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Gender differences in prodynorphin but not proenkephalin mRNA expression in the striatum of adolescent rats exposed to prenatal cocaine

Annelyn Torres-Reveron^{1,a}, Yasmin L. Hurd^{2,b}, and Diana L. