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Specificity of prenatal cocaine exposure effects on cortical interneurons is independent from dopamine D1 receptor co-localization

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Abstract

Gestational cocaine exposure in a rabbit model leads to a persistent increase in parvalbumin immunoreactive cells and processes, reduces dopamine D1 receptor coupling to Gs α by means of improper trafficking of the receptor, changes pyramidal neuron morphology, and disrupts cognitive function. Here, experiments investigated whether changes in parvalbumin neurons were specific, or extended to other subpopulations of interneurons. Additionally, we examined dopamine D1 receptor expression patterns and its overlap with specific interneuron populations in the rabbit prefrontal cortex as a possible correlate for alterations in interneuron development following prenatal cocaine exposure. Analysis of calbindin and calretinin interneuron subtypes revealed that they did not exhibit any differences in cell number or process development. Thus, specific consequences of prenatal cocaine in the rabbit appear to be limited to parvalbumin positive interneurons. Dopamine D1 receptor expression did not correlate with the selective effects of cocaine exposure, however, as both parvalbumin and calbindin cell types expressed the receptor. The findings suggest that additional, unique properties of parvalbumin neurons contribute to their developmental sensitivity to *in utero* cocaine exposure.

Keywords

rabbit; *in utero*; parvalbumin; calbindin; calretinin

Introduction

Prenatal drug exposure in human fetuses affects brain development, although the specific effects reported vary widely (for review (Thompson *et al.*, 2009)). It is well-established that there are long-term consequences of exposure, from mild to severe disruption of brain architecture and function (Dow-Edwards *et al.*, 1999; Gingras and O'Donnell, 1998; Karmel

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and Gardner, 1996; Karmel *et al.*, 1996; Mayes *et al.*, 1998; Richardson, 1998; Singer *et al.*, 2004). Consistent with this heterogeneity, different animal models, designed to mimic human drug use during gestation, have reported complex developmental phenotypes in animals as consequences to *in utero* exposure to cocaine (Jones *et al.*, 1996; Lidow, 1998; Morrow *et al.*, 2003, 2005; Ren *et al.*, 2004; Stanwood and Levitt, 2004; Wang *et al.*, 1995a; Zhen *et al.*, 2001). A rabbit model of intravenous prenatal cocaine exposure that we and others have used has revealed persistent alterations in neuronal morphology and pharmacology within dopamine-rich cortical and sub-cortical brain regions (Jones *et al.*, 1996; Jones *et al.*, 2000; Wang *et al.*, 1995b). Specific cellular changes include increased parvalbumin immunostaining in layers 2/3 of the prefrontal cortex, correlated with increased dendritic expression of parvalbumin (Stanwood *et al.*, 2001a; Stanwood *et al.*, 2001b). Additionally, detailed neuroanatomical studies revealed an increase in apical dendrite length of pyramidal neurons in dopamine-rich cortical areas (Jones *et al.*, 2000; Stanwood *et al.*, 2001a). *In vitro* studies demonstrated increased cellular outgrowth from cells prenatally exposed to cocaine and provide evidence of the role of the dopamine D1 receptor (DAD1R) in these changes (Jones *et al.*, 2000; Stanwood and Levitt, 2007). At the molecular level, prenatal cocaine exposure results in a permanent reduction of G α coupling to DAD1R (Friedman *et al.*, 1996; Jones *et al.*, 2000). The reduction in coupling is a result of DAD1R sequestration and reduced plasma membrane trafficking, thereby reducing possible interactions with G α (Stanwood and Levitt, 2007). These cellular and molecular changes correlate well with changes in behavior mediated by dopamine-rich cortical and sub-cortical areas following prenatal cocaine exposure, including deficits in attention, conditioned place preference to cocaine, and psychostimulant induced stereotypies, but no effects on short term memory or locomotion (Simansky and Kachelries, 1996; Stanwood and Levitt, 2003; Thompson *et al.*, 2005a; Thompson *et al.*, 2005b).

