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Adolescent Maturation of Cocaine-Sensitive Neural Mechanisms

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Both clinical and animal studies have shown that adolescents undergo a late maturation of the central nervous system, which may underlie adolescent typical behaviors. In particular, decreased behavioral response to cocaine has been found in adolescents as compared to adults. In the present study, cocaine was used as a tool to explore adolescent brain maturation. Juvenile (postnatal day (P) 27), adolescent (P37), and adult (P90) male Sprague–Dawley rats were treated acutely with cocaine (750 μg/kg/injection × 2, i.v.), and c-fos mRNA expression, a marker of neuronal activation, was evaluated by *in situ* hybridization. Cocaine-induced c-fos mRNA was similar across ages in the dorsal caudate putamen (CPu), nucleus accumbens, and lateral bed nucleus of the stria terminalis. In contrast, there was a diminished response in juvenile/adolescent ventral CPu and in juvenile central nucleus of the amygdala, and an increased response in juvenile/adolescent cortex. Further studies evaluated the mechanism of the late maturation of cocaine response in ventral CPu. No significant age differences were observed in regional dopamine (DA) transporter binding. Although striatal DA content was significantly reduced at P27 as compared to adult, there was no difference between dorsal and ventral subregions. In contrast, basal- and cocaine-induced extracellular DA overflow, as measured by *in vivo* microdialysis, was lower in juvenile ventral CPu than in the adults. This age difference was not observed in dorsal CPu. These findings suggest that impulse activity in DA afferents to ventral CPu is immature in adolescents. In conclusion, the present study showed that cocaine-sensitive neuronal circuits continue to mature during adolescence.
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INTRODUCTION

Adolescence is defined as a period of unique behaviors, characterized by novelty seeking, risk taking, and increased self-consciousness (Spear, 2000; Pechmann *et al*, 2005). Adolescents are more impulsive than adults (Adriani and Laviola, 2003), and are susceptible to inhibitory control disorders such as substance abuse (Kandel and Logan, 1984) and eating disorders (Nicholls and Viner, 2005). The onset of several neuropsychiatric disorders, including major depression, bipolar illness, and schizophrenia, occurs mainly during adolescence (Kovacs *et al*, 1984; Lewinsohn *et al*, 2003; van Nimwegen *et al*, 2005). Other syndromes with a childhood onset, such as attention deficit hyperactivity disorder and Tourette's, frequently either remit or change symptamotology during the adolescent period (Peterson, 1996; Biederman *et al*, 2000; Wolraich *et al*, 2005).

Both clinical and animal studies have shown that adolescents experience a late maturation of the brain, which may underlie specific behavioral changes. Cortex matures substantially, in a region-specific manner, with myelination-induced increases in white matter and pruning-induced decreases in gray matter (Durston *et al*, 2001; Gogtay *et al*, 2004). Sex-dependent alterations in hippocampal and amygdala volume are also observed (Durston *et al*, 2001; Koshibu *et al*, 2004). The interactions between frontal cortex and amygdala, which are critical in the cognitive processing of emotional information, also continue to develop throughout adolescence (Cunningham *et al*, 2002). In addition to these morphological changes, monoamine systems, which integrate numerous neural functions and critically regulate action, emotion, motivation, and cognition, also continue to mature. Although inconsistent developmental profiles have been reported, dopamine (DA) signaling in cortex, especially frontal cortex, is not mature in adolescents (Lewis, 1997; Andersen *et al*, 2000; Tseng and O'Donnell, 2005). In striatum, DA transporter (DAT) expression increases during early

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adolescence (Tarazi *et al*, 1998), and there is a sex-dependent overproduction and subsequent pruning of DA receptors (Tarazi *et al*, 1999; Andersen and Teicher, 2000). Together, these studies have shown that the central nervous system continues to develop throughout adolescence.

There is substantial evidence that adolescents exhibit unique responses to addictive drugs. Some drugs, such as nicotine and alcohol, are more likely to produce addiction during adolescence than at later ages (Chen *et al*, 2006; Spear and Varlinskaya, 2005). In contrast, numerous clinical and animal studies have shown that adolescents are hyposensitive to the actions of other drugs that activate DA systems, including cocaine (reviewed by Spear, 2000). A lower percentage of adolescents than adults have ever used cocaine (Department of Health and Human Services, 2002), and the rates of cocaine dependence are lower in adolescents, especially in males (Chen and Kandel, 2002). Early adolescents report negligible subjective responses to cocaine exposure (Weiss *et al*, 1994). Similarly, animal studies have shown that adolescents have diminished locomotor responses to acute cocaine treatment (Spear and Brake, 1983; Laviola *et al*, 1995; Collins and Izenwasser, 2002). Although cocaine induces conditioned place preferences to a similar extent in adolescents and adults (Campbell *et al*, 2000), adolescent rats show decreased cocaine self-administration (Belluzzi *et al*, 2005) and cocaine-induced locomotor sensitization (Laviola *et al*, 1995; Collins and Izenwasser, 2002) as compared to adults. In contrast, serotonin systems may be hypersensitive to cocaine during adolescence, with adolescent drug exposure increasing aggression resulting from disrupted serotonergic cascades (Ricci *et al*, 2004).

Given the immature behavioral response to cocaine in adolescents, the present study was designed to evaluate whether cocaine-sensitive neural circuits in rat brain have matured by adolescence. We examined age differences in cocaine-induced forebrain neuronal activation using the immediate early gene, c-fos as a marker (Kovacs, 1998). Adolescence in rats was defined as the fifth and sixth postnatal weeks, according to the definition of Spear (2000). The present detailed regional analysis of cocaine-induced brain activation revealed significant differences between adolescent and adult brain responses. *In vivo* microdialysis and other neurochemical approaches were used for further analysis of mechanisms underlying observed age differences in striatal drug responsiveness.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats were maintained in a temperature (21°C) and humidity (50%) controlled room on a 12-h light–dark cycle (lights on 0700–1900), with unlimited access to food and water. All experiments were carried out in accordance with the Institutional Animal Care and Use Committee at the University of California, Irvine, and were consistent with Federal guidelines. All efforts were made to minimize animal suffering, and to reduce the number of animal used. Young animals, aged postnatal day (P) 27-P28 or P37 on the experimental day, were used as in previous behavioral studies (Belluzzi *et al*, 2005). To minimize prenatal stress effects on the offspring (Graham *et al*, 2006), young animals were delivered with dams at P16 and habituated in the vivarium for 5 days before weaning at P21. Only one animal per litter was assigned to each experimental group. Adults, aged P90 on the experimental day, were habituated to the vivarium for a minimum of 1 week before use. All animals were group housed except those used for microdialysis, which were single housed after surgery to avoid damage to the cranial guide cannula.

