do not influence the rate of extinction (Zavala et al., 2003), suggesting that new learning is important for extinction. Prelimbic PFC lesions, however, do reduce reinstatement of a preference for cocaine-associated environments following a priming injection of cocaine (Zavala et al., 2003). The D1 dopamine receptor within the plPFC is involved in extinction and reinstatement of cocaine-associated cues. Microinjection of the D1 antagonist SCH-23390 into the plPFC after conditioning to cocaine delays extinction in place preferences (Hacik, 1978) or prevents reinstatement following self-administration (Ciccocioppo et al., 2001). Similarly, D1 expression on glutamate neurons projecting to the accumbens correlates with increased reinstatement to extinguished cocaine cues in adult rats (McFarland et al., 2003). The D1 receptor in the plPFC is transiently over-expressed during adolescence relative to juveniles and adults (Andersen et al., 2000), including specific over-expression on the glutamatergic output neurons to the accumbens (Brenhouse et al., 2008). The relative elevation in D1 receptors may explain the protracted extinction of adolescents relative to adults (Brenhouse and Andersen, 2008) and has yet to be determined; in juveniles, neither the profile of extinction and reinstatement to cocaine cues nor its relationship to D1 receptors is known.

In the current study, we examined whether juvenile males rats show similarly strong associations to cocaine-conditioned cues as adults using a place conditioning paradigm. Specifically, we determined whether immature (juvenile) rats would show the same prolonged extinction as we have shown in adolescent rats (Brenhouse and Andersen, 2008) relative to adult rats and how this relates to D1 dopamine expression (Experiment 1). We genetically increased D1 dopamine receptors via a lentiviral vector with a CamKIIα (CK) promoter specifically within glutamatergic neurons in the plPFC (Sonntag et al., 2014). We have previously shown that increased D1 by viral transduction increases cocaine place preferences. Second, we examined whether sex differences exist in the time to extinction to cocaine-associated environments in juvenile rats (Experiment 2). Experiment 3 examined all of these groups for differences in reinstatement following a priming dose of cocaine.

2. Materials and methods

2.1. Subjects

Male Sprague–Dawley rats were obtained from Charles River (Boston, MA); litters arrived at postnatal day (P)15 and adult rats arrived at P80 to allow for acclimation. Rats were P19 (juvenile) at the time of surgery or P85 (adulthood) based on established milestones (Andersen, 2003). Food and water were made available *ad libitum* in constant temperature and humidity conditions on a 12-h light/dark cycle (light period 0700–1900). All experiments were conducted in accordance with the 2011 Guide for the Care and use of Laboratory Animals (NIH) and McLean-approved IACUC protocols. Rats were handled for a minimum of three days before conditioning started.

2.2. Lentiviral vector

A third generation lentiviral vector system was used. The system expressed the rat D1R or green fluorescent protein; GFP and was driven by a CamKIIα promoter, with full methods found in Sonntag et al. (2014). The resulting viruses are CK.GFP and CK.D1. Virus production, concentration by ultracentrifugation, and qRT-PCR-based titer determination were performed by the Massachusetts General Hospital Vector Core.

2.3. Lentiviral injections

Rats were anesthetized with a ketamine/xylazine mixture (80/12 mg/kg, respectively) and received 0.6 μl of virus (10^6–10^7 transducing units per μl) bilaterally into the plPFC at stereotaxic coordinates (P85 adults: AP: +2.8, ML: 0.4; DV: −2.7 (Paxinos and Watson, 1986) and P19 juveniles: AP: 2.6, ML: 0.4; DV: −2.6 (Sherwood and Timeras, 1970)). Animals began behavioral assessments 5 days after surgery (at P90 and P24) to allow for viral expression (Sonntag et al., 2014). Expression was stable throughout all experiments and placement and D1 over-expression confirmed by immunohistochemistry for D1 receptors. We have previously confirmed that this virus is specific for glutamatergic neurons (Sonntag et al., 2014). Subjects who did not demonstrate D1 overexpression within the plPFC were not used in the analyses.

