Behavioral/Systems/Cognitive

Transient D_1 Dopamine Receptor Expression on Prefrontal Cortex Projection Neurons: Relationship to Enhanced Motivational Salience of Drug Cues in Adolescence

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Adolescence is a transitional period during development that is associated with a greater likelihood of addiction to drugs than any other age. In the prefrontal cortex (PFC), D_1 dopamine receptors mediate motivational salience attribution, which plays a role in addiction. Here, we investigated the relationship of age-related D_1 dopamine receptor expression in the PFC with the maturation of cocaine place conditioning. Confocal microscopy revealed that retrogradely traced cortical output neurons to the nucleus accumbens express higher levels of D_1 receptors during adolescence compared with younger and older ages. D_1 expression does not change on GABAergic interneurons across age. Adolescent differences in D_1 expression occur independently of cortical-accumbens connectivity, which proliferates through adulthood. Behaviorally, adolescent rats are more sensitive to cocaine place conditioning than younger and older rats. However, microinjections of the D_1 antagonist SCH23390 into the PFC blocked adolescent place preferences, whereas microinjections of D_1 agonists dose-dependently increased preferences for cocaine-associated environments previously not preferred by juveniles. These results suggest that the heightened expression of D_1 receptors on cortical-accumbens projections may help explain increased sensitivity to environmental events and addictive behaviors during adolescence, whereas the paucity of D_1-expressing projections may reduce risk in juveniles.

Key words: **adolescence; cocaine; place conditioning; retrograde tracer; D_1 dopamine; PFC**

Introduction

Adolescence is an important transitional stage that comprises many of the motivated behaviors that characterize substance use (Spear, 2000; Laviola et al., 2003), placing adolescents at increased risk for drug abuse and addictions (O'Brien and Anthony, 2005). Drug-cue associations play a major role in the drug-seeking behavior that leads to addiction (Goldstein and Volkow, 2002). Drug-seeking depends on motivational circuits in the frontal cortex (Grant et al., 1996; Maas et al., 1998; Tzschentke, 2000; Volkow et al., 2005; Hyman et al., 2006), and is modeled in animals through repeated pairings of drug and a particular environment (Carr et al., 1988; Everitt et al., 1991; Tzschentke and Schmidt, 1998; Carlezon, 2003). Adolescent rats display heightened responding in such models, requiring lower doses of cocaine than adults to form preferences for cocaine-associated environments (Badanich et al., 2006). Psychostimulants are argued to increase the salience of environmental stimuli through activation of cortical circuits (Volkow et al., 2002). Similarly, heightened salience attribution may explain the ability of nicotine to produce place preferences during adolescence, but not adulthood, in rats (Belluzzi et al., 2004; Leslie et al., 2004) and its high abuse in teenagers (Kandel and Chen, 2000). Although growing evidence implicates cortical maturation in the high vulnerability to addiction in adolescence (Crews et al., 2007), little is known about the developmental changes in the receptor systems that mediate incentive salience.

Dopaminergic (DA) regulation of glutamatergic output from the prefrontal cortex (PFC) to subcortical systems, including the nucleus accumbens (NAc), mediates drug seeking (Piazza et al., 1991; Robinson and Berridge, 1993; Kalivas et al., 1998; Blum et al., 2000; Bjork et al., 2004; Matthews et al., 2004). DA D_1 receptors enhance this cortical drive, as their role in self-administration (Alleweireldt et al., 2002; Kalivas et al., 2005) and reinstatement of conditioned place preference (Sanchez et al., 2003) suggests in adult rats. Under addictive states, drug-associated stimuli become prepotent at the expense of other information through selective D_1 modulation of glutamatergic neurons (Everitt and Wolf, 2002; Kalivas et al., 2005). During development, PFC D_1 density and associated second messenger activity rises dramatically between postnatal day 25 (P25; juvenile, preadolescent stage) and P40 (adolescence), with a subsequent reduction by P100 (full adulthood) in rats (Andersen et al., 2000).

Cocaine-seeking behavior was assessed here in a place-conditioning paradigm across ages with low PFC D_1 expression (juveniles and adults) compared with an age of relatively higher D_1 expression (adolescence). Retrograde track-tracing was used to assess the development of PFC-NAc connectivity. To deter-

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mine whether D_1 receptors on projections to the NAc, which are particularly important in salience attribution (Kalivas et al., 2003; Seamans and Yang, 2004), are uniquely expressed in adolescence, age-dependent localization of PFC D_1 expression on GABAergic interneurons or glutamatergic output neurons to the NAc core was examined with confocal microscopy. Finally, to investigate the role of PFC D_1 in drug-seeking through development, the effects of cortical microinjections of D_1 agonists and antagonists on cocaine place conditioning were evaluated in juveniles and adolescents.

Materials and Methods

Subjects

Male Sprague Dawley rats, obtained from Charles River Laboratories (Boston, MA), were used in these studies. Rats were housed with food and water available *ad libitum* in constant temperature and humidity conditions on a 12 h light/dark cycle (light period, 7:00 A.M. to 7:00 P.M.). These experiments were conducted in accordance with the National Institutes of Health 1996 *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee at McLean Hospital. Three age groups were used. Litters of eight males at P18–P19 were obtained from Charles River Laboratories, were weaned at P21, and were tested at P27 (juveniles). One rat per litter was used in each group. Adolescents and adults were obtained directly from Charles River Laboratories and were allowed 1 week to acclimate to our facilities.

Experiment 1: determine the effect of age on cocaine place conditioning across three doses

Subjects. Rats in all three age groups were used ($n = 5–8$). Juveniles were P27 on test day, adolescents were P44 on test day, and adults were age P105 on test day.