Surgical Implantation of Intravenous Catheters

Catheter construction and implantation was as described previously (Belluzzi *et al*, 2005). Animals were anesthetized with Equithesin (2.5 ml/kg adolescents, 3 ml/kg adults, i.p.) and a chronic catheter was surgically implanted into the right external jugular vein. The catheter was passed subcutaneously from the animal's back to the jugular vein where the tubing was inserted. The cannula assembly was mounted on the animal's back and was sealed to prevent clogging and to keep a closed system. The wounds were closed with wound clips, antiseptic ointment was applied to the wounds, and Baytril (0.1 ml/150 g, i.m.) was injected to prevent infection. The animals were kept in a warm cage for postsurgical observation until they emerged from anesthesia.

Drug Treatment

For 3 days before the experiment, rats were handled daily for 3 min to decrease stress, and catheters were flushed with 0.2 ml of a heparinized saline solution (600 or 300 U of heparin in 30 ml saline for adult and young animals, respectively) to prevent clogging. Each animal was habituated for 5 min daily in a single cage with home cage bedding. Propofol (10 mg/ml), a fast-acting, short-lived intravenous anesthetic, was administered (0.1 ml for adults and 0.05 ml for juveniles) to test catheter patency 1 day before the experiment. Only rats that were immediately anesthetized by propofol and recovered from anesthesia within 10 min were used. On the experimental day, rats (aged P27, P37, and P90) were habituated to the test environment for 120 min before drug injection. Cocaine was administered intravenously at a dose of 750 μg/kg/100 μl injection, with two injections spaced 1 min apart, whereas control animals were injected with the same volume of 0.9% saline. This paradigm is designed to mimic conditions in which we have found age differences in cocaine self-administration (Belluzzi *et al*, 2005). After drug administration, animals were placed for 30 min into the cages to which they had been habituated and were then decapitated. Brains were extracted immediately and frozen in 2-methylbutane at −20°C for 30 s. Samples were stored at −70°C until use for c-fos mRNA *in situ* hybridization (Samaha *et al*, 2004).

In Situ Hybridization

Coronal sections were cryostat cut at a thickness of 20 μm at −20°C. Sections were mounted onto poly L-lysine-coated glass slides and fixed with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) for 1 h at room tempera-

ture. Tissue sections were washed in 0.1 M PBS for 3×5 min, dried, and stored in airtight boxes at -20°C until use.

Tissue sections were processed for *in situ* hybridization according to the method of Winzer-Serhan *et al* (1999). [^{35}S]-labeled riboprobes were transcribed in antisense and sense directions from a pGEM-3Z plasmid containing a 680 bp fragment of c-fos cDNA between T7 and SP6 promoter sites (kindly provided by Dr Stanley Watson, University of Michigan). Sections were pretreated with proteinase K (0.05 μg/ml), acetylated, dehydrated through graded ethanols (50, 75, 95, and 100%), and air dried, then incubated for 18 h at 60°C with hybridization solution containing ^{35}S-labeled sense or antisense riboprobes (1×10^7 c.p.m./ml). After hybridization, sections were treated with RNase A and washed at high stringency. Tissue sections were dehydrated and exposed to Kodak Biomax film for 1 day with ^{14}C standards of known radioactivity (Winzer-Serhan *et al*, 1999).

DAT Radioligand Binding

Brains from naïve animals, aged P27, P37, and P90, were cryostat sectioned at 20 μm thickness at -20°C. Sections were mounted onto poly L-lysine-coated slides, dehydrated at 4°C for 2 h, and stored at -20°C until use. The method of Pradhan *et al* (2002) was used to measure binding of [^{125}I] RTI-55 to DAT. Sections were thawed, dried, and incubated for 2 h at room temperature with buffer containing 10 mM Na_2HPO_4, 0.1 M sucrose, 10pM [^{125}I] RTI-55, 50 nM citalopram HBr, and 5 nM desipramine to block binding to serotonin and norepinephrine transporters (NETs), respectively. Alternate sections were also incubated with the DA uptake inhibitor, GBR12909 (10 μM) to determine nonspecific binding. Sections were rinsed in ice-cold buffer 1×1 and 2×20 min. After a brief rinse with cold distilled water, slides were blown dry, and exposed to Kodak Biomax film for 48 h with ^{14}C standards of known radioactivity.

Quantitative Analysis of Autoradiograms

Autoradiographic images were quantified using a computer-based image analysis system (MCID, Image Research Inc., St Catharines, ON, Canada). Brain areas on autoradiograms were identified with reference to adjacent brain sections processed for fast cresyl violet stain (Paxinos and Watson, 1986). Corresponding sections were chosen from different ages.