2.4. Cocaine place conditioning

Subjects were conditioned to 10 or 20 mg/kg cocaine (McKesson, Inc., Boston, MA) using an unbiased place conditioning protocol, as previously described (Brenhouse and Andersen, 2008). Ten mg/kg dose of cocaine does not produce consistent preferences in juveniles and adults, although 20 mg/kg cocaine produces reliable place preferences (Badanich et al., 2006; Brenhouse and Andersen, 2008). The conditioning apparatus consisted of two large (24 × 18 × 33 cm) side

compartments and a small (12 × 18 × 33 cm) middle gray compartment, with the large compartments differing in color, lighting, and floor texture (Med Associates, Georgia, VT). On Day 1 subjects explored the entire apparatus for 30 min after a 5-min adaptation period of confinement to the middle compartment. Subjects demonstrating an initial side preference (e.g., >18 of 30 min) were eliminated from testing. Rats were injected with saline (1 ml/kg, i.p.) and placed in a side chamber for 1 h, and 4 h later injected with cocaine (10 or 20 mg/kg, i.p.) and placed in the opposite chamber for 1 h across Days 2 and 3. On Day 4, rats were allowed access to the entire apparatus in a drug-free state for 30 min to test for conditioned preferences. Preference scores were determined by calculating the ratio of time spent in the drug-paired side to the total time spent in both the drug and saline-paired sides (Brenhouse and Andersen, 2008). Subjects with a score of greater than 0.54 were allowed to progress to the extinction studies, with the rationale that a preference cannot be extinguished if it does not exist in the first place. Subjects were then allowed to explore the entire chamber for successive days until a preference score of 0.54 or less was attained two days in a row, thereby reaching extinction (Brenhouse and Andersen, 2008; Sanchez et al., 2003). Subjects were given a priming dose of 5 mg/kg cocaine and tested for reinstated preferences the following day.

2.5. Immunohistochemistry (IHC) to verify lentiviral placements

Transduced rats were deeply anesthetized and intracardially perfused and the tissue was processed with standard methods (Sonntag et al., 2014). Briefly, sections were incubated overnight in rat anti-D1 DAR IgG (1:250; targeting the intracellular C-terminus; Sigma), washed, and incubated for 60 min with anti-rat Alexa 488-coupled IgG (1:200; Molecular Probes). GFP expressed sufficiently as to not warrant staining (images of our viruses can be found in (Sonntag et al., 2014)). Sections were washed, mounted on slides, and placement verified through the use of a Zeiss AxioScop microscope. Only the data presented here had viral placements within the plPFC.

2.6. Data analysis

All data were analyzed using SPSS, v.20. Preference scores were calculated as described above. Place preference data were analyzed with a mixed ANOVA, with Age, Cocaine, and Sex as between-subjects factors, and conditioning (pre/post) as a within-subject variable. We note that a three-way interaction with conditioning (noting that Age and Sex were between-subject variables in different experiments) was unlikely as all subjects were originally selected as having a positive preference score. Significance was set at $P < 0.05$, with any necessary post-hoc tests corrected by Bonferroni. The number of days to extinction was determined for each individual subject and was the dependent variable. Kaplan–Meier survival analysis was used to estimate the number of days where 50% of the subject had extinguished their preferences, and thus provides the most accurate mathematical description of these differences (these data are found in Table 1). Reinstatement data were also analyzed with ANOVAs as described.

3. Results

Rats that demonstrated a significant place preference (ratio ≥ 0.54) were repeatedly exposed to the testing chamber to extinguish their preferences in an unbiased manner. The number of those rats initially screened for a preference (preference ratio >0.54) and those that achieved a ratio of 0.54 or greater are presented in Fig. 1. While not all control subjects had an initial preference for cocaine-associated environments, all CK.D1 rats met criterion or were eliminated due to missed targeting of virus.

Table 1
Extinction Kaplan–Meier survival analyses.

Age (days)	Condition	Cocaine (mg/kg)	Sex	Days to extinction (Mean ± SE)	Statistical differences
P21	Control/CK.GFP	10	Male	9.17 ± 1.38	Age control; Sex × Coc; Age × Coc
			Female	5.14 ± 1.22	Sex × Coc
		20	Male	5.33 ± 1.38	Age control; Sex × Coc; Age × Coc
			Female	7.11 ± 1.33	Sex × Coc
P90	Control/CK.GFP	10	Male	3.92 ± 0.42	Age control; Age × Coc
		20	Male	5.0 ± 0.66	Age × Coc
P21	CK.D1	10	Male	3.20 ± 0.37	Age CK.D1
P90	CK.D1	10	Male	7.29 ± 1.46	Age CK.D1

Age control: $F1,24 = 5.14$, $P < 0.05$.
Age × Cocaine: $F1,24 = 3.85$, $P = 0.06$.
Age × Virus: $F1,21 = 18.93$, $P < 0.001$.
Sex × Cocaine interaction: $F1,24 = 4.52$, $P < 0.05$.

