Developmental Effects of Prenatal Cocaine Exposure on 5-HT$_{1A}$ Receptors in Male and Female Rat Offspring

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Abstract
Prenatal cocaine exposure results in behavioral abnormalities throughout development in rats, but little is known regarding the biological mechanisms underlying these abnormalities. Pregnant rats received subcutaneous twice-daily injections (1 ml/kg) of normal saline or 15 mg/kg of cocaine hydrochloride throughout gestation (gestation days 1–20). Following delivery, pups were placed with untreated surrogates. Male and female pups were killed on postnatal days 30, 60 or 120 for assessment of 5-HT$_{1A}$ receptor development in the forebrain, diencephalon, midbrain and pons using radiolabel immunocytochemistry. Findings revealed gender and age differences in developmental regulation of 5-HT$_{1A}$ receptors, indicating that male rats are more susceptible to long-term consequences of prenatal cocaine exposure in comparison to females. This study also demonstrates gender-specific development of serotonin (5-HT$_{1A}$) receptors across postnatal ages, demonstrating a fundamentally different pattern of development of 5-HT$_{1A}$ receptors between males and females.

Introduction
Although the magnitude of the physiological and neurobehavioral deficits engendered by prenatal exposure to cocaine continues to be debated [Lyons and Rittner, 1998], there seems to be general agreement that prenatal cocaine exposure can lead to multiple anatomical and physiological impairments, including decreased birth weight, intrauterine growth restriction, decreased head circumference, premature delivery [Bresnahan et al., 1991; Kelley et al., 1992; Koren et al., 1998; Scafidi et al., 1996], ophthalmic and auditory defects [Church et al., 1998], cardiac dysfunction, e.g. fetal tachycardia and heart rate variability [Hume et al., 1989], and gastrointestinal maladies [Plessinger and Woods, 1998]. There is less consensus regarding the extent of cognitive and behavioral insults suffered by children prenatally exposed to cocaine, including delayed developmental milestones, deficits in alertness and attentional abilities,
poor quality of sleep, decreased intelligence, delayed language acquisition, poor impulse regulation, increases in aggressive behavior, greater levels of anxiety and depression, as well as social problems [Chasnoff et al., 1998; Koren et al., 1998; Neuspiel et al., 1991; Richardson et al., 1996; Scafidi et al., 1996; Wasserman et al., 1998]. Deciphering the role of cocaine in these putative cognitive and behavioral deficits is a task made more difficult by confounding and mediating variables, such as inadequate maternal nutrition during pregnancy, socioeconomic considerations, maternal education, polydrug abuse, and home environment. While recent studies have controlled for some of these intervening variables [Chasnoff et al., 1998; Koren et al., 1998], the role of cocaine in the potential pathophysiology of such complex phenomena remains unclear.

Animal (largely rodent) models have been employed to evaluate the effects of prenatal exposure to cocaine under controlled experimental conditions. In rats prenatally exposed to cocaine, recent data have revealed altered stress responsivity [Molina et al., 1994], decreased acoustic startle response [Hughes et al., 1996], deficiencies in spatial memory tasks [Cutler et al., 1996], and deficits in acquiring and blocking Pavlovian conditioning in young pups and adolescents [Wilkins et al., 1998], respectively. There have also been reports of altered social interaction, including decreased elicitation of play behavior by same-sex conspecifics [Wood et al., 1995; Johns and Noonan, 1995], as well as increases in aggressive behavior in adolescent and adult rats [Wood and Spear, 1998] in competition tasks and tasks using a resident intruder model [Johns et al., 1994; Johns and Noonan, 1995].