Striatum was analyzed at three levels (rostral, middle, and caudal levels), with nucleus accumbens (NAc) core and shell evaluated at the rostral level. Given the anatomical heterogeneity of caudate putamen (CPu) (McGeorge and Faull, 1989; Willuhn *et al*, 2003), CPu at the middle level was further divided into dorsal, ventrolateral, and ventromedial regions and, at the caudal level, into dorsal and ventrolateral regions (Figure 1). Cortical regions with substantial projections to striatum (medial prefrontal, orbital, motor, and somatosensory cortices) and extended amygdala (lateral bed nucleus of the stria terminalis (BSTL) and central nucleus of the amygdala (CeA)) were also examined. Optical densities in discrete brain regions were measured and the corresponding values of radioactivity were determined by interpolation from a standard curve, generated from ^{14}C standards of known radioactivity (Broide *et al*, 1995). In each brain region, mRNA expression and specific radioligand binding were quantified by subtracting corresponding regional measures of sense hybridization and nonspecific binding, respectively. mRNA expression was expressed as d.p.m./mg wet weight, and radioligand binding as fmol/mg wet weight. Averages were obtained from readings of the right and left hemispheres for each brain region.

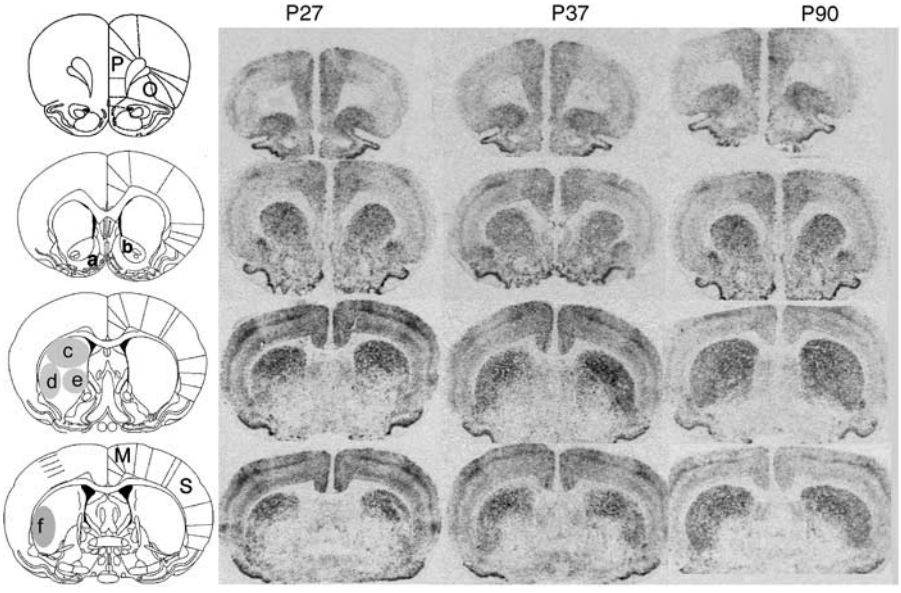


Figure 1 Representative autoradiographic images of cocaine-induced c-fos mRNA expression in coronal forebrain sections of male rats aged P27, P37, and P90. Quantified areas are labeled on representative atlas sections: a, NAc shell; b, NAc core; c, dorsal CPu; d, ventrolateral CPu; e, ventromedial CPu; f, caudal ventrolateral CPu; M, motor cortex; O, orbital cortex; P, prefrontal cortex; S, somatosensory cortex.

In Vivo Microdialysis

Stereotaxic surgery. Immediately after implantation of their intravenous catheter, animals for microdialysis were implanted with a cranial guide cannula. Animals were placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA, USA), their skulls revealed and drilled to expose the dura. A chronic guide cannula (20 gauge; CMA/Microdialysis AB, Stockholm, Sweden) was stereotaxically implanted 2.0 mm above the target area, fixed to the skull with acrylic dental cement, and sealed with a dummy cannula. Anatomical coordinates for adult animals were established from the atlas of Paxinos and Watson (1986). The coordinates for P27–P28 animals were estimated from the adult atlas and adjusted for body weight, then empirically determined in a preliminary experiment with histological confirmation. The following guide cannula coordinates were measured from the dura; adult dorsal CPu: AP, +1.8 mm; ML, ±2.5 mm; DV, −3.6 mm; adult ventrolateral CPu: AP, −0.2 mm; ML, ±4.1 mm; DV, −4.7 mm; P27–28 dorsal CPu: AP, +1.2 mm; ML, ±2.1 mm; DV, −3.2 mm; P27–28 ventrolateral CPu: AP, 0; ML, ±3.8 mm; DV, −4.3 mm.

Microdialysis. Animals were given 2 days to recover with daily handling after surgeries. Catheters were flushed daily and animals were habituated to the microdialysis chambers for 5 min per day. Intravenous catheter patency was tested by propofol 1 day before the experiment.

On the experimental day, the dummy cannula was replaced with a 2 mm microdialysis probe (CMA/12). The quality of probes was tested *in vitro* before the experiment with an average recovery of 10.8±1.7%, $n=16$. Microdialysis was carried out under a free-moving condition, with the probe continuously perfused with artificial cerebrospinal fluid (CMA Microdialysis, N Chelmsford, MA, USA) at a constant flow rate of 1.1 μl/min delivered by a microinfusion pump (CMA/100 Microdialysis, N Chelmsford, MA, USA). After a 4 h equilibration period, samples were collected every 20 min. When DA levels reached a stable baseline, with a variance of <5% in three consecutive collections, animals were given two 100 μl injections (i.v.) of saline, 1 min apart. After 40 min, cocaine (750 μg/kg/100 μl injection, i.v.) was injected twice at a 1 min interval and samples were collected for another 100 min. DA and its metabolite levels were quantified by high-performance liquid chromatography with electrochemical detection (HPLC-ED). The position of microdialysis probes was verified histologically and mapped onto relevant atlas sections (Paxinos and Watson, 1986).

HPLC-ED detection. Microdialysate samples (20 μl) were automatically injected by an ESA 542 refrigerated autosampler onto a 150 × 3.2 mm ODS C^{18} column (ESA Inc., Chelmsford MA) connected to an ESA 580 HPLC pump. The column was kept at 35°C and perfused by MD-TM mobile phase (ESA, Chelmsford, MA) at a rate of 0.6 ml/min. DA and metabolite levels were determined by an electrochemical ESA 5600 detector with an ESA 5020 guard cell with the dominant potential of 300 mV. The sensitivity of the detector is 500 fg. Measurements were analyzed using CoulArray for Windows[32] Software 2.0 (ESA Inc., Chelmsford, MA, USA). Standard curves were generated with catecholamine (ESA, Chelmsford, MA), DOPAC, and HVA (Sigma-Aldrich, St Louis, MO) standards, and levels in experimental samples were determined from the curve and expressed as pg per 20 μl, unadjusted for recovery, as there were no significant differences in probe recovery. Basal levels of DA and its metabolites were determined by averaging the samples before cocaine injection. Cocaine-induced changes in DA and its metabolite levels were expressed as area under the curve (AUC).

Tissue Catecholamine Levels

Brains from naïve animals, aged P27, P37, and P90, were dissected on an ice-chilled rat brain matrix (Plastic One, Roanoke, VA, USA). One-millimeter sections of striatum were obtained and quickly frozen on dry ice. Tissues samples from dorsal and ventral CPu were dissected using a 1.19 cm diameter tissue punch (Stoelting, Wood Dale, IL, USA), expelled into 300 μl of ice-cold 0.1 M perchloric acid, and homogenized. Samples were centrifuged at 10 000*g* for 10 min, and the resulting pellets resuspended in 100 μl of 0.1 M NaOH overnight before measuring the protein content using a BCA protein assay kit (Pierce, Rockford, IL, USA). The supernatants were used for the measurement of DA and metabolites using HPLC-ED as described above. Remaining tissue sections were examined anatomically after tissue puncture to verify correct localization of tissue samples.

Statistics

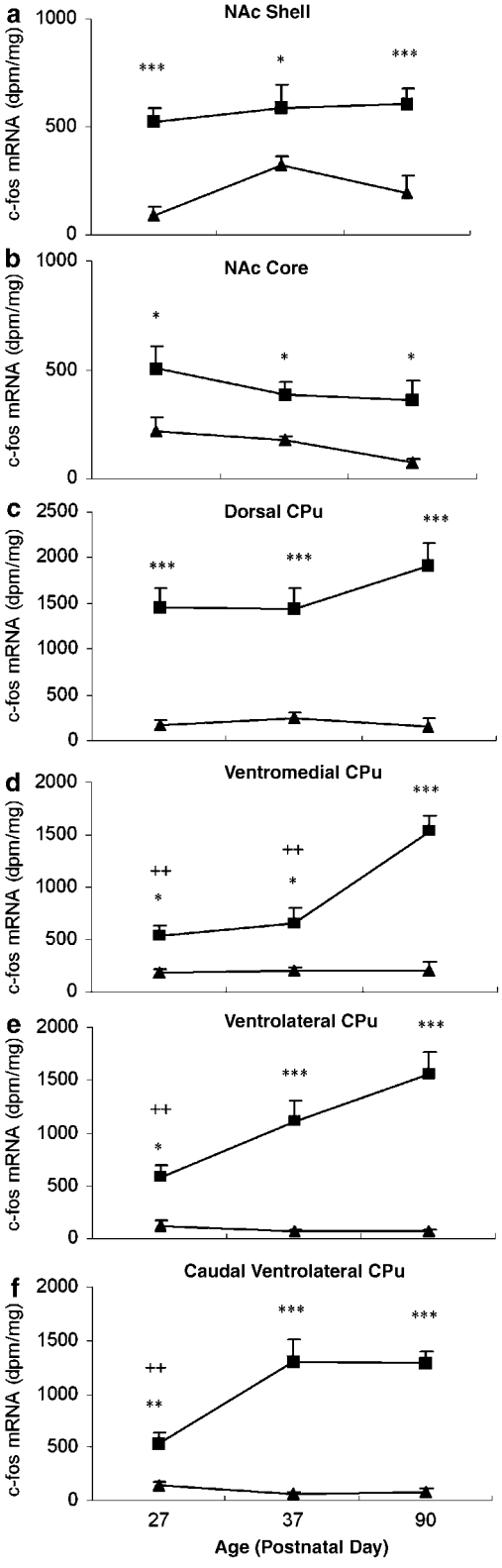
C-fos mRNA and DAT binding for each cluster of brain regions studied (striatum, cortex, or extended amygdala) were analyzed using a three-way ANOVA for Age × Drug × Brain area, with repeated measures on Brain area. Following a finding of overall significance, individual brain regions were examined using a two-way ANOVA on Age × Drug. Microdialysate samples, and total tissue contents, were analyzed by a two-way ANOVA for Age × Area. Significant main effects or interactions were further tested by *t*-test with the Bonferroni adjustment for multiple comparisons.

RESULTS

c-fos mRNA Expression

Striatum. Significant cocaine-induced c-fos mRNA expression was observed in striatum at all ages studied (Figures 1 and 2). More detailed regional analysis showed no significant age differences in cocaine-induced c-fos mRNA expression in NAc shell, core, and dorsal CPu (Figure 2 a–c), or in rostral CPu (quantitative data not shown). In contrast, there were marked age differences in cocaine-induced activation of ventral CPu. In this region, drug-induced c-fos mRNA expression showed a significant lateral to medial gradient of maturation (Figure 2 d–f): cocaine-induced c-fos mRNA in the ventrolateral CPu was lower at P27 than at P37 or P90 ($p<0.01$), whereas activation of c-fos mRNA expression in the ventromedial CPu was significantly lower at both P27 ($p<0.01$) and P37 ($p<0.01$), as compared to adult.

Cortex. Cocaine also induced significant activation of all cortical regions examined (Figures 1 and 3). Whereas there was a significant overall drug effect on medial prefrontal cortex ($F_{1,18} = 5.461$, $p < 0.05$), *post hoc* analysis did not reveal a significant effect at any specific age (Figure 3a). In contrast, cocaine induced significant c-fos mRNA expression in orbital ($p < 0.05$) and somatosensory cortices ($p < 0.01$) at P27, but not at later ages (Figure 3b and c). In motor cortex, there were significant cocaine effects at P27 ($p < 0.05$) and P37 ($p < 0.01$) but not in adults (Figure 3d).