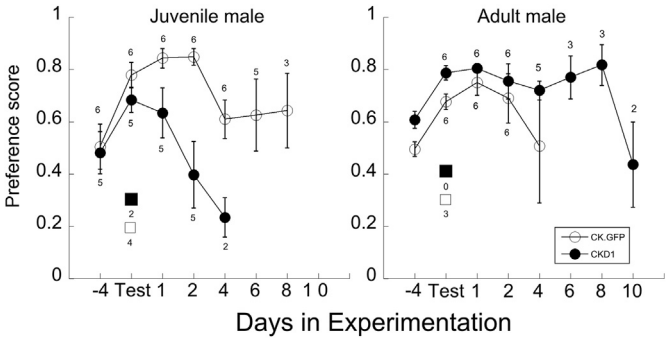

Fig. 1. Days to extinction as a function of age and D1 over-expression by virus. The number of days required to extinguish a place preference score of 0.54 or less for two consecutive days is shown above. Males only were transduced with the CK.GFP/control or CK.D1 virus. Means ± SE were presented across days, with the first data point representing baseline preferences. The number of subjects in each data point is located above. Test represents the preference score upon initial assessment; the number of subjects that did not have a preference score of 0.54 or more are shown for CK.GFP (open box) and CK.D1 (closed box). The remaining scores show the progressive change in preferences in subjects during extinction, with the SE increasing as fewer subjects maintain their preferences and progressively drop out of inclusion.

3.1. Experiment 1: effect of age and D1 over-expression on the rate of extinction in male rats

The number of days to extinguish an original preference score of 0.54 or more was determined for each individual subject. An initial comparison demonstrated no difference between control rats and those transduced with CK.GFP ($p > 0.8$); we collapsed these data into a single control group. Differences in days to extinction were analyzed with an ANOVA that included Age (P21 and 90) and Cocaine (10 and 20 mg/kg) in males only. Statistically, the average number of days to reach extinction is shown in Table 1 below as determined by Kaplan–Meier survival analysis. Days to extinction for individual subjects revealed a significant effect of Age between control P21 males and control P90 males at 10 and 20 mg/kg

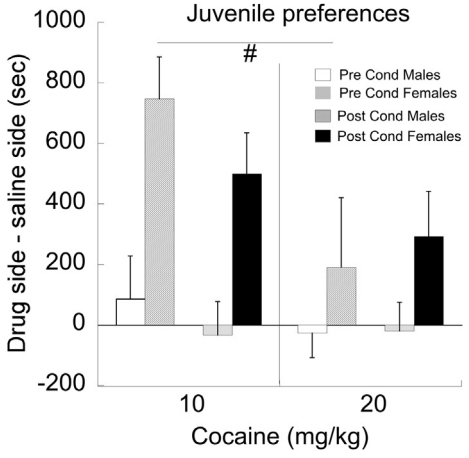

Fig. 2. Place preferences of the juvenile subjects in Experiment 1. Means ± SE are presented for subjects achieving a preference score above 0.54; * indicates $P < 0.05$ differences between pre- and post-conditioning effects; # indicates a significant differences between the post-conditioning effect of 10 and 20 mg/kg in males.

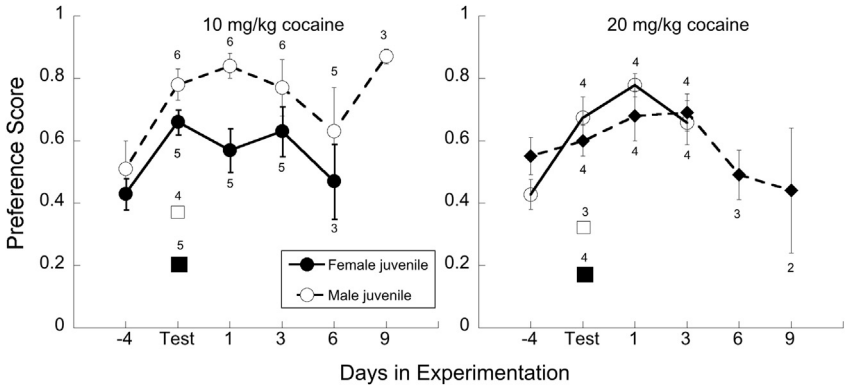

Fig. 3. Changes in preference scores across days of extinction. Means ± SE are presented for male and female P21 subjects when groups were greater than n = 3. The number of subjects in each data point is located above. Individual preferences progressively decline and subjects are eliminated from the average when their preference score is < 0.54. Test represents the preference score upon initial assessment; the closed and open boxes represent the number of subjects that did not have a preference score of 0.54 or more for CK.GFP (open box) and CK.D1 (closed box).

cocaine doses (Age: $F1,24 = 5.14$, $P < 0.05$) that modestly differed by dose (Age × Cocaine: $F1,24 = 3.85$, $P = 0.06$). Extinction took the longest at the 10 mg/kg dose of cocaine in juveniles relative to adults, and decreased at the higher 20 mg/kg dose to near adult levels.