In many of the cognitive and behavioral tasks evaluated in animal models of cocaine exposure, differential behaviors, dependent on the gender of the offspring, have emerged at variable developmental time points and in some cases, later returned to control levels [Hughes et al., 1996; Johns and Noonan, 1995; Johns et al., 1992b, 1994; Henderson and McMillen, 1993; Overstreet et al., 2000]. Generally, the extent and number of behavioral alterations are less evident in females compared to males prenatally exposed to cocaine. For example, Johns and Noonan [1995] have shown that male offspring prenatally exposed to 30 mg/kg of cocaine daily throughout gestation were less social (less likely to interact with same-age conspecifics) and were hypoactive at postnatal day (PND) 30 relative to saline-exposed controls [Johns et al., 1992a]. Males were also neophobic at PND 60, less social at PND 90, and at PND 180 [Johns et al., 1992a, 1992b, 1994], were more aggressive, had a stronger response to acoustic startle following administration of phencyclidine [Overstreet et al., 2000], and were still somewhat neophobic compared to offspring prenatally exposed to saline [Johns et al., 1992b]. On the other hand, female offspring prenatally exposed to 30 mg/kg of cocaine throughout gestation, had a somewhat different pattern of behavioral changes. They were more active than males and showed little neophobia at PND 30 or 60 and, depending on the types of social interaction tests, showed altered social behavior [Johns and Noonan, 1995; Overstreet et al., 2000]. Compared to offspring prenatally exposed to saline, cocaine-exposed females were hyperresponsive to tactile stimuli (air puff) on PND 180, but unlike cocaine-exposed males they habituated to the stimulus normally [Johns et al., 1994]. Aggressive behavior towards conspecifics across different postnatal ages has not been thoroughly explored in female offspring prenatally exposed to cocaine so that comparisons between male and females cannot yet be made.

Cocaine-induced alterations in cognitive and behavioral tasks are likely to be multiply determined in prenatally exposed animals. By blocking the uptake of dopamine, serotonin and norepinephrine into monoaminergic presynaptic neurons, cocaine causes increased concentrations of these neurotransmitters to remain in the synaptic cleft. In development and adulthood these increased synaptic monoamine concentrations can result in prolonged activation of postsynaptic cells. Investigators have begun to closely examine the effects of prenatal cocaine exposure on subsequent levels of monoamine neurotransmitters and receptors.

A number of the behaviors altered by prenatal cocaine exposure in both rats and humans (depression, aggression, social behavior) are most closely associated with reduced functioning of the serotoninergic (5-HT) system. Cocaine has been shown to inhibit nonspecific 5-HT uptake and synthesis and to suppress firing of 5-HT neurons in adult rats [Lakoski and Cunningham, 1988; Ritz et al., 1990]. Studies with selective serotonin uptake inhibitors have implicated serotonergic involvement in cocaine-induced behavioral changes in adult rats [Walsh and Cunningham, 1997; Carey et al., 2000]. Henderson and McMillen [1993] found that prenatal cocaine exposure resulted in decreased 5-HT and/or metabolites in the striatum and hypothalamus at PND 60 but not at PND 30 relative to saline controls. In the fetus, altered levels of serotonin may lead to significant, persistent alterations in the cytoarchitecture of the developing brain [Lauder, 1995; Levitt et al., 1998]. Serotonin has been implicated in maturation of the central nervous system where it acts as a
Semi-Quantitative Immunocytochemical Analysis of 5-HT1A Receptors

Effects of prenatal cocaine on 5-HT1A receptors in offspring were analyzed using radiolabel \([^{125}\text{I}]\) protein A immunocytochemistry (immunobinding, IB) performed on cryostat sections [adapted from Hunt and Mantyh, 1984]. At each postnatal age, animals were perfused with cold 4% paraformaldehyde and 0.1% glutaraldehyde in 0.07-M phosphate buffer (pH 6.8), preceded by phosphate-buffered saline (PBS). Brains were removed and post-fixed with the same fixative overnight, then washed in 3 changes of PBS and immersed in 30% sucrose in PBS until they sank. Brains were serially sectioned on a cryostat at 20 μm. Matched anatomical sections through the pons, midbrain, diencephalon and forebrain were chosen for IB from a Nissl-stained cryostat section series. These sections contained the following anatomical landmarks: pons (dorsal and median raphe nuclei), midbrain (ventral hippocampus/substantia nigra), diencephalon (dorsal hippocampus/habenula), and forebrain (basal ganglia/forebrain). Sections were permeabilized with Triton-X in normal sheep serum (NSS), rinsed in NSS and incubated overnight at 4°C with a primary antibody (5-HT1A rabbit polyclonal, gift of John Raymond) as described by Raymond et al. [1993] at a dilution of 1:500, or with diluent (bovine serum albumin/PBS/nitrocellulose). Sections were rinsed in PBS followed by NSS, then incubated for 2 h at room temperature in NSS containing \([^{125}\text{I}]\) protein A (400,000 cpm/ml), which binds to the primary antibody. Following this, sections were rinsed thoroughly in several changes of PBS, followed by deionized water, and air dried. Dried slides were placed in a phosphorimaging cassette at room temperature overnight. Phosphorimaging of sections was done directly from exposed phosphorimaging screens using a molecular dynamics storm phosphorimager, utilizing the same-size sampling area for all brain regions and treatment groups. Background label (sections exposed to diluent in place of primary antibody) was adjusted using the object-averaging mode of the Imagequant software. Specificity of the 5-HT1A antibody was determined previously by blocking antibody with the peptide antigen [Moiseiwitsch and Lauder, 1995].