Extended amygdala. BSTL and CeA constitute major components of the extended amygdala, which has been implicated in the regulation of motivated behavior (Davis and Shi, 1999; Koob, 1999). Cocaine significantly increased c-fos expression in CeA in adult ($p < 0.01$) and P37 ($p < 0.001$), but not at P27, whereas there were significant drug effects in BSTL at all ages studied (Figure 4).

Mechanistic Studies

Further studies were conducted to evaluate the mechanisms underlying the late maturation of cocaine-induced c-fos mRNA expression in the ventral CPu. As DA transmission plays an important role in cocaine-induced c-fos expression in the striatum (Graybiel *et al*, 1990; Ruskin and Marshall, 1994; Zhang *et al*, 2004), these studies were focused on this neurochemical system.

In vivo *microdialysis.* The effects of cocaine on presynaptic DA transmission in dorsal and ventral CPu were compared in juveniles, aged P27–28, and adults, by evaluating extracellular levels of DA and its metabolites (Figure 5, Table 1). Owing to the observed age differences in c-fos expression, these analyses focused on the ventrolateral CPu. As saline injections did not affect extracellular DA levels in any group, basal DA was determined by averaging all samples before cocaine injections. In the dorsal CPu, a region in which no age differences in cocaine-induced c-fos mRNA expression were detected, there were no significant differences in basal DA levels in juveniles (13 ± 4 pg/20 µl) and adults (13 ± 3 pg/20 µl), or in cocaine-induced DA overflow (AUC = 10 ± 2 pg/20 µl and 12 ± 3 pg/20 µl, respectively). In contrast, basal DA overflow in ventrolateral CPu was significantly lower in juveniles than adults (1 ± 0.2 pg/20 µl and 9 ± 0.8 pg/20 µl, respectively; $p < 0.05$). Similarly, cocaine-induced DA overflow in ventrolateral CPu was significantly lower in juveniles (AUC = 2 ± 1.0 pg/20 µl and 12 ± 2.8 pg/20 µl, respectively; $p < 0.01$).

Neither cocaine nor saline injections altered extracellular DOPAC or HVA levels in either dorsal or ventrolateral CPu (data not shown). As shown in Table 1, there were no age differences in basal DOPAC or HVA overflow in the dorsal

Figure 2 Age differences in striatal c-fos mRNA expression. Data are from animals aged P27, P37, and P90 following i.v. injection of cocaine (750 µg/kg/injection × 2; squares) or saline (triangles), $n = 3$–5 per group. Overall three-way ANOVA for Age × Drug × Striatal region showed significant effects of drug ($F_{1,16} = 185.9853$, $p < 0.0001$), age ($F_{2,16} = 5.2492$, $p < 0.02$), and an interaction of age by drug ($F_{2,16} = 6.0924$, $p < 0.02$), with significant regional differences ($F_{5,80} = 35.4691$, $p < 0.0001$). Nucleus accumbens (NAc) (a and b) and dorsal CPu (c) were significantly activated by cocaine at all ages. Although ventral CPu (d, e, and f) was activated by cocaine at all three ages, drug-induced c-fos mRNA levels were significantly lower in young animals as compared to adults. *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$, significantly different from saline treatment at the same age. $^{++}p < 0.01$, significantly different from adult cocaine treatment.

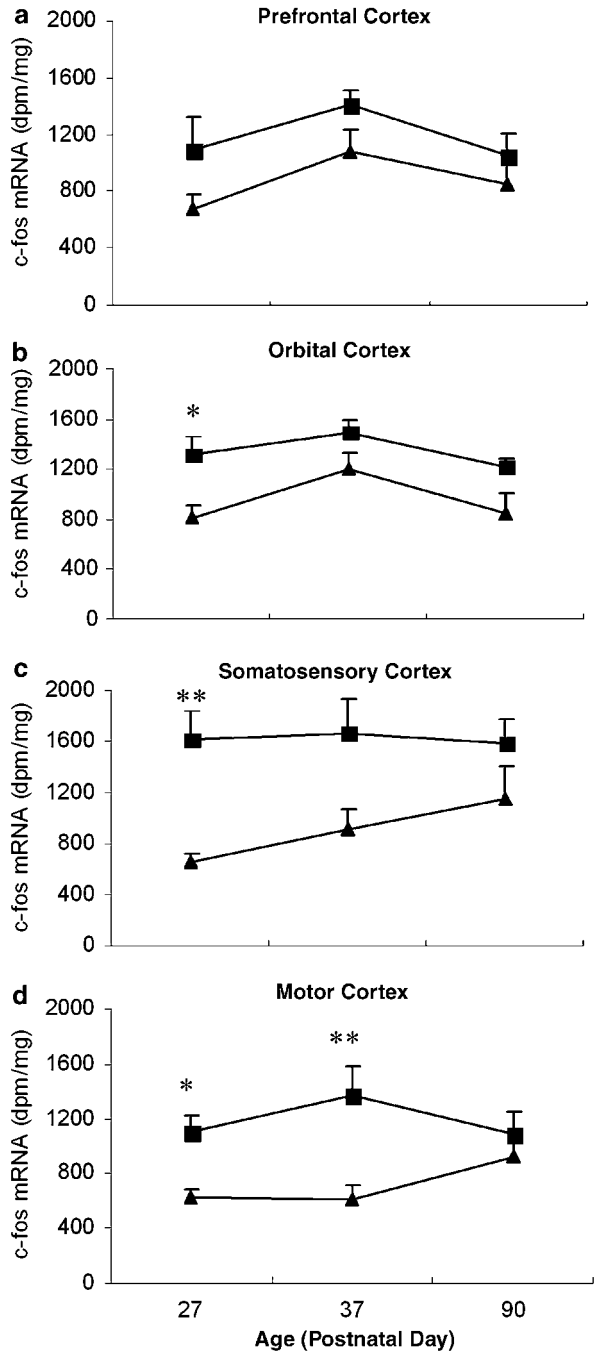


Figure 3 Age differences in cortical c-fos mRNA expression. (a) prefrontal cortex, (b) orbital cortex, (c) somatosensory cortex and (d) motor cortex. Data are from animals aged P27, P37 and P90 following i.v. injection of cocaine (750μg/kg/injection × 2; squares) or saline (triangles), $n = 3$–5 per group. Overall three-way ANOVA for Age × Drug × Cortical area showed a significant drug effect ($F_{1,18} = 17.162$, $p < 0.001$) and regional differences ($F_{4,72} = 15.429$, $p < 0.0001$). Whereas cocaine induced significant c-fos mRNA expression in most cortical regions in young rats, there was no significant activation in adults. *$p < 0.05$, **$p < 0.01$ significantly different from saline treatment at the same age.