Experimental design. An unbiased place conditioning protocol was used, as previously published (Andersen et al., 2002b). Conditioning was done in a three-chamber apparatus, consisting of two large (24 × 18 × 33 cm) side compartments that differed in lighting and floor texture, and a small (12 × 18 × 33 cm) middle compartment. Rats were habituated to the entire apparatus for 30 min on day 1. Rats with a preference to either side (>18 of 30 min) were eliminated from further testing, and groups were organized such that average baseline preferences were minimized. Two days of conditioning, with two sessions per day, were conducted, during which rats were injected with saline (1 ml/kg, i.p.) in the morning and placed in one side, and 4 h later injected with one of three doses of cocaine (10, 20, or 40 mg/kg, i.p.) and placed into the opposite side. Moderate-to-high doses were chosen to parse age effects on responsivity to a moderate dose, as well as to provoke preferences for a high dose at ages of low sensitivity. On the fourth day, rats were permitted to freely explore the entire apparatus for 30 min in a drug-free state. To determine the effect of age on conditioning, two-way age by conditioning (pre vs post) ANOVA was performed within each dose of cocaine, with conditioning as a repeated measure. Where significant interactions were found, *post hoc* Student's *t* test comparisons were used to determine which age groups showed significant conditioning effects. To determine age-dependent shifts in the dose–response curve, the ED_{50} of cocaine at each age was analyzed using a sigmoidal dose–response curve-fitting program (Prism; GraphPad, San Diego, CA) and compared with each other using one-way ANOVA.

Experiment 2: ascertain developmental shifts in D_1 receptor distribution on GABAergic interneurons and excitatory projection neurons in the prefrontal cortex

Subjects. Six juvenile rats (which were P21 on surgery day and P27 when brains were harvested), six adolescent rats (which were P39 on surgery day and P44 when brains were harvested), and five adult rats (which were P100 on surgery day and P105 when brains were harvested) were used.

Retrograde tracer microinjections. Rats were anesthetized with a ketamine/xylazine mixture, and a Hamilton syringe was stereotaxically lowered into the NAc. Coordinates for juveniles were anteroposterior (AP) +2.1, mediolateral (ML) +1.3, and dorsoventral (DV) −6.0, and coordinates for adolescents were AP +1.8, ML +0.7, and DV −6.0 (Sherwood and Timeras, 1970). Adolescent injections were angled at 3° to avoid the sinus, which was a problem in pilot surgeries. Coordinates for adults were AP +1.6, ML +1.2, and DV, −6.8 (Paxinos and Watson, 1998). One microliter of 660λ fluospheres (Invitrogen, Eugene, OR) was injected over 2 min, after which the needle remained in the brain for 1 additional minute to ensure diffusion into the surrounding tissue. Fluo-

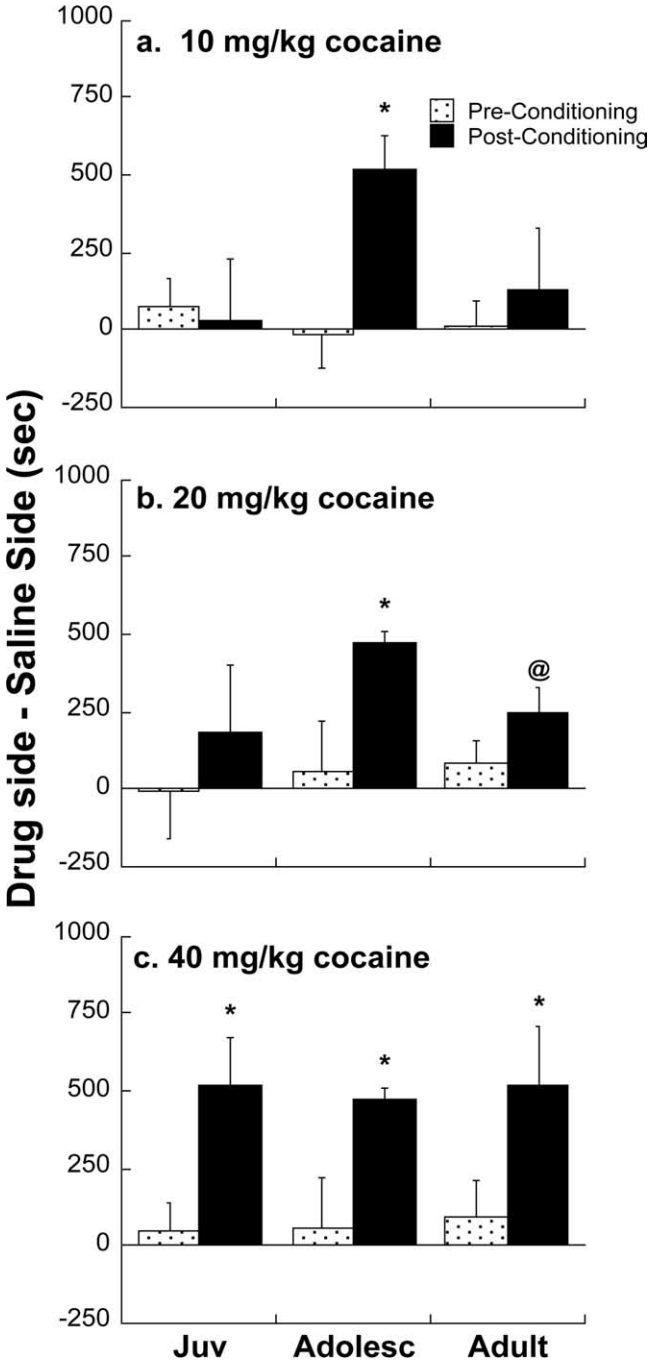

Figure 1. Effect of age on cocaine place conditioning across three doses of cocaine. ***a***, A significant interaction of age and conditioning was found at 10 mg/kg cocaine ($F_{(2,20)} = 3.63$; $p < 0.05$) with adolescents (P41; $^*p < 0.01$), but neither juveniles (P27) nor adults (P100), forming a conditioned preference at this dose. ***b***, A significant interaction of age and conditioning was also found at 20 mg/kg cocaine ($F_{(2,13)} = 4.87$; $p < 0.05$), with adolescents ($^*p < 0.05$), but neither juveniles nor adults ($^@p = 0.07$), forming a conditioned preference at this dose. ***c***, All three age groups formed a robust conditioned place preference for a context paired with 40 mg/kg cocaine ($^*p < 0.05$).