Statistics

Statistics employed were analyses of variance (ANOVA) for treatment group × postnatal day (PND) × brain region × sex (4 way), followed by post hoc analyses using Tukey HSD tests for significant differences. Significance was assumed at the 0.05 level or less. Means and standard errors were calculated from raw phosphorimaging values [corrected for background against the negative control (no primary antibody)] from IB assays. IB was used rather than Western blot analyses because it has been shown to be more accurate for separate brain regions than Western blotting [Grobín et al., 2000]. Each of 4–6 animals had multiple (2–3) coronal sections scanned for each brain region and each IB run. Two to three IB runs were repeated on adjacent sections to control for procedural differences between runs. Any run that produced values significantly different from the other runs within a specific treatment and PND was considered an outlier and excluded from the analysis. Counts were averaged for each run and all the runs were then averaged for each animal at each PND for each brain region. The final average level of IB (in phosphor units) was then statistically compared by ANOVA as described above.

Methods

Animals and Procedures

Virgin female Sprague-Dawley rats (225–250 g; Charles River, Raleigh, N.C., USA) were group-housed (4 rats per cage) during a two-week habituation period. Rooms were on a 12:12 timed reverse light/dark cycle and animals had free access to rat chow (Purina) and water. Females were bred and the morning a sperm plug was found was designated as gestational day 0. Sperm-positive females were assigned to each of the two treatment groups or as a surrogate and housed singly in plastic rat cages. The treatment groups received subcutaneous injections of either 15 mg/kg of cocaine-HCl (Sigma Chemical Co., St. Louis, Mo., USA) in 0.9% normal saline (1 ml/kg) or an equal volume of normal saline twice daily at approximately 9:00 a.m. and 4:00 p.m. from gestational days 1–20. Surrogates received no treatment. Rat dams remained on the reverse light cycle for 8 days and were then changed to a normal 12:12 light/dark cycle, which results in most dams delivering in the afternoon rather than early morning. Cocaine, saline and surrogate dams were fed ad lib. Treatment dams were weighed daily, whereas surrogate dams were weighed and separated into same-sex litters of male or female pups. Pups were given to a surrogate dam that had delivered within 24 h of delivery. Brains were weighed and separated into same-sex litters of male or female pups. All pups were culled to 8 per cage and then further separated to 4 per cage at PND 30 until testing. One pup from each litter was selected for receptor assessment at each developmental time point. Six males and females (1 male/1 female per litter) from each of the two treatment groups were employed for each PND assessed. Ages were chosen to correspond roughly to juvenile (PND 30), puberty (PND 60) and adult (PND 120) periods.
Results

Gestational Variables

ANOVA indicated no significant differences between groups in maternal weight gain or gestation length. There were also no differences in litter weight, number of pups per litter or the male/female sex ratio of pups.