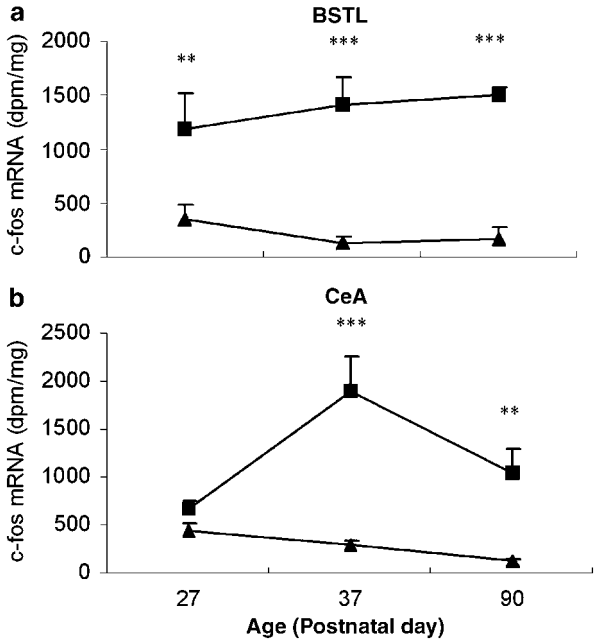


Figure 4 Age differences in c-fos mRNA expression within the extended amygdala. (a) Lateral bed nucleus of the stria terminalis (BSTL) and (b) central nucleus of the amygdala (CeA). Data are from animals aged P27, P37, and P90 following i.v. injection of cocaine (750μg/kg/injection × 2; squares) or saline (triangles), $n = 3$–5 per group. Overall three-way ANOVA of these regions showed a significant effect of drug ($F_{1,17} = 76.8192$, $p < 0.0001$), an interaction of drug by age ($F_{2,17} = 5.0101$, $p < 0.02$) and an interaction of area by age ($F_{2,17} = 5.1957$, $p < 0.02$). Whereas cocaine induced significant c-fos mRNA expression in BSTL at all ages studied, it did not induce significant neuronal activation of juvenile CeA. **$p < 0.01$, ***$p < 0.001$, significantly different from saline treatment at the same age.

CPu (Table 1). In contrast, in ventrolateral CPu, extracellular levels of both DOPAC ($p < 0.005$) and HVA ($p < 0.05$) were significantly reduced in juveniles as compared to adults.

DAT binding and tissue DA levels. Given the evidence, from *in vivo* microdialysis, that DA transmission in ventral CPu continues to mature throughout adolescence, additional biochemical studies were undertaken to evaluate age-related changes in the status of presynaptic DA terminals. DAT-binding site levels in dorsal, ventrolateral, and ventromedial CPu were quantified using [^{125}I] RTI-55 (Table 2). No significant age differences were observed in DAT binding to any of these striatal subregions ($F_{2,8} = 2.887$, $P > 0.1$), although there was a trend towards a generalized increase in binding from P27 to P37 across all regions studied.

DA and metabolite levels were also measured in tissue punches taken from dorsal and ventral CPu of juveniles, aged P27–28, and adults (Table 3). DA levels were significantly lower in juveniles than adults ($p < 0.01$); however, this age difference was observed in both dorsal and ventral CPu. Although there was a trend towards lower DOPAC levels in juveniles than adults, in both dorsal and ventral CPu, this did not reach significance (Table 3). No significant age differences in HVA levels were observed.

DISCUSSION

Although cocaine-induced neuronal activation, as measured by c-fos gene expression, has been extensively studied in

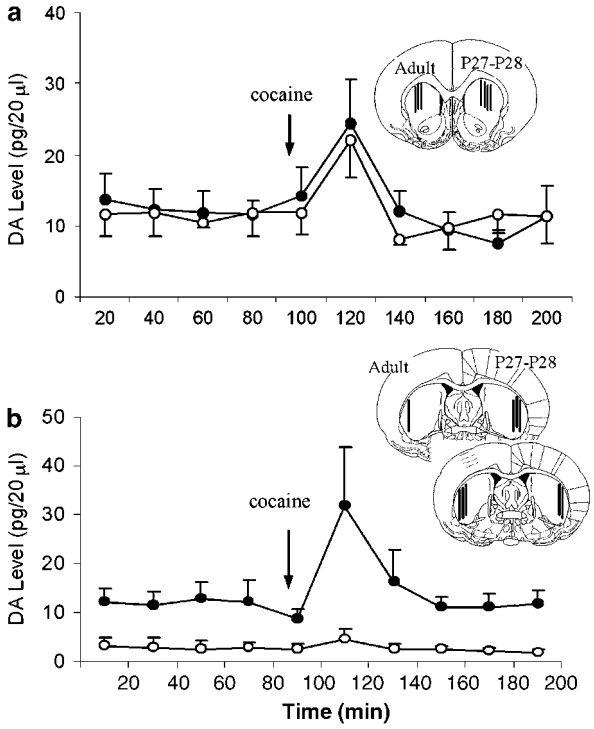


Figure 5 Extracellular DA levels in (a) dorsal CPu and (b) ventrolateral CPu in adult (closed circles) and P27–P28 rats (open circles), $n = 3$–5 per group. Figure inserts show probe placement in adult (left side) and P27–P28 (right side). Samples were collected every 20 min. Saline and cocaine (750µg/kg/injection × 2, i.v.) were injected at 60 and 100 min, respectively, after the initiation of sampling. No age differences were found in the dorsal CPu; however there were significant age differences in ventrolateral CPu with a lower DA baseline ($p < 0.05$) and decreased cocaine-induced DA overflow ($p < 0.05$) at P27–P28 as compared to adult.