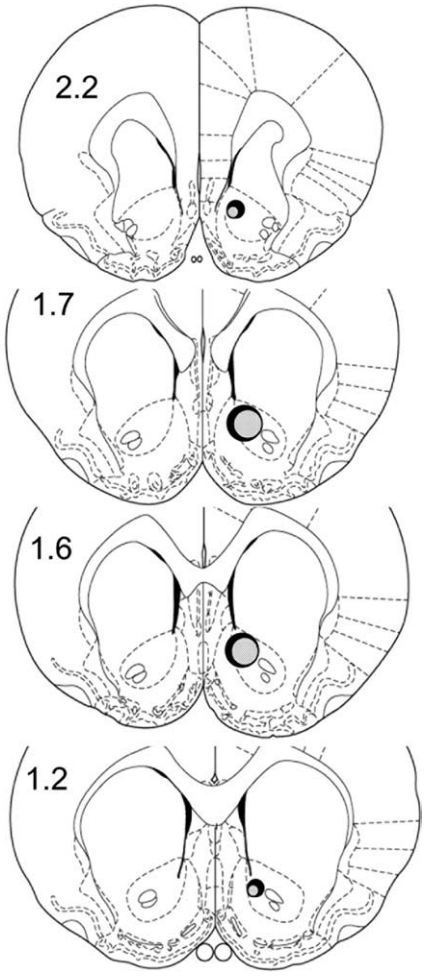

Figure 2. Histological analysis of intra-NAc fluosphere injection sites. Numerals indicate millimeters from bregma. The largest (solid) and smallest (shaded) bolus sizes of all animals included in analysis are indicated. All injection sites were within ±5% in size.

spheres were used because fibers of passage are not affected such that only direct projections are stained (Katz et al., 1984). Correct placement within the dorsomedial region of the NAc core was verified under 10× magnification using a Cy5 filter to view the bolus of fluosphere injection. Serial sections (at 320 μm intervals) throughout the NAc were examined, the number of sections with evidence of injection bolus was recorded, and the area of the fluosphere spread was quantified using Leica (Heidelberg, Germany) confocal imaging software. All brains contained evidence of the injection bolus in four to five sections, with the cross-sections of the widest part ranging from 390,000–410,000 μm^2.

Fluorescence immunohistochemistry. Five to 7 d after retrograde tracer microinjections, rats were deeply anesthetized and were perfused with ice-cold PBS, pH = 7.4, and then 4% paraformaldehyde. Brains were removed and postfixed overnight, then saturated in 30% sucrose. 40 μm sections were washed three times for 5 min in PBS, blocked for 60 min in 10% donkey serum in PBS, and incubated at 4°C overnight with mouse anti-GAD67 IgG (1:2000; Millipore, Billerica, MA) and rat anti-D1R IgG (1:250; targeting the intracellular C-terminus; Sigma, St. Louis, MO) diluted in 2% normal goat serum, 2% bovine serum albumin, and 0.2% Triton X in PBS. After washing three times for 5 min with 0.2% Triton X in PBS, sections were incubated for 60 min with anti-mouse tetramethylrhodamine isothiocyanate (TRITC)-coupled IgG (1:200; Invitrogen) and anti-rat Alexa 488-coupled IgG (1:200; Invitrogen). Sections were then washed a final time and mounted on gelatin-coated slides with a mounting medium that contained anti-fading agents (Gel/Mount; Biomeda, Foster City, CA). A subset of cortical sections was treated as above only using a primary mouse antibody to α type-II calcium calmodulin-dependent kinase (CAMK-II) IgG (1:1000; Millipore) and an anti-mouse Alexa 568-coupled secondary IgG (1:200; Invitrogen). This was done to positively identify retrogradely traced cells as glutamatergic projection neurons (Liu and Jones, 1996).

Confocal microscopy and analysis. High-magnification digitized images of neurons showing retrograde tracer were made with a 40× oil-immersion lens and confocal microscopy (Leica). A *z*-series stack of images was acquired using 2 μm step intervals for 7–10 consecutive focal planes. Regions of interest (ROIs) were chosen within the medial PFC (mPFC) based on prominence of retrograde fluosphere-labeled cells as identified by scanning at 10× (which fell within the prelimbic region of the mPFC). Within each ROI, *z*-series stacks were generated for each individual cell with the FITC [for D_1-like immunoreactivity (IR)], TRITC (for GAD67-like IR), and Cy5 filters (to view retrograde fluospheres), respectively, to guard against false positives. Three ROIs were generated per animal. Within each region, individual tracer- or GAD67-labeled cells were counted, selected and measured for D_1 IR across focal planes. The density of D_1-labeled cells that were colocalized with either tracer or GAD67 was recorded, and these values were compared between ages using ANOVA with least-significant difference (LSD) *post hoc* analysis. As well, the number of each cell-type was compared between ages using one-way ANOVA with LSD *post hoc* analysis.

Experiment 3: determine the effects of D_1 agonist microinjections in the PFC on preadolescent and adolescent drug-seeking

Subjects. Nine groups of juvenile rats (n = 5–7) and four groups of adolescent rats (n = 6–7) were used in this experiment. Juveniles were P21 on day of cannula implantation and P27 on test day, and adolescents were P38 on day of cannula implantation and P44 on test day.

Cannula implantation surgery. Rats were anesthetized with ketamine/xylazine and were implanted with bilateral 26-gauge stainless-steel guide cannulas (Plastics One, Roanoke, VA) above the mPFC (stereotaxic coordinates for juveniles: AP +2.8, ML ±0.6, DV −1.6; adolescents: AP +3.8, ML ±0.6, DV 1.6) (Sherwood and Timeras, 1970). Animals were given 2 full days after surgery to recover before experimental procedures commenced.