5-HT1A Receptors

Effects of prenatal cocaine on levels of 5-HT1A receptor IB (5-HT1A IB) at matched anatomical levels of the brain (forebrain, diencephalon, midbrain, pons) at 3 developmental periods are illustrated in figures 1, 2. Analyses indicated there were no significant differences in 5-HT1A IB levels between brain regions in any of the experimental groups resulting from treatment. Separate analyses for each region were examined (although there were no differences between the regions) and they were all quite similar. Therefore treatment effects on 5-HT1A IB levels are reported for whole brain (combined data from brain regions) rather than for individual brain regions. There was, however, a significant gender × PND × treatment interaction effect [F(2,114) = 16.50; p < 0.01]. Tukey HSD post hoc tests revealed significant differences in levels of 5-HT1A IB between controls and cocaine-exposed animals at different postnatal ages, and between genders as described below (fig. 1, 2).

Gender Effects

Overall, males in both control and cocaine exposed groups had significantly lower levels of receptors than did females [F(1,114) = 325; p < 0.01], regardless of age. This resulted in a different pattern of 5-HT1A receptors throughout development. As shown in figure 1, females had a higher but steadily declining receptor pattern while receptor numbers in males peaked at PND 60 and dropped at PND 120 to levels similar to those seen at PND 30.

Treatment, Gender and PND Effects

Differences between age [F(2,114) = 133.6; p < 0.01] and treatment [F(1,114) = 46.2; p < 0.01] are presented in figure 2 and discussed below.

PND 30. As illustrated in figure 2a, levels of 5-HT1A IB in cocaine-exposed males (219,282 ± 36,876 phosphor units) were not significantly different from saline controls (271,070 ± 36,876) whereas in females (fig. 2b), levels were significantly lower (p < 0.01) in the cocaine-exposed females (991,866 ± 46,644) relative to saline control females (1,419,157 ± 46,644).

PND 60. As males approached adulthood (fig. 2c), levels of 5-HT1A IB were now reduced (p < 0.01) in all brain regions analyzed in cocaine-exposed offspring (281,094 ± 46,644) compared to saline-exposed controls (758,558 ± 46,644) whereas in females, 5-HT1A IB levels in cocaine-exposed offspring had reached control levels by PND 60 (fig. 2d).

PND 120. At adulthood, 5-HT1A IB was still reduced (90,577 ± 42,018) in all brain regions (p < 0.01) of cocaine-exposed males (fig. 2e) compared to saline controls (158,642 ± 51,462) whereas in cocaine-exposed females (fig. 2f), 5-HT1A IB levels were slightly (\(\bar{x} = 363,066 ± 49,474\)), but not significantly, greater than controls (297,235 ± 45,163).

Discussion

This is the first study demonstrating that prenatal exposure to a moderate dose of cocaine results in gender- and age-specific abnormalities in the postnatal development of 5-HT1A receptors in rats. Patterns of 5-HT1A receptor development, as measured by 5-HT1A IB, dif-
Fig. 2. Prenatal cocaine treatment significantly decreased 5-HT$_{1A}$ expression (levels of 5-HT$_{1A}$ IB) in 60- (c) and 120-day-old (e) males in the whole brain (forebrain, diencephalon, midbrain, and pons) compared to controls (* $p < 0.01$). Reduced 5-HT$_{1A}$ expression is seen in females prenatally exposed to cocaine compared to control offspring at 30 days of age only (b), returning to control levels by 60 (d) and 120 days of age (f).

fered dramatically between male and female offspring prenatally exposed to cocaine, indicating that cocaine has very different effects on developmental regulation of 5-HT$_{1A}$ receptors in the two genders. The dynamics of change in receptor levels over the postnatal period were strikingly different in male and female offspring. While cocaine-exposed offspring of both sexes differed from controls according to treatment, males and females differed significantly in the pattern of change in 5-HT$_{1A}$ receptors. As shown in figure 1, males exhibited lower levels of IB at PND 30 and 120, with a peak at PND 60, whereas females had a distinctly different pattern, with IB levels decreasing from PND 30 to 120. As well, prenatal cocaine caused a greater magnitude of change in 5-HT$_{1A}$ receptors in males than in females (fig. 2).