Table 1 Age Differences in Striatal Extracellular DOPAC and HVA Levels

		DOPAC and HVA levels (pg/20 µl)	
	Brain areas	**P27–P28**	**Adult**
DOPAC	d	4056 ± 69	3802 ± 71
	v	2196 ± 28**	4494 ± 65
HVA	d	2082 ± 43	2021 ± 26
	v	1208 ± 7*	2175 ± 31

DOPAC and HVA levels are expressed as pg/20 µl dialysate and shown as mean ± SEM of $n = 3$–5 per group. There were no age differences in dorsal CPu (d), whereas both DOPAC and HVA levels were significantly lower in ventral CPu (v) at P27–P28 as compared to adults. *$p < 0.05$, **$p < 0.01$ significantly different from adults.

adult animals, there have been few such studies in adolescents. Whereas a previous study did not detect any differences between adolescents and adults (Kosofsky *et al*, 1995 a, b), our present, more detailed regional analysis has shown that cocaine-sensitive neural pathways in cortex, ventral CPu and amygdala continue to mature during adolescence. We have found a late developmental maturation, in a lateral to medial gradient, of cocaine-sensitive pathways in the ventral CPu. Whereas cocaine-induced

Table 2 [^{125}I] RTI-55 Binding to DAT in Subdivisions of Caudate Putamen

	DAT site densities (fmol/mg tissue)		
Age (postnatal day)	**dCPu**	**vlCPu**	**vmCPu**
27	1.29 ± 0.12	2.23 ± 0.14	1.81 ± 0.04
37	1.66 ± 0.05	2.82 ± 0.30	2.33 ± 0.24
90	1.64 ± 0.09	2.54 ± 0.17	2.10 ± 0.14

DAT binding is expressed as fmol/mg tissue and shown as the mean ± SEM of $n = 3$–4 per group. No significant age differences were found in dorsal CPu (dCPu), ventrolateral CPu (vlCPu), or ventromedial CPu (vmCPu).

Table 3 Age Differences in Striatal Tissue DA and Metabolite Levels

		DA and its metabolites levels (ng/mg protein)	
		P27–P28	**Adult**
DA	d	59.7 ± 3.3**	126 ± 15.8
	v	47.5 ± 8.2**	106 ± 9.5
DOPAC	d	10.6 ± 0.7	14.2 ± 1.8
	v	6.8 ± 0.7	11.7 ± 2.5
HVA	d	11.0 ± 1.0	9.6 ± 1.3
	v	9.7 ± 1.2	8.0 ± 1.2

DA and metabolites are expressed as ng/mg protein and shown as mean ± SEM of $n = 5$–6 per group. Tissue DA levels at P27–P28 were significantly lower than adult in both dorsal (d) and ventral CPu (v). There were no significant age differences in DOPAC or HVA levels. **$p < 0.01$ significantly different from adults.

c-fos mRNA expression in ventrolateral CPu was fully mature by mid-adolescence (P37), neuronal activation in the ventromedial quadrant continued to mature after this time. The response of CeA to cocaine also emerged during adolescence but was fully mature by P37. Observed age differences are unlikely to have resulted from differences in drug availability to the brain, as there were no significant age differences in cocaine-induced c-fos expression in medial prefrontal cortex, dorsal CPu, and NAc. A summary of these findings is illustrated in Figure 6.

Mechanisms Underlying Developmental Changes in Cocaine Response

Cortex. In both limbic and sensorimotor cortices, age-related declines in cocaine-induced neuronal activation were observed. In early adolescence, there was robust activation of all cortical regions analyzed, with the exception of medial prefrontal cortex. Significant drug-induced cortical c-fos expression was not observed in adult, however. Other studies (Thiriet *et al*, 2000; Uslaner *et al*, 2001) which demonstrated cortical activation in response to cocaine differed in route of administration and, importantly, environmental context. Cocaine causes much greater

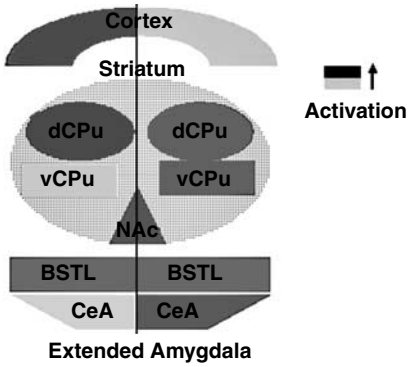


Figure 6 Summary diagram of age differences in cocaine-induced c-fos mRNA expression in cortex, striatum, and extended amygdala of juvenile (left side) and adult (right side) rats. Solid colors, darker and lighter, represent relatively higher and lower activation, respectively.

c-fos expression in both prefrontal and somatosensory cortices when drug is administered in a novel environment as compared to the home cage (Uslaner *et al*, 2001). To better elucidate the pharmacological effects of cocaine on brain circuitry, all animals in the present study were thoroughly habituated to the test environment before the experiment, thus eliminating the influence of novelty.

Whereas DAT blockade has been implicated in cocaine-induced neuronal activation in adult cortex (Trinh *et al*, 2003), it is not clear what mechanisms underlie cocaine-induced c-fos expression at earlier ages. Nisoxetine binding to the norepinephine transporter (NET) in rat cortex peaks immediately before adolescence and then declines (Moll *et al*, 2000), in parallels with the time course observed for cocaine-induced c-fos expression. Thus, the strong cocaine-induced activation of c-fos expression in immature cortex may partially reflect occupancy of NET.