Place conditioning. An unbiased place conditioning protocol was used, as described in experiment 1. During conditioning days, animals were injected with saline (1 ml/kg, i.p.) in the morning and placed in one side, and 4 h later injected with cocaine (10 mg/kg, i.p.) and placed into the opposite side, of the conditioning apparatus. Ten minutes before the afternoon injections, juvenile rats were infused bilaterally with vehicle, 1 of 3 doses of SKF38393 (partial D_1 agonist; Sigma, St. Louis, MO; 0.1 μg/0.3 μl/side, 0.3 μg/0.3 μl/side, or 1.0 μg/0.3 μl/side), or 1.0 μg/0.3 μl/side of the full D_1 agonist SKF81297 (Sigma), using an injector cut to protrude 1 mm beyond the guide cannula (Plastics One). Three groups of adolescent rats were conditioned identically, but were microinjected with vehicle, 1.0 μg/0.3 μl/side of SKF81297, or 1.0 μg/0.3 μl/side of the D_1 antagonist SCH23390 (Sigma). To rule out the possibility that D_1 agents themselves caused preferences or aversions, three additional groups of juvenile rats were treated with the three doses of SKF38393 and injected with saline (1 ml/kg) instead of cocaine during the afternoon place conditioning. One additional group of adolescents was treated with SCH23390 and given saline in place of cocaine.

Placement verification. After testing, rats were infused bilaterally with 1% toluidine blue (Sigma; 0.3 μl/side) and 10 min later were deeply anesthetized and intracardially perfused with PBS and then 4% paraformaldehyde. Brains were cut into 40 μm sections and stained with cresyl violet. The placement and spread of infused dye was observed with a light microscope.

Analysis. The dose-dependent effects of SKF38393 on cocaine place conditioning were analyzed using a two-way (dose SKF38393 by conditioning) ANOVA with repeated measures for conditioning. Treatment effects of D_1 drugs on adolescent place conditioning were also analyzed using two-way (treatment by conditioning) ANOVA with repeated measures for conditioning (pre vs post). Where significant interactions were found, Student's *t* test comparisons were performed to find effects of conditioning after each treatment.

Results

Experiment 1: effect of age on place conditioning

Figure 1 illustrates that rats of different ages respond differently to the place conditioning effects of cocaine. Two-way ANOVA revealed a significant interaction of age and conditioning when rats were administered either 10 mg/kg cocaine (Fig. 1*a*) ($F_{(2,20)} = 3.63$; $p < 0.05$) or 20 mg/kg cocaine (Fig. 1*b*) ($F_{(2,15)} = 5.35$; $p < 0.05$). Specifically, only adolescents formed a significant preference for a drug-associated environment when conditioned to 10 mg/kg cocaine ($p = 0.002$) or 20 mg/kg cocaine ($p = 0.026$). Adult conditioning to 20 mg/kg cocaine approached significance ($p = 0.07$). No interaction was seen at the 40 mg/kg dose (Fig. 1*c*), with all three age groups forming a preference for environments paired with 40 mg/kg cocaine ($p < 0.05$ at all ages). When comparing the ED_{50} of the conditioning effects of cocaine across ages, a significantly lower ED_{50} ($F_{(2,15)} = 149.3$; $p < 0.01$) was found in adolescents, compared with younger and older rats.

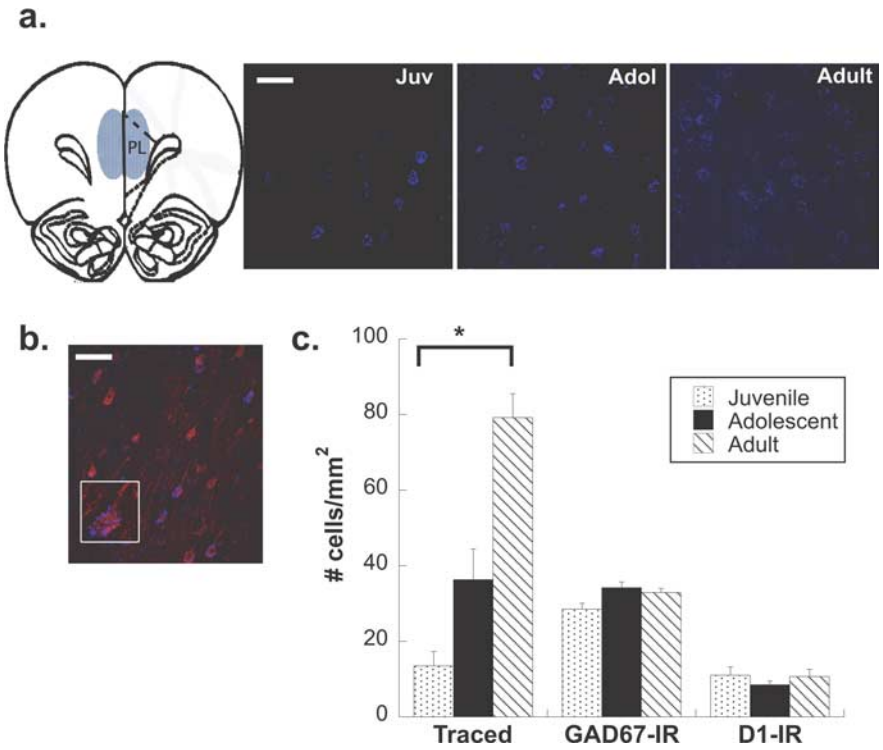

Figure 3. Age-dependent density of projection neurons terminating in the NAc core, as well as GABAergic interneurons and D_1-expressing neurons, in the PFC. ***a***, Representative ROI from each of three analyzed ages (juvenile, P27; adolescent, P44; adult, P105) showing age-related changes in retrogradely traced neurons. Left, Atlas plate illustrating the area of PFC within which all ROIs resided (prelimbic region of the PFC). Scale bar, 50 μm. ***b***, Representative ROI showing double-labeling with retrograde tracer (blue) and CAMK-II IR (red), demonstrating that traced cells are glutamatergic projection neurons. Inset, A single double-labeled cell. Scale bar, 50 μm. ***c***, Graphic representation of the density of retrogradely traced projection neurons, and GAD67- and D_1-immunoreactive neurons, across three ages. As pictured in ***a***, density of traced neurons increased progressively with age ($F_{(2,13)} = 24.8$; $^*p < 0.001$).

Experiment 2: development of PFC microcircuitry

Retrograde fluospheres injected into the dorsomedial quadrant of the NAc core (Fig. 2) were found in the prelimbic region of the PFC at all age groups (Fig. 3 a), and were identified as glutamatergic via CAMK-II-like IR (Fig. 3*b*). All rats included in analysis had similar sized (within ± 5%) fluosphere injection sites that were restricted to the NAc core. Figure 3*c* illustrates the density of each cell type analyzed across ages. The density of traced projection neurons increased with age (Fig. 3*a*,*c*) ($F_{(2,13)} = 24.8$, $p < 0.001$) with adolescents showing more traced neurons than juveniles ($p = 0.026$) and adults having more traced neurons than adolescents ($p = 0.001$). Age had no effect, however, on the overall density of GAD67- or D_1-immunoreactive neurons in the prelimbic PFC (Fig. 3*c*).

D_1 was colocalized on retrogradely traced neurons to a significantly greater extent in adolescents compared with juveniles and adults ($F_{(2,13)} = 13.4$; $p = 0.001$), with an average of 44% of all D_1 labeling being distributed on traced projection neurons in adolescents (3.4 ± 0.5 cells/mm^2), compared with only 2% (0.4 ± 0.3 cells/mm^2) and 6% (0.5 ± 0.4 cells/mm^2) in juveniles and adults, respectively (Fig. 4*a*,*d*). However, distribution of D_1 on GABAergic interneurons remained consistent across age groups (Fig. 4*b*,*d*). A third population of cells was identified as those that were D_1-immunoreactive, but not costained with retrograde tracer or GAD67 (Fig. 4*c*). The percentage of D_1 that fell into this category also did not differ significantly across ages, yet appeared slightly higher in juveniles compared with the older two ages.

Experiment 3: PFC D_1 effects on juvenile and adolescent drug seeking

All animals included in analysis had microinjection placements verified in the prelimbic region of the PFC, with a small degree of spread into the infralimbic region (Fig. 5). Administration of the partial D_1 agonist SKF38393 during conditioning dose-dependently increased the amount of time juveniles spent in a cocaine-paired context during a drug-free test ($F_{(3,20)} = 3.36$, $p < 0.05$) (Fig. 6*a*). Only at the high dose of SKF38393 (1.0 μg) did rats spend significantly more time in the drug-paired chamber after conditioning compared with preconditioning ($p = 0.015$). The full D_1 agonist SKF81297 had similar effects; administration of a high dose (1 μg) during conditioning also resulted in significantly more time spent in the cocaine-paired chamber after conditioning ($p = 0.033$) (Fig. 6*b*) compared with vehicle-infused animals ($F_{(1,13)} = 5.0$; $p < 0.05$). There was no significant difference between the effects of 1.0 μg of the partial and full agonists. Additionally, rats did not form a place preference with SKF38393 when the environment was paired with vehicle (Fig. 6*c*).

Figure 7 shows that D_1 manipulation also significantly effected adolescent place conditioning to 10 mg/kg cocaine ($F_{(2,17)} = 3.7$; $p < 0.05$). Whereas adolescents administered vehicle into the PFC before cocaine conditioning formed significant place preferences for the drug-paired chamber ($p = 0.02$), those administered the D_1 antagonist SCH23390 did not form conditioned place preferences ($p = 0.67$). When adolescents were administered the D_1 agonist SKF81297, average preferences for the drug-paired chamber were comparable with vehicle-infused animals, although variability was decreased and, therefore, conditioning effects were greater ($p = 0.001$).

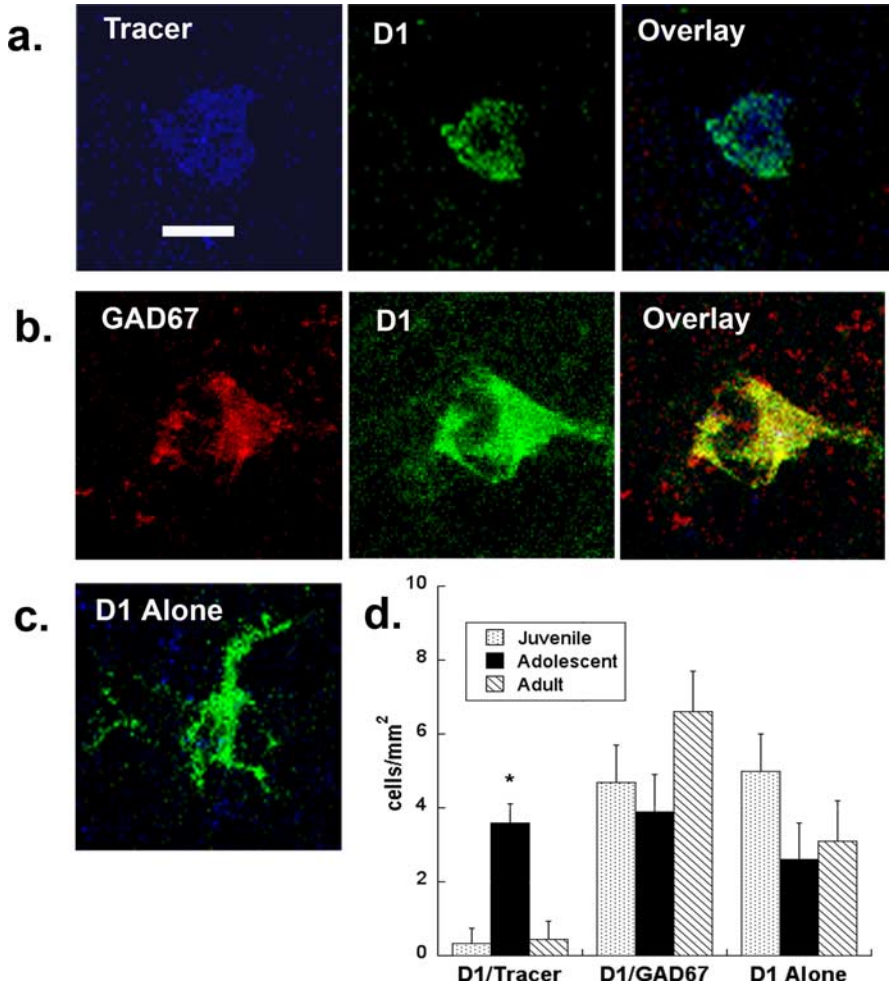

Figure 4. Differential distribution of D_1 on PFC projection neurons and GABAergic interneurons across three ages. Sections already labeled with retrograde fluospheres were double-stained for the D_1 dopamine receptor and GAD67 (a marker for GABAergic interneurons). ***a***, ***b***, Example of a neuron colocalized with retrograde tracer (blue) and D_1 (green; ***a***); and a neuron colocalized with GAD67 (red) and D_1 (green; ***b***), with an overlay of both channels shown in right panels. ***c***, Example of a D_1-immunoreactive cell not colocalized with either GAD67 or tracer. ***d***, Graphic representation of the density of D_1-labeled cells that were colocalized with tracer, GAD67, or neither, across three ages (P27, P44, and P105). D_1 expression on retrogradely traced projection neurons was significantly different across ages ($F_{(2,13)} = 15.4$; $^*p < 0.01$), with a peak colocalization during adolescence. Scale bar, 5 μm.

Discussion

These results suggest that developmental changes in PFC D_1 receptor expression influence cocaine-cue associations during adolescence. Relative to younger and older subjects, adolescents show a leftward-shift in the dose–response to the conditioning effects of cocaine. Connectivity alone between the PFC and NAc fails to explain the degree of sensitivity to cocaine; PFC-NAc projections increase in density between preadolescence and adulthood, consistent with the maturation of DAergic and glutamatergic innervation from areas such as the VTA (Kalsbeek et al., 1988) and amygdala (Cunningham et al., 2002). Previous research in adult rats suggests that cortical D_1 receptors on glutamatergic output neurons play a role in the formation of drug–cue associations (Kalivas et al., 2005). Although neither GABAergic interneuron density nor its colocalization with D_1 changed across age, we found elevated D_1 expression on PFC-NAc projection neurons during adolescence relative to juveniles and adults. Finally, direct PFC D_1 inactivation blocked place preferences in adolescents, whereas D_1 activation elicited the formation of preferences for environments associated with a moderate dose of cocaine in juvenile rats.

Age effects on cocaine place conditioning

Preadolescent rats (P27) did not form a preference for a cocaine-paired context (10 and 20 mg/kg) using a 2 d unbiased conditioning paradigm. Comparatively, adolescents (P44) formed a strong preference for a context paired with the lowest (10 mg/kg) dose of cocaine used, corroborating findings from Schramm-Sapyta et al. (2006). This is consistent with the notion that adolescence is characterized by a high sensitivity to reward (Chambers et al., 2003; Tirelli et al., 2003; Ernst et al., 2006), although this sensitivity may partly be caused by enhanced cortical drive as suggested here. Adolescents also maintain context-drug associations 75% longer than adults during extinction of cocaine-conditioned place preferences and demonstrate stronger drug-primed reinstatement (Brenhouse and Andersen, 2008). Together, these data suggest greater salience attribution to cocaine-paired cues during adolescence.

Place preference tests have an extensive underlying theoretical framework (Tzschentke, 1998; Bardo and Bevins, 2000) involving the processes by which neutral stimuli acquire incentive salience (Berridge and Robinson, 2003). The assessment of motivational responsiveness to stimulants during development is not well defined in the literature for a number of reasons. Inconsistencies in paradigms used, handling stressors, ages, sex (Campbell et al., 2000), drug doses, and the use of stimulants with different behavioral sequelae, such as stereotypy, yield variable conclusions, ranging from decreased amphetamine place conditioning to a heightened sensitivity to cocaine place conditioning in adolescence (Campbell et al., 2000; Tirelli et al., 2003; Badanich et al., 2006; Balda et al., 2006; Schramm-Sapyta et al., 2006) (for review, see Brenhouse and Andersen, 2008). For example, handling is one such variable to consider. Handling history may account for enhanced preferences for lower doses of cocaine, as suggested by comparisons across several studies with and without previous handling (Andersen et al., 2002b; Aberg et al., 2007; Brenhouse and Andersen, 2008). Together, age-related differences in the place-conditioning effects of cocaine reported here are consistent with an enhanced sensitivity to these effects in adolescent rats, when examining male animals in an unbiased place-conditioning paradigm.

PFC D_1 receptor distribution over development

Previous studies have shown that D_1 density peaks in adolescence (Andersen et al., 2000). The present study expands on this observation by showing qualitative differences in the neuronal phenotype where D_1 is expressed in the PFC, but did not quantify D_1. In general, no change in the number of D_1-expressing cells implies that the overall rise is caused by an upregulation of the protein within each neuron. Although D_1 IR on the number of GABAer-

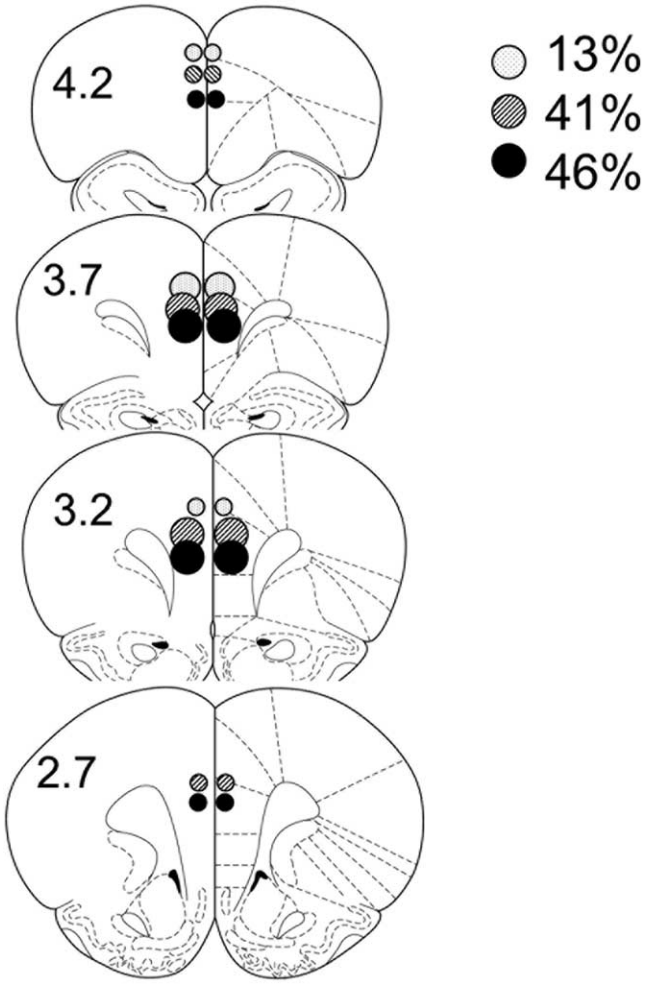

Figure 5. Histological analysis of intra-PFC bilateral cannulas placements. Numerals indicate mm from bregma. All injection sites, as verified by dye spread, fell within the prelimbic region of the PFC, with minimal spread into area 1 of the cingulate cortex and infralimbic regions. Shading indicates percentage of subjects with dye boluses within specified boundaries. Placements located outside of these anatomical regions were excluded from analysis.

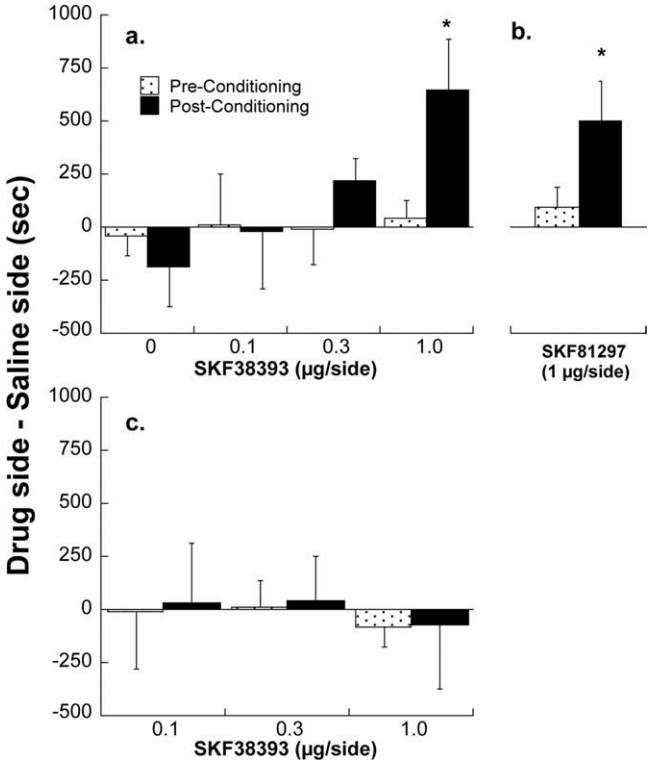

Figure 6. Provocation of drug-seeking in preadolescents with PFC microinjection of D_1 agonists. ***a***, Infusion of the partial D_1 agonist SKF38393 directly into the PFC dose-dependently increased cocaine place preference to 10 mg/kg in juvenile rats ($F_{(3,20)} = 3.36$; $p < 0.05$). Juveniles infused with vehicle failed to display a preference for the cocaine-paired chamber, whereas those administered the high dose of SKF38393 during conditioning showed a significant postconditioning preference for the cocaine-paired chamber (*$p < 0.05$). ***b***, Infusion of the full D_1 agonist SKF81297 also provoked a significant place preference for 10 mg/kg cocaine (*$p < 0.05$). ***c***, SKF38393 did not generate a preference for a saline-paired chamber.

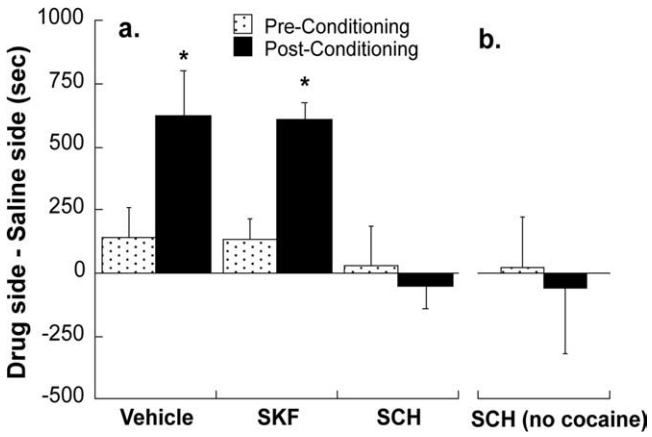

Figure 7. Effects of cortical D_1 activation and inactivation on cocaine conditioned place preference in adolescents. ***a***, Infusion of either vehicle (*$p < 0.05$) or the D_1 agonist SKF81927 (*$p < 0.01$) resulted in significant place preferences for 10 mg/kg cocaine. Infusion of the D_1 antagonist SCH23390 blocked these preferences, resulting in no conditioning effects of the same dose of cocaine. ***b***, Infusion of the D_1 antagonist SCH23390 into the PFC had no conditioning effect on its own when saline was substituted for cocaine.