The effect of the over-expression of D1 on glutamate neurons by the CK.D1 virus was then examined at the single dose of 10 mg/kg of cocaine between P21 and P90 males. ANOVA revealed an Age × Condition (D1/Control) interaction ($F1,21 = 18.93$, $P < 0.001$), where CK.D1 facilitated extinction at P21, but prolonged extinction at P90 (Fig. 1). At the 10 mg/kg dose of cocaine, control juveniles took longer to extinguish their preferences relative to adults ($F1,9 = 6.38$, $P < 0.05$) and relative to CK.D1 subjects ($F1,9 = 19.41$, $P < 0.0001$). In contrast, CK.D1 males seemed to extinguish longer, although this was not significant ($F1,9 = 2.35$, $P = 0.16$).

3.2. Experiment 2: sex differences in rate of extinction to preferences for 10 and 20 mg/kg cocaine-associated environments

Shown in Fig. 2 are the conditioned place preferences for male and female juveniles who met the preference score criterion. To be clear, only subjects that had significant place preferences are included. A mixed ANOVA of Cocaine (10/20) × Sex (2) × (conditioning [pre/post]) was used to analyze the data. A lack of a 3-way interaction with Cocaine × Sex × [conditioning] is not surprising given that all subjects were selected for their preferences. A Cocaine × Sex interaction was observed ($F1,24 = 7.66$, $P = 0.01$) and the within-subjects factor of conditioning was also significant ($F1,24 = 29.6$, $P < 0.001$), where each group demonstrated significant conditioning. The interaction was driven by the male post-conditioning group between the 10 and 20 mg/kg doses of cocaine as indicated by post-hoc tests with Bonferroni correction.

Of the subjects that were included in the extinction study, a Sex × Cocaine interaction was evident in the number of days required to extinguish place preferences ($F1,24 = 4.52$, $P < 0.05$). These data are shown in Fig. 3. Preferences to the 20 mg/kg dose extinguished more rapidly in males relative to females and the reverse was true in the 10 mg/kg dose.

The number of days required for each group to reach extinction was analyzed with Kaplan–Meier survival analysis. Table 1 shows survival estimates for each group and the statistical significance as determined by ANOVAS.

3.3. Experiment 4: reinstatement of place preferences following a priming dose of 5 mg/kg cocaine

Reinstatement in D1 over-expressing subjects (Fig. 4) demonstrated a Condition × Age interaction ($F1,20 = 17.13$, $P = 0.001$). Post-hoc comparisons show that juvenile CK.D1 subjects had less reinstatement than their age-matched control, whereas no effect was observed between the adults.

A mixed ANOVA of Sex × Cocaine × (reinstatement [preference scores from last day of extinction/reinstatement]) was used to

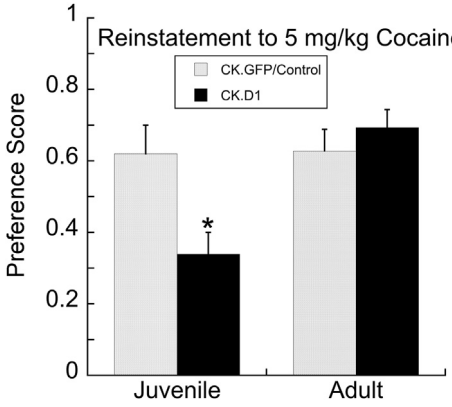

Fig. 4. Reinstatement as a function of D1 over-expression. Means ± SE presented for preference scores; *indicates post-hoc comparison between P21 controls and CK.D1 following a significant interaction at the P < 0.05 level.