Extended amygdala. The extended amygdala, including CeA and BSTL, is an important mediator of the stress response, fear and anxiety, and the rewarding and aversive aspects of addictive drugs (Herman and Cullinan, 1997; Davis and Shi, 1999; Koob, 1999). Whereas norepinephine (NE) and serotonergic afferents (Fallon *et al*, 1978; Phelix *et al*, 1992) may contribute to cocaine's effects in these regions, the CeA and BSTL receive intense DA projections (Fallon *et al*, 1978; Freedman and Cassell, 1994). Activation of DA afferents to the extended amygdala is essential in mediating many of the actions of addictive drugs (Caine *et al*, 1995; Hurd *et al*, 1997; Epping-Jordan *et al*, 1998). Cocaine and other addictive drugs have been shown to induce phosphorylation of ERK, an intermediary in the induction of c-fos expression (Zhang *et al*, 2004), in adult CeA and BSTL via activation of D1 receptors (Valjent *et al*, 2004).

Whereas cocaine-induced c-fos expression was at adult levels in BSTL by P27, the response of the CeA emerged during early adolescence and was not fully mature until P37. Further studies will be required to determine whether this reflects the late development of the DA system in this brain region, as we have found for the ventral CPu.

Striatum. In adult striatum, the mechanisms underlying cocaine induction of c-fos expression have been well studied. Whereas serotonergic and noradrenergic systems contribute little (Graybiel *et al*, 1990), DA plays a key role. Cocaine-induced c-fos expression in striatum is inhibited by systemic or intrastriatal administration of D1 receptor antagonists (Graybiel *et al*, 1990), and in D1 receptor mutant animals (Zhang *et al*, 2004). Psychostimulant-induced c-fos expression is also mediated by D2 receptor activation (Ruskin and Marshall, 1994) and facilitated by excitatory input (Torres and Rivier, 1993).

Whereas no significant age differences were observed in cocaine-induced c-fos expression in the dorsal CPu and NAc, we have found a later developmental maturation, in a lateral to medial gradient, of cocaine-sensitive pathways in the ventral CPu. Whereas cocaine-induced c-fos mRNA expression in the ventrolateral CPu was fully mature by mid adolescence (P37), neuronal activation in the ventromedial quadrant was still immature at this time. Although both pre- and postsynaptic DA elements may contribute to the late maturation of cocaine-induced c-fos expression in ventral CPu, we have focused our present mechanistic analysis on presynaptic mechanisms. Using *in vivo* microdialysis, we have shown that cocaine-induced DA overflow in dorsal and ventral CPu exhibited developmental profiles that paralleled maturation of drug-induced c-fos expression. Consistent with the c-fos data, there were no age differences in basal- or cocaine-induced DA overflow in dorsal CPu. In contrast, basal- and cocaine-induced DA overflow were significantly lower in ventral CPu of juveniles than adults. Such findings did not reflect increased metabolism of extracellular DA in juveniles, as dialysate levels of DOPAC and HVA were also significantly lower.

In an earlier microdialysis study, Laviola *et al* (2001) reported diminished basal and amphetamine-induced DA overflow in adolescent striatum as compared to adults. Although not specified, evaluation of their probe coordinates suggests that they were also targeting ventral CPu. In contrast, a recent study has reported no differences in basal- or cocaine-stimulated DA overflow in the NAc of adolescents and adults (Frantz *et al*, 2006). This finding is consistent with our present observation of early maturation of cocaine-induced c-fos expression in this component of the ventral striatum.

Although there is a late maturation of cocaine-induced DA overflow in ventral CPu, our biochemical data suggest that this does not result from a late ingrowth of mesostriatal fibers. Consistent with an earlier report (Tarazi *et al*, 1998), we have found that, although striatal DAT-binding site levels are slightly lower at P27 than in adult, there is no regional differentiation in this maturation pattern. Similarly, tissue DA and DOPAC content is also lower in juveniles than adults in both dorsal and ventral CPu. Thus, although these data suggest that presynaptic DA terminals are not fully mature by early adolescence, this cannot explain the regional differences that we have observed in the maturation of cocaine-induced c-fos expression and extracellular DA overflow. As cocaine-induced DA overflow depends on neuronal activity, the diminished drug response in adolescent ventral CPu may result from lower impulse activity in DA afferents to this region. Ventral CPu is anatomically distinct from dorsal CPu, receiving different

inputs from DA cells (Fallon and Moore, 1978) and cortex (Voorn *et al*, 2004). Thus, our findings may reflect developmental differences in the maturation of afferent activity in dorsal and ventral CPu.

Functional Correlates of Late Maturation of Cocaine-Sensitive Pathways

The immature activity of DA afferents to ventral CPu during adolescence may underlie some of the unique behavioral responses that have been observed during this developmental period (Spear, 2000). Dopaminergic input to the ventrolateral CPu activates oral stereotypy (Delfs and Kelley, 1990; Dickson *et al*, 1994; Baker *et al*, 1998), and is important in feeding related behaviors (Kelley *et al*, 1989; Salamone *et al*, 1993). This brain region has also been implicated in mediating the rewarding effects of amphetamine (Baker *et al*, 1998). In contrast, ventromedial CPu is associated with locomotion and rearing (Dickson *et al*, 1994) and conditioned responding (Kelley and Delfs, 1991). The late maturation of the DA input to ventromedial CPu may underlie decreased locomotor responding to acute and/or chronic cocaine (Spear and Brake, 1983; Laviola *et al*, 1995; Collins and Izenwasser, 2002; Frantz *et al*, 2006). Chronic cocaine has also been reported to induce oral stereotypy and reduced food intake in adults, but not adolescents (Laviola *et al*, 1995), a finding that is consistent with late maturation of DA input into the ventrolateral CPu. Further studies will be required to determine whether the late maturation of monoamine inputs to both ventral CPu and CeA may underlie the decreased sensitivity of adolescents to the addictive effects of cocaine (Department of Health and Human Services, 2002; Chen and Kandel, 2002).

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