The current study utilized immunohistochemistry techniques to determine whether the previously documented interneuron changes were limited to the parvalbumin subtype, or whether additional subpopulations of interneurons were similarly affected. To determine whether there is a relationship between neuronal disturbances and dopamine D1 receptor (*Drd1*) expression, specific mapping of *Drd1* transcript with GABA-ergic interneuron subpopulations is documented here for the first time in the rabbit prefrontal cortex. Thus, using double-labeling strategies, we examined whether interneuron changes were correlated with co-localization of *Drd1*.

Materials and Methods

Animals and tissue collection

Adult proven breeder male and female Dutch-belted rabbits from Myrtle's Rabbitry (Thompson Station, TN) were bred in-house. The dams were then individually housed for the duration of their pregnancy (30 days). Day of birth was designated postnatal day 0, and offspring lived with their litter and dam until sacrificed. For the immunohistochemistry studies, pregnant dams were administered cocaine as described in previous studies (Stanwood *et al.*, 2001a; Thompson *et al.*, 2005b). Briefly, cocaine hydrochloride (3 mg/kg, supplied by the National Institute of Drug Abuse) or saline was administered through the marginal ear vein, to pregnant dams twice daily from embryonic days 16–25. As previously reported, drug treatment had no effect on the weight of the dams or on gross parameters such as kit weight and litter size (Murphy *et al.*, 1997; Thompson *et al.*, 2005b). All experiments conformed to the guidelines set forth by the Vanderbilt University School of Medicine Institutional Animal Care and Use Committee.

Immunohistochemistry

Tissue collection—On postnatal day 20, offspring (n=4 prenatal saline and n=4 prenatal cocaine) were sacrificed by an overdose of sodium pentobarbital and then transcardially perfused with saline followed by 4% paraformaldehyde. Brains were extracted, cryoprotected in increasing sucrose concentrations (10, 20, and 30%) for approximately 12 hours each, and then sectioned at 50 μ m using a sledge microtome. Free-floating sections were stored in cryopreservative solution (45% phosphate buffered saline (PBS), 30% ethylene glycol, and 25% glycerol) at -20° C until processed.

Procedure—All reactions were performed at room temperature, unless otherwise noted. For calretinin immunohistochemistry, free-floating sections were rinsed in Tris-Glycine, incubated in blocking solution (4% Blotto (Carnation Milk), and 0.2% Triton X in PBS) to minimize non-specific antibody binding, and then incubated for 48 hours in polyclonal (antiserum lyophilized) antibody against calretinin (1:200) at 4° C (Swant Cat.No.7699/3H, Bellinzona, Switzerland). Sections were rinsed and then incubated in donkey cy³ anti-rabbit antibody (1:1000) for 90 minutes (Jackson Immuno Research Laboratories Cat.No.711167003, West Grove, PA). After rinsing, sections were incubated in DAPI (0.01 mg/ ml) (Molecular Probes, Carlsbad, CA), mounted on slides, dehydrated in ethanol, and cover-slipped with DPX (Fisher). Double labeling of calbindin with Gad-67 immunohistochemistry was performed to exclude calbindin positive pyramidal neurons. Free-floating sections were first rinsed in 30% hydrogen peroxide to inactivate endogenous peroxidases prior to treatment with Tris-Glycine. Sections were then blocked in 4% Blotto, 0.3% Tween 20 in PBS to minimize non-specific binding, and then incubated in polyclonal (antiserum lyophilized) antibody against calbindin (1:2000) (Swant Cat.No.CB38, Bellinzona, Switzerland) and monoclonal antibody against Gad67 (1:500) (Chemicon Cat.No MAB 5406, Billerica, MA) for 48 hours at 4° C. Sections were rinsed and then incubated in donkey cy² anti-rabbit (calbindin) (Jackson Immuno Research Laboratories Cat.No.711227003, West Grove, PA), and donkey cy³ anti-mouse (Gad-67) (Jackson Immuno Research Laboratories Cat.No.715167003, West Grove, PA) antibodies (1:1000) for 90 minutes. Sections were incubated in DAPI (0.01 mg/ ml), mounted on slides, dehydrated in ethanol, and cover-slipped with DPX (Fisher). Processed slides were stored at -20° C until analysis.

Data analysis—For analysis, slides were coded so the experimenter was blind to prenatal treatment groups. Images were collected using a Zeiss AxioPlan II microscope and a Zeiss AxioCam HRc camera with AxioVision 4.1 software. Two regions of interest that included layers 2/3 and corresponding to ventral and dorsal anterior cingulate cortex regions were captured per hemisphere using a 10 $\times$ magnification lens. Cell counts were averaged across 4 sections from each animal, each spaced 150 μ m apart. Cells expressing calretinin in layers 2/3 and cells expressing both calbindin and Gad-67 in layers 2/3 were used for analyses. DAPI staining was used for confirmation of layers 2/3 location. All cell counts were corrected using the Abercrombie method (Abercrombie, 1946) which corrects for differences in cell profile size or profile splitting. Within each region of interest for both calbindin and calretinin interneurons, analysis of dendritic morphology was performed by counting the number of cells that exhibited immunoreactive primary, secondary and tertiary dendrites and comparing the two prenatal treatment groups. Data are presented as mean $\pm$ standard error of the mean.

Statistics—Differences in the number of interneuron subpopulations and dendritic branching in saline-and cocaine-exposed groups were evaluated by a two-tailed t-test, using animal number as sample size. A p-value <0.05 was considered statistically significant.

Fluorescent in situ hybridization

Tissue collection—On postnatal day 20, offspring (n=10) from naïve dams were anesthetized with a saturating vapor of isoflurane, and brains were extracted and flash frozen in isopentane using RNase-free techniques. Using a cryostat, brains were sectioned at 20 µm and brain slices were mounted on permafrost slides (VWR) and then stored at −80° C until tissue processing began. Brain sections on each slide were a minimum of 100 µm apart from each other as slices were collected in a minimum of 5 slides per series. This allowed for multiple sections on a single slide to be used for quantification, and eliminated the potential for cell count duplication. A total of 10 animals from 8 litters were used for these experiments.

Probe synthesis—Rabbit probe templates were made by RT-PCR amplification of adult rabbit cortex cDNA. For these co-localization studies double-label fluorescent *in situ* hybridization was performed, as at the time of experiments there were no specific DAD1R antibodies available. As such, novel rabbit probes were synthesized, using either existing rabbit sequence for calbindin (*Calb1*), guinea pig sequence for parvalbumin (*Pvalb*), or the rabbit trace library (NCBI, trace archives) for *Drd1* and calretinin (*Calb2*). Human, rat, and mouse sequence homology was compared for designing rabbit probes from the rabbit trace library. For *Drd1*, rabbit trace sequence corresponding to nucleotides 26 to 661 (636 base pairs) of G757P62810RC3.T0 was amplified. For *Calb2*, rabbit trace sequence corresponding to nucleotides 1 to 101 (101 base pairs) of 610603307 was amplified. For *Calb1*, rabbit sequence corresponding to nucleotides 885 to 1607 (722 base pairs) of AF391152 was amplified. For *Pvalb*, rabbit sequence corresponding to nucleotides 4 to 273 (270 base pairs) of U26677 (guinea pig cDNA) was amplified. Probes were transcribed and incorporated with digoxigenin-UTP nucleotides into *Drd1* antisense, or fluorescein-UTP nucleotides into *Pvalb*, *Calb1*, and *Calb2* antisense. Custom primers (Integrated DNA Technologies, Coralville, IA) were designed for each probe; *Drd1* forward (CAGCGTGTGGGATCAGATAA) reverse (ACTGTGACTCCAGCCTGAGC), *Calb1* forward (ATAAATGGCTTTGTCCGGATGG) reverse (TGTGTCATCTGGTGCTGTTT), *Pvalb* forward (TGCTCAACGCTGAGGACAT) reverse (CGTCCCCATCCTTGTCAC), *Calb2* forward (AGGAGATGAGCATCCAGCAG) reverse (GCAGAGCACAATCTCCAGGT). PCR amplification was accomplished using rabbit brain cDNA library as template. Following successful PCR amplification of the rabbit-derived probe targets, the DNA fragments were TA-cloned into pSTBlue-1 (Novagen Cat. No 70573, Gibbstown, NJ). Direct re-sequencing of probe templates confirmed cloning of rabbit DNA segments with high homology and specificity to orthologous genes in mouse and human.