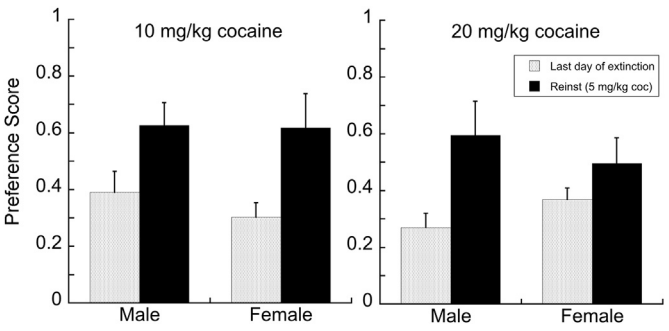

Fig. 5. Reinstatement in P21 control males and females. Means ± SE are presented of the preference scores from the last day of extinction and reinstatement following a priming dose of 5 mg/kg cocaine.

determine the degree of reinstatement (Fig. 5). At P21, reinstatement to cocaine-associated cues after a priming dose was significant in controls, independent of Sex and Cocaine dose (within subjects effect of Reinstatement from last day of extinction: $F1,17 = 17.50$, $P = 0.001$).

4. Discussion

Investigation into the development of the underlying mechanism(s) of extinction training and reinstatement is important for further understanding addictive processes. Extinction, or the gradual decline in behavior when that behavior is no longer reinforced, occurs following the learning of new associations that predict the absence of drug outweigh the previous drug-cue association. We have previously demonstrated that adolescent rats show more resistance to extinction of the drug-cue association unless the absence is made more salient through either explicit pairing of saline and context or through pharmacological manipulation (Brenhouse et al., 2010). Normal adolescent rats also show greater reinstatement to cocaine-associated cues at a low (10 mg/kg) dose of cocaine, but not a higher (20 mg/kg) dose, relative to adult rats (Brenhouse and Andersen, 2008). Greater sensitivity to cocaine reinstatement by D1-overexpressing adolescent rats is reminiscent of the findings by Ciccocioppo et al. (2001) who demonstrated that elevated D1 receptors in the plPFC were associated with high relapse rates following extensive cocaine exposure. In the current study, D1 receptors were elevated on plPFC glutamatergic projection neurons by lentiviral vectors in both juvenile and adult males. The results show developmentally-divergent effects of D1 overexpression which: 1) facilitates extinction in juveniles; and 2) prolongs extinction in adult rats (which was not significant) similar to adolescent rats (Brenhouse et al., 2008). These results suggest that the maintenance of the drug-cue association is mediated in part by D1 receptors – but on the background of other systems whose maturation is neither fully understood nor investigated here.

Typical juveniles used in this study (who notably are distinct from a majority of their peers by forming a preference for cocaine-associated environments [>0.54 preference score]) show prolonged extinction relative to adults most strongly at 10 mg/kg cocaine. Both the plPFC and the amygdala (basolateral; BLA) are involved in cocaine-associated memories (Miller and Marshall, 2004, 2005). Activity in both of these regions is observed during extinction learning (Nic Dhonnchadha et al., 2012). Cocaine-associated memories are susceptible to disruption during both retrieval and reconsolidation, suggesting either process or both could be altered by D1 expression. A recent study shows that a systemic injection of the D1 antagonist SCH-23390 blocks the post-conditioning retrieval of extinction memories, resulting in prolonged extinction (Fricks-Gleason et al., 2012). Normal juveniles may have relatively low levels of activity in general to dopamine stimulation due to PFC immaturity (Andersen et al., 2001) and/or low D1 receptor expression (Andersen et al., 2000) that may prevent new learning required in extinction processes.

D1 over-expression in juvenile males facilitated extinction and reduced reinstatement in contrast to controls and adolescents (Brenhouse and Andersen, 2008) and adults, suggesting that immaturity reduced the ability to make enduring drug-cue associations or to express these associations in the same way as more mature rats. Consistent with the first hypothesis, juveniles require more cocaine than adults to form an initial place preference to cocaine (Brenhouse et al., 2008) or simultaneous elevated D1 activity in the plPFC and dopamine activity-presumably in the accumbens – for such associations to form. Neuronal activity within the plPFC, but not the ilPFC, is observed during self-administration and extinction of cocaine and its cues (West et al., 2014). These two regions influence extinction in opposing ways as described by the oversimplified Go/Stop model (Moorman et al., 2014; Peters et al., 2009). Localized overexpression of the D1 dopamine receptor in the plPFC may facilitate the acquisition of the new (extinction) memory of the context that is now paired with the absence of cocaine; this context itself may itself gain heightened motivational salience (Brenhouse and Andersen, 2008; Waylen and Wolke, 2004). This new extinction memory could be formed in an unbiased context (where the rat is free to explore both saline- and drug-paired sides of the chamber) as D1 is sufficiently elevated. The prediction follows that D1 over-expressing juveniles would show more rapid extinction in a biased context, consistent with what we previously observed in adolescents who naturally over-express D1 (Brenhouse and Andersen, 2008).