gic interneurons did not change significantly across ages and was consistent with previous reports in adults (Vincent et al., 1995), D_1 IR on neurons projecting to the NAc rose from <4% of total D_1-labeled cells to >40% in adolescence, then returned to <5% in adulthood. The mechanism for this transient expression is unknown, but is unlikely caused by hormonal changes because striatal overproduction of D_1 receptors occurs independently of gonadal hormones (Andersen et al., 2002a). It is, however, consistent with the peak in DA afferent density to pyramidal (but not nonpyramidal) cells in the PFC around puberty (Lambe et al., 2000). In contrast, postadolescent D_1 expression appeared largely on GABAergic interneurons, which has been previously found in fluorescent ligand binding studies (Vincent et al., 1995). Davidoff and Benes (1998) suggest, rather, that D_1 is evenly distributed on pyramidal and nonpyramidal cells in the adult rat PFC. The current adult data suggests that more interneurons than NAc-terminating neurons express D_1. Therefore, some D_1-expressing pyramidal neurons may project to other structures and would have been included in the "D_1 alone" category.

Alternatively, the D_1 alone category in juveniles may represent a portion of D_1-expressing projection neurons that have not yet reached their NAc target. As PFC-NAc connectivity increased steadily from preadolescence through adulthood, their phenotype is clarified with the uptake of tracer. More than half of the D_1-expressing cells in juveniles were otherwise unlabeled and appeared to have a pyramidal morphology (see Fig. 4*c*). Notably, we aimed the bolus of fluospheres into the core of the NAc; pro-

jections to different regions of the NAc from the PFC may arise earlier than others. The maturation in PFC connectivity alone, however, does not appear to explain the developmental trajectory of drug-seeking behavior.

D_1 effects on cocaine place conditioning

Direct cortical D_1 receptor inactivation during conditioning blocked the enhanced place preference for cocaine in adolescents. In contrast, D_1 activation provoked conditioned place preference for cocaine in juveniles at a dose that was previously ineffective. Although these results do not implicate pyramidal D_1 per se in adolescent place conditioning, they corroborate the idea that peak levels of D_1 are critically involved with an enhancement of motivational salience attribution in adolescence. In adults, D_1 antagonism in the PFC attenuates the reinstatement of both conditioned place preference (Sanchez et al., 2003) and cocaine self-administration (Alleweireldt et al., 2002), further implicating the D_1 receptor in salience attribution. The current anatomical results complement this idea with observations that increased D_1 in adolescence is selectively expressed on projections to the NAc, where the D_1 receptor reportedly regulates the "perceived" significance of stimuli by gating NAc activation (Seamans and Yang, 2004; Robbins, 2005).

It is important to interpret these findings in the context of other developmental brain changes. It is unlikely that age differences in cocaine's effect on extracellular DA levels underlie these differences, as cocaine produces comparable increases between adolescents and adults in the NAc and striatum (Cao et al., 2007; Frantz et al., 2007). Microinjected D_1 agonist into the juvenile PFC may provide aberrant stimulation to immaturely innervated receptors, as DAergic projections to the mPFC are still progressively increasing between preadolescence and adulthood (Kalsbeek et al., 1988). Moderate DA stimulation in juveniles would be predicted to affect a greater preponderance of GABAergic interneurons containing D_1 IR (Fig. 4). These findings, and those of others (Miller and Marshall, 2004), illustrate the importance of distinguishing the population of cells that are activated. For example, adult rats exposed to cocaine-paired cues have greater immediate early gene activity in the GABAergic inhibitory neurons of the PFC, and not in CAMK-II-expressing output neurons (Miller and Marshall, 2004). Similarly elegant studies have not been done in juveniles. However, as DA receptor activity mediates c-*fos* expression within the cortex (LaHoste et al., 1996), studies in juveniles that have previously reported age effects on c-fos after stimulant exposure in the PFC (Andersen et al., 2001; Cao et al., 2007) may need to be re-interpreted. Based on the current data, it is possible that increased c-fos, in juveniles and adults, occurs on GABAergic neurons rather than PFC-NAc projections.

Conclusions

Adolescence is a transition in information processing through the pruning and potential re-focusing of cortical networks (Crews et al., 2007), which involves functional rearrangement of inhibitory and excitatory control within the PFC (Tseng and O'Donnell, 2007). The present data suggest that adolescence is associated with a shift in distribution of the D_1 receptor between cell types within the PFC microcircuitry that regulates salience attribution. Typically, activation of D2 receptors on pyramidal neurons allows multiple inputs to effectively compete with drug-associated cues, thereby tempering the salience assigned to these cues (Seamans and Yang, 2004; Kalivas et al., 2005). Repeated exposure to cocaine in adult animals increases D_1-like activity selectively on these NAc-targeting projection pyramidal neurons and enhances craving (Kalivas et al., 2005). Therefore, the scarcity of D_1 receptors colocalized on these projection neurons in juveniles and adults (Fig. 4*d*) may explain a lower propensity for drug addiction (O'Brien and Anthony, 2005). However, the results from experiment 3 indicate that this balance can be disrupted with an overactivation of D_1 in juveniles. By recruiting the smaller percentage of D_1 on mature projection neurons, microinjected D_1 agonist may have brought an otherwise immature cortical mediation of mesolimbic activity on-line. In contrast, inactivating D_1 in adolescence blocked preferences for cocaine-associated environments.

The clinical significance of these findings could extend to other disorders that involve motivated behavior, such as attention deficit hyperactivity disorder (Volkow et al., 2005) or depression (Ernst et al., 2006). Specifically, early life events that cause perturbation of the developing D_1 profile within the PFC could disrupt the synaptic remodeling described here and thereby lock in vulnerability for a range of dysfunctional behaviors at older ages.

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