Procedure—Double-label fluorescent *in situ* hybridization was performed as described by Campbell et al (Campbell and Levitt, 2003) with modifications. All washes were done at room temperature unless otherwise noted. Sections were fixed in 4% paraformaldehyde and then rinsed in PBS. Slides were incubated in triethanolamine- hydrochloric acid with acetic anhydride, followed by incubation in a solution containing 1% IGEPAL CA-630, 1% SDS, 0.5% sodium deoxycholate, 50mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), and 150mM NaCl, followed by a PBS wash. Slides were immersed again in 4% paraformaldehyde, followed by PBS washes. Slides were then pre-hybridized at 55° C in a humidified oven, followed by hybridization in the pre-hybridization solution plus ~ 200 ng/ml of each probe for 16 hours at 55° C. Pre-hybridization solution contained: 50% formamide, 1% sodium dodecyl sulfate, 5× standard sodium citrate, pH 4.5, 50 µg/ml heparin, 50 µg/ml yeast tRNA, and 19% DEPC water. Post-hybridization washes began with a wash in solution × containing 50% formamide, 2× SSC pH 4.5, and 0.5% sodium dodecyl sulfate at 55° C. Slides were then incubated in RNase buffer (500 µl 1M PIPES, 5 ml 5M NaCl, 50 µl Tween 20) and then in RNase buffer plus 50 µg/ml RNase A at 37° C. Slides were incubated in solution × at 55° C, followed by washes in TBST (25 mM Tris, pH7.5, 136 mM sodium chloride, 2.68 mM potassium chloride, and 1% Tween 20), and then incubated in 3% hydrogen peroxide in methanol. Slides were

rinsed in PBS, then incubated in 100mM Tris, pH 7.5, 150mM sodium chloride, and 1.5% blocking reagent (Roche Applied Science). Anti-DIG-POD (1:1000) and anti-fluorescein-AP (1:500) antibodies (Roche Applied Science) were diluted in the pre-incubation solution, and then sections were incubated in antibodies at 4° C. After antibody incubation, sections were rinsed in 100mM Tris, pH 7.5 and 150mM sodium chloride. Sections were then washed in TNT (1M Tris, pH 7.5, 150 mM sodium chloride, and 0.05 % Tween 20). Sections were incubated in biotinyl tyramide in amplification diluent (1:50) (Perkin Elmer, Waltham, MA), rinsed in TNT, and then washed in PBS. Slides were incubated overnight at 4° C in ABC complex (Vector Laboratories, Burlingame, CA) according to manufacturer's instructions. The following day, slides were washed in PBS, followed by TNT and then incubated in fluorescein tyramide in amplification diluent plus (1:500) (Perkin Elmer). Slides were rinsed in TNT and then washed in HNPP buffer (0.1M Tris, pH8.0, 100mM sodium chloride, 10mM magnesium chloride). Slides were then incubated in HNPP buffer plus 1:100 each component of HNPP fast red (Roche Applied Science). Slides were again washed in HNPP buffer, followed by washes in PBS, and then cover-slipped with Vectashield (Vector Laboratories). Slides were stored at 4° C until image acquisition. Each set of experiments included slides treated with negative-control sense strand probes (transcribed from the same cDNA rabbit template as the experimental anti-sense strand probe) to ensure hybridization specificity.

Data Analysis—For all double label *in situ* experiments *Drd1* was labeled with digoxigenin-UTP nucleotides and the interneuron probes were labeled with fluorescein-UTP nucleotides. Data collection of cell counts for each *in situ* combination (*Drd1* with *Pvalb*, or *Drd1* with *Calb1*) were combined across three independent staining experiments. Images were captured using a Zeiss LSM 510Meta confocal microscope. Pixel size was 0.45 $\mu\text{m} \times 0.45 \mu\text{m} \times 0.48 \mu\text{m}$. Maximum and minimum depth for capturing cells was determined, and Z-stacks for each region of interest were acquired. Regions of interest included left and right, dorsal and ventral layer 2/3 anterior cingulate cortex from 2 different sections on each slide. Counts were made for interneurons with a single label and cells positive for co-expression of two transcripts (*Drd1* with *Pvalb* or *Calb1*). In our sampling, a total of 169 cells from 8 animals were positive for *Pvalb*, and 156 cells from 10 animals were positive for *Calb1*. Cells were considered positive for *Drd1* if the accumulated punctate labeling over a cellular profile was more than double the background neuropil staining. Background levels in antisense labeled sections were comparable to that seen using a sense probe (Figure S1). Cells positive for double labeling are expressed as percent of total number of either *Pvalb* or *Calb1* interneurons. Data are presented as mean $\pm$ standard error of the mean.