The second hypothesis, which is not necessarily independent from the first hypothesis, is related to the role of immature circuitry. In addition to the plPFC, the infralimbic PFC (ilPFC) plays a role in the *consolidation* and *expression* of extinction memories, including cocaine cues (LaLumiere et al., 2010). Notably, the role of the ilPFC and cocaine cue extinction has not been investigated in immature systems. In addition, BLA activity also facilitates extinction (Schroeder and Packard, 2003, 2004). As the PFC innervates the BLA by 28 and 45 days (Cressman et al., 2010), elevated D1 in the plPFC could facilitate extinction by modulating the BLA. D1 enhancement of the function of these connections could increase extinction in the juveniles. Consistent with enhanced BLA activity, a recent fMRI study shows that viral-mediated enhancement of D1 receptors in the juvenile plPFC elevated BLA activity in response to cocaine-associated cues (Lowen et al., submitted for publication).

The hypothesis of prolonged extinction and reinstatement in D1 overexpressing adult rats was initially based on greater reinstatement of self administration in subjects that had a chronic history was related to D1 receptors in the PFC (Ciccocioppo et al., 2001) or the reinstatement of place preferences that could be blocked by a microinjection of a D1 antagonist into the PFC (Sanchez et al., 2003). Later studies localized the self-administration effects to increased glutamate activity into the accumbens from the plPFC (McFarland et al., 2003) that could be blocked with a microinjection of the D1/D2 antagonist fluphenazine (McFarland et al., 2003). Here, D1 subjects had minimal (two conditioning days plus one prime) cocaine exposure and showed increases in days to extinction, but had no significant effect on reinstatement in adults. One plausible reason may be due to the selection of subjects for the controls. Only adults with *a priori* preferences were selected (preference ratio >0.54), suggesting they may possibly have elevated D1 naturally or some addiction-biasing mechanism that would increase their reinstatement scores. Elevation of only D1 in the plPFC in adults prolonged the number of days to extinction. It has been proposed that the ilPFC indirectly inhibits the plPFC circuits to the nucleus accumbens to decrease cocaine-seeking behavior (Peters et al., 2008). Under high plPFC D1, this inhibitory mechanism may be over-ridden and extinction protracted.

Surprisingly, we observed modest sex differences in extinction, but not reinstatement. An interaction was evident in the days to extinction. Developing females are equally sensitive as males to the locomotor effects of cocaine pre-pubertally (Kuhn et al., 2001), and more sensitive to low doses of cocaine than males during adolescent place conditioning (Brenhouse et al., 2009; Zakharova et al., 2009). This increased sensitivity to cocaine in females, we believe, played a role in noticing cocaine's absence, and extinguished faster. The reverse was observed in males. Reasons for this modest difference are not known, although these data suggest that increases in gonadal hormones likely play a role. Estrogen levels increase as juvenile females progressed through the behavioral

paradigms across ~12 days. By P28, plasma estrogen levels reach a peak (Hacik, 1978). Estrogen increases cocaine sensitization by modulating D1 receptors in the PFC (Zhen et al., 2007). The estrogen/D1 interaction could explain the protracted extinction of cocaine place preferences in adult female mice compared with males (Hilderbrand and Lasek, 2014), although no such sex difference was observed in the degree of extinction in adult rats (Bobzean et al., 2010). Estrogen increases the rate of self-administration of cocaine (Becker and Hu, 2008), whereas ovariectomy reduces activity in females relative to males (Kuhn et al., 2001). We have observed both increased sensitivity to cocaine and self-administration in D1 over-expressing males (Sonntag et al., 2014) – independent of estrogen.

In conclusion, juvenile male rats show prolonged extinction and increased reinstatement to cocaine-associated contexts relative to adult males. Males took significantly longer than females to extinguish preferences in response to a low dose of cocaine, whereas female preferences extinguished in fewer days at the higher dose of cocaine. When D1 dopamine receptors were over-expressed in the plPFC, a region implicated in the formation of associations and their expression, juvenile males had fewer days to extinction and less reinstatement than adult males. This observation suggests a D1-mediated facilitation in new learning that is mediated by the plPFC. In contrast, the prolonged extinction that was observed in D1 over-expressing adult males may reflect contributions of the ilPFC to this process where strong original drug-cue associations prevented new learning. Additional studies should focus on how D1 and maturation interact at different points in extinction consolidation and retrieval processes.

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