Results of studies reported earlier [Johns et al., 1998] suggested that postnatal development of 5-HT$_{1A}$ receptors might be permanently dysregulated by prenatal cocaine exposure in males, as indicated by increased IB levels in neonates but decreased levels in adulthood. The pattern of dysregulation was different in females, where neonates had decreased 5-HT$_{1A}$ receptors [Johns et al., 1998] and, as also shown in the present study, reached control levels by adulthood. The present results describe the long-term
Consequences of prenatal cocaine exposure and confirm our original interpretation that prenatal exposure dysregulates postnatal 5-HT<sub>1A</sub> receptor development differently in males and females [Johns et al., 1998]. In males, although 5-HT<sub>1A</sub> IB reached control levels by PND 30, IB levels were significantly decreased by PND 60 and 120. In contrast, females, which were previously found to have decreased 5-HT<sub>1A</sub> receptors as neonates, continued to exhibit decreased receptor levels at PND 30, but reached control values by PND 60, where they remained at PND 120.

The present results indicate that prenatal cocaine exposure not only has opposite effects on postnatal 5-HT<sub>1A</sub> receptor development in male and female offspring, but also results in more serious long-term consequences for males. This prenatal insult causes dysregulation of postnatal receptor development in both sexes, but in males, where 5-HT<sub>1A</sub> receptors are increased neonatally by prenatal cocaine, receptor levels are permanently decreased by adulthood. Females on the other hand, which have reduced levels of 5-HT<sub>1A</sub> receptors as neonates, appear to regulate receptor expression by adulthood. In a recent study, prenatal exposure of pregnant mice to cocaine during a similar gestational period resulted in elevated concentrations of 5-HT and dopamine in fetal brain [Jun et al., 2002]. Serotonin levels were not determined in the fetal brains of our cocaine-exposed rats, so it is not known whether concentrations were increased. However, if this did occur it could lead to decreased levels of 5-HT<sub>1A</sub> receptors prior to birth [Whitaker-Azmitia et al., 1987]. In this case, gender differences in levels of 5-HT<sub>1A</sub> receptors in neonates [Johns et al., 1998] could indicate early differences in the plasticity of receptor development in males and females.

When attempting to interpret the results of the present study, it is useful to consider the developmental dynamics of 5-HT<sub>1A</sub> receptor expression. Transcripts for these receptors are maximally expressed prenatally in both rat and human brain. In rats there is a peak of 5-HT<sub>1A</sub> mRNA expression in whole brain at about gestational day 16 [Lauder et al., 1996]. Expression levels then gradually decrease until the time of birth, and then increase to adult levels during the postnatal period [Bar-Peled et al., 1991; Del Olmo et al., 1998; Hillion et al., 1993; Lauder et al., 1996]. Increased levels of 5-HT<sub>1A</sub> receptors seen in prenatally exposed neonatal male offspring [Johns et al., 1998] could indicate that cocaine led to retarded prenatal development of these receptors in males, such that the prenatal peak was delayed until around the time of birth. Similarly, reduced levels of 5-HT<sub>1A</sub> receptors in prenatally exposed neonatal females [Johns et al., 1998] suggests that the prenatal peak of 5-HT<sub>1A</sub> expression may have been accelerated by prenatal cocaine, resulting in lower levels of these receptors at birth. These are possibilities that must be addressed in future studies.

The present findings are consistent with previous reports of functional alterations in 5-HT<sub>1A</sub> receptors following prenatal cocaine exposure [Battaglia and Cabrera, 1994; Cabrera et al., 1993, 1994] where differences in male and female offspring were reported with respect to changes in hormone release following agonist stimulation of these receptors. Although those studies suggested that altered 5-HT<sub>1A</sub> and 5-HT<sub>2A</sub> receptor function following prenatal cocaine exposure was only measurable in relatively mature animals [Battaglia et al., 2000], our previous results [Johns et al., 1998] indicate that consequences of prenatal cocaine exposure are evident at birth, when levels of 5-HT<sub>1A</sub> receptors already differ in male and female offspring. The present results also demonstrate that in response to prenatal cocaine, patterns of 5-HT<sub>1A</sub> receptor development continue to change in a dynamic, gender-specific manner throughout the postnatal period.