Results

Immunohistochemistry

Quantitative analysis of immunohistochemistry experiments revealed no differences in cellular labeling for calbindin or calretinin following prenatal cocaine exposure. Total cell counts from layers 2/3 were not different between the two prenatal treatment groups (Figure 1). Furthermore, no differences were found between dorsal versus ventral regions for either calcium binding protein, so the data across the two regions was combined into a single score. The average number of calbindin positive cells per mm^2 in prenatal saline exposed tissue was 3.0 ± 0.5 and in prenatal cocaine exposed tissue was 2.8 ± 0.2 ($t(1,5) = 0.31$, $p > 0.05$). The average number of calretinin positive cells per mm^2 in prenatal saline exposed tissue was 4.8 ± 0.2 and in prenatal cocaine exposed tissue was 5.5 ± 0.7 ($t(1,6) = -0.98$, $p > 0.05$). For both subtypes of interneurons, immunoreactive dendritic processes and branching patterns were far less complex than observed previously for parvalbumin-positive neurons. Quantitative analyses of the number of primary, secondary, and tertiary dendritic branches failed to distinguish between the two prenatal treatment groups (Table 1). This is in contrast to previous

findings revealing significant changes in the parvalbumin interneuron subpopulation after prenatal cocaine exposure (Stanwood *et al.*, 2001a; Stanwood *et al.*, 2001b), indicating a cell-type specific effect of drug exposure in the rabbit.

Fluorescent in situ hybridization

We next determined whether *Drd1* expression patterns correlate with the cell type- specific effect of prenatal cocaine exposure. Probe specificity and general expression patterns of each transcript were determined using ³⁵S single label *in situ* hybridization techniques (see (Campbell and Levitt, 2003) for detailed protocol) (Figure 2). Analysis of *Drd1* expression patterns reveal that similar to other mammalian species, rabbit *Drd1* expression is dense in the striatum, but far less dense in cortical areas, making localization more challenging. *Drd1* displays a laminar pattern, with the most dense labeling in deeper cortical layers (Figure 2a) as previously described (Stanwood *et al.*, 2001a). Interneuron subtypes differed in their distribution patterns in the rabbit forebrain. Both *Pvalb* and *Calb1* expressing interneurons were distributed in layers 2/3 of the anterior cingulate cortex. *Pvalb* also was expressed densely in somatosensory cortex (Figure 2b). *Calb1* was expressed in deeper cortical layers and neurons in the striatum (Figure 2c). *In situ* hybridization for *Calb2* revealed very low expression throughout the brain, with sparse labeling in the cortex, but dense staining in the medial thalamus, and lateral anterior thalamic nucleus in more caudal sections (Figure 2d).

We next examined whether expression of *Drd1* exhibited unique patterns of expression in the interneuron subtypes. Analysis of double labeling for each calcium-binding protein with *Drd1* revealed that the majority of each interneuron subtype expresses both transcripts (Figure 3). Quantification showed that 88.2 ± 3.5 percent of *Pvalb* positive interneurons express *Drd1* (Figure 3e and 4), whereas *Drd1* and *Calb1* double labeling comprised 61.6 ± 6.2 percent of the *Calb1* positive population (Figure 3f and Figure 4). These findings are similar to previous reports of both primate and rodent co-expression studies with DAD1R and parvalbumin co-expression more dominant than DAD1R co-expression by other calcium binding proteins (Glausier *et al.*, 2009; Le Moine and Gaspar, 1998). Because detection of *Calb2* transcript in the cortex was near background, co-expression with *Drd1* was not analyzed further.

Discussion

The present study combines with previous neuroanatomical data to indicate that *Drd1* is expressed on multiple cortical interneuron subtypes in the rabbit cortex. The absence of detectable changes in morphology or staining of the calbindin and calretinin subtypes provides strong evidence that the cortical parvalbumin interneuron in DA-rich regions is targeted uniquely by prenatal cocaine exposure. However, the widespread expression of *Drd1*, even on interneuron subtypes that exhibit normal morphological features indicates that other properties contribute to the permanent impact of prenatal cocaine exposure on the parvalbumin subpopulation in the cortex.

The present study also reports for the first time single- and double-label expression patterns for *Drd1*, *Pvalb*, *Calb1*, and *Calb2* transcripts in the rabbit brain. These studies revealed that transcript expression patterns for *Drd1* in the rabbit brain is similar to what has been reported in primate and rodent brain (Glausier *et al.*, 2009; Le Moine and Gaspar, 1998; Muly *et al.*, 1998). Not surprisingly, the striatum displayed dense labeling of *Drd1* transcript, with modest staining in the medial frontal cortex, though more dense in the deeper cortical layers. Co-localization patterns of *Drd1* with specific interneuron subpopulations were analyzed, and we show here that similar co-localization patterns exist for the rabbit as that published previously for both non-human primate and rodent brains. Specifically, more than 80% of cells that were positive for *Pvalb* transcript were also positive for *Drd1* transcript. In comparison, 60% of cells that expressed *Calb1* transcript also expressed *Drd1* transcript. To further demonstrate the

unique features of each subpopulation, immunohistochemistry co-localization studies revealed that parvalbumin and calbindin mark essentially non-overlapping interneuron subpopulations in P20 rabbit cortex (Figure S2). Thus the relatively high co-occurrence of *Drd1* transcript and *Calb1* cannot be due to overlap in the interneuron subpopulations defined by these calcium binding proteins.

While significant co-localization of *Drd1* with interneurons was found, there was an abundant number of single-label *Drd1* cells present, but not quantified in this study. These are likely excitatory pyramidal neurons that express *Drd1*, as reported in other species (Bergson *et al.*, 1995; Gaspar *et al.*, 1995; Maurice *et al.*, 2001; Santana *et al.*, 2009; Smiley *et al.*, 1994).

Our analysis of *Drd1* knockout mice (Stanwood *et al.*, 2005) and multiple studies from different laboratories showing alteration of dendritic growth of developing cortical neurons *in vitro* by modulating dopamine signaling (Crandall *et al.*, 2007; Jones *et al.*, 2000; Reinoso *et al.*, 1996; Stanwood and Levitt, 2007), suggested that this specific dopamine receptor is a major contributor to the altered parvalbumin neuron development reported previously (Stanwood *et al.*, 2001a; Stanwood *et al.*, 2001b). We recognize that the morphological and neurochemical analyses provide negative data, but are nonetheless essential to more clearly define the putative cellular targets of prenatal cocaine exposure. Thus, based on the co-localization patterns for both parvalbumin and calbindin subpopulations in the rabbit brain, and the lack of detectable changes to calbindin and calretinin subtypes, the alterations in parvalbumin dendritic labeling following prenatal cocaine exposure cannot be due solely to a DAD1R- mediated mechanism. One alternative explanation for the reported selective neurodevelopmental changes in parvalbumin and pyramidal neurons in dopamine-rich regions of the cerebral cortex are differences in the balance in expression of dopamine D1 and D2 (DAD2R) receptors may contribute to altered differentiation. While our previous studies have shown DAD2R coupling to its cognate G-protein to be intact, a second possibility is that increased synaptic dopamine could affect subsequent DAD2R function. Finally, unique electrophysiological and circuit properties of parvalbumin neurons in DA-rich cortex may contribute to their selective vulnerability to prenatal cocaine exposure. We have previously hypothesized that the balance between the two receptor subtypes is critical for the maintenance of cellular homeostasis between DAD1R and D2R mediated events, and prenatal cocaine exposure might disrupt this balance (Stanwood and Levitt, 2004). This study provides evidence that the presence of *Drd1* itself does not fully explain the neurodevelopmental consequences of prenatal cocaine exposure. Rather, a combination of phenotypic features that ultimately impose unique differences in the balance between DAD1R and DAD2R signaling on individual neurons during development, could mediate the effects. Future electrophysiological and developmental studies will address this hypothesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Calbindin and calretinin immunoreactivity following prenatal cocaine exposure
 Calbindin (A) and calretinin (B) immunoreactive cells in layers 2/3 of postnatal day 20 rabbit brain. Quantification of immunoreactivity for calbindin (C) and calretinin (D) reveals no differences in the number of immunolocalized interneuron sub-groups or in basic dendritic branching following prenatal cocaine exposure. Because calbindin also marks some layer III pyramidal neurons, only cells that were also immunopositive for GAD-67 were included in the quantification. Values are presented as Mean $\pm$ SEM.

Figure 2. Expression patterns of *Drd1*, *Pvalb*, *Calb1*, and *Calb2*

In situ hybridization autoradiograms of *Drd1* (A), *Pvalb* (B), *Calb1* (C), and *Calb2* (D) expression patterns in coronal sections of a postnatal day 20 rabbit brain. Note the very dense labeling of the neostriatum for *Drd1*, contrasting with the very sparse labeling in the overlying cortex. The bilaminar accumulation of *Pvalb*-expressing neurons in the cortex is evident. *Calb1* is evident throughout all layers of neocortex, but accumulates mostly in layers 2/3 in cingulate cortices. *Calb2* is very sparse in cerebral cortical regions, but very dense in the dorsal thalamus. The solid lines in panel B indicate the boundaries of cortical layers 1 through 6. The cortical layers above the dashed line are supragranular, whereas those below the dashed line are infragranular. Scale bar represents 400 μ m. Abbreviations in panel B: MFC (medial prefrontal cortex), SSC (somatosensory cortex), STR (striatum).

Figure 3. Confocal images of fluorescent *in situ* hybridization for *Drd1*, *Pvalb*, and *Calb1*
 Fluorescent *in situ* hybridization depicted in photomicrographs of double-labeling for *Drd1* and *Pvalb* (A, C, E) or *Drd1* and *Calb1* (B, D, F) in layers 2/3 in the medial frontal cortex of postnatal day 20 rabbit. Arrowheads point to cells that co-express *Drd1* and *Pvalb* (E) or both *Drd1* and *Calb1* (F). Full arrows point to cells with only a single label either of *Drd1*, *Pvalb*, or *Calb1*. Scale bar in panel B represents 28 μ m. Accumulated punctate labeling over cellular profiles is readily distinguishable from more diffuse background puncta, which is similar to background labeling using the sense strand.

Figure 4. *Drd1* is detected in both *Pvalb* and *Calb1* expressing interneurons

Quantification of double-label fluorescent *in situ* hybridization reveals a majority of *Pvalb* and *Calb1* interneurons co-express *Drd1*. Values are presented as Mean $\pm$ SEM.

Table 1
Prenatal cocaine exposure does not influence dendritic branching of calretinin or calbindin immunoreactive interneurons

Data presented as Mean $\pm$ SEM percentage of cells within the region of interest with zero, one, two, or three dendritic branches expressing the marker of interest.

		Dendritic Branches			
		0	1	2	3
Calbindin	Prenatal Saline	63.7 $\pm$ 0.8	27.8 $\pm$ 2.6	6.7 $\pm$ 1.8	1.7 $\pm$ 0.2
	Prenatal Cocaine	63.5 $\pm$ 3.5	24.7 $\pm$ 2.2	9.5 $\pm$ 1.0	1.7 $\pm$ 0.8
Calretinin	Prenatal Saline	54.2 $\pm$ 8.7	23.6 $\pm$ 5.3	7.2 $\pm$ 1.3	0.9 $\pm$ 0.5
	Prenatal Cocaine	53.1 $\pm$ 6.3	25.3 $\pm$ 5.4	9.9 $\pm$ 1.5	1.3 $\pm$ 0.5