Accumulating evidence suggests that prenatal serotonin receptors, developing on serotonin neurons, associated glia and along serotonergic pathways [Hellendall et al., 1993; Lauder, 1993, 1995], are functional and therefore vulnerable to maternal drug exposure [De Caballos et al., 1985; Lauder et al., 2000; Whitaker-Azmitia et al., 1987]. The results of the present study support and extend this evidence. This raises the interesting possibility that prenatal cocaine could impact regulation of 5-HT<sub>1A</sub> receptor transcriptional mechanisms [Meijer et al., 2000; Ou et al., 2000; Wissink et al., 2000]. Cocaine binds largely to 5-HT and DA transporters in the adult rat brain, whereas the majority of cocaine binding in fetal brain appears to be to the 5-HT transporter [Meijer et al., 2000; Whitaker-Azmitia, 1998]. Since cocaine may largely target the 5-HT transporter early in development, maternal cocaine exposure should impact prenatal development of the serotonergic system, with behavioral and structural consequences.

Serotonin is thought to be a growth regulatory signal in brain development. During embryogenesis, 5-HT regulates differentiation and growth of raphe 5-HT neurons and their target cells developing along serotonergic pathways [Lauder, 1990; Lauder et al., 1982; Whitaker-Azmitia et al., 1990]. Therefore, exposure to cocaine during critical periods of prenatal brain development could interfere with the development of serotonergic neurons and...
their targets. Neonatal rat pups prenatally exposed to cocaine have been found to exhibit a transient decrease in serotonin terminal density in the cortex and hippocampus that returns to normal levels by 4 weeks following birth [Akbari et al., 1992]. Such effects may be explained by the finding that prenatal cocaine exposure decreases S-100β, a trophic factor for 5-HT neurons that is released by astroglial cells in response to activation of 5-HT1A receptors [Akbari et al., 1994]. Prenatal cocaine also delays maturation of astroglia [Clarke et al., 1996]. Such disruption of trophic support for 5-HT neurons could have behavioral consequences, since delayed development of serotonergic nerve terminals has been reported to result in behavioral deficits in adult animals [Shemer et al., 1991]. Others have reported hyperinnervation by serotonergic terminals in brain regions, such as the striatum, following prenatal cocaine exposure [Snyder-Keller and Keller, 1993].

In previous studies, we found that rats prenatally exposed to the same dose regimen of cocaine as used in the present study exhibited a typical developmental pattern of behavioral abnormalities, including decreased social activity, aggressive behavior towards conspecifics, immobility in water, hypactivity, neophobia, and enhanced responses to tactile [Johns et al., 1992a, 1992b, 1994; Johns and Noonan, 1995; Overstreet et al., 2000; Wood and Spear, 1998] and decreased responses to auditory stimuli, primarily with drug challenge [Hughes et al., 1996; Overstreet et al., 2000]. Such behavioral abnormalities occurred differentially with respect to age, task and sex [Johns and Noonan, 1995; Hughes et al., 1990; Molina et al., 1994; Overstreet et al., 2000; Smith et al., 1989; Wood and Spear, 1998] and were, in many cases, more apparent in response to stress [Molina et al., 1994]. While some behavioral alterations were transient, other behaviors only began to appear at or just after puberty (PND 60), and continue into adulthood. These patterns of behavioral alterations are generally consistent with the developmental changes in 5-HT1A receptors following prenatal cocaine exposure seen in the present study. Of course, behavioral differences in both sexes may be attributed to various other developmental alterations and this may be particularly true in cases where behavioral changes continue to occur in adulthood in females. These behavior alterations are also similar to those observed in a strain of rats selectively bred to have increased sensitivity to a 5-HT1A receptor agonist, 8-OH-DPAT [Overstreet et al., 1996].

Abnormal functioning of the serotonergic system has been implicated in various disorders, including depression, autism, schizophrenia and compulsive behaviors. The results of our previous behavioral studies indicate that prenatal cocaine may cause alterations in social, depressive and aggressive behaviors, some of which are characteristics of psychopathological disorders. It is possible that changes in development of the serotonergic system could underlie many of these cocaine-induced behavioral alterations. Future studies will investigate effects of prenatal cocaine exposure on other 5-HT receptor subtypes in brain regions of interest.

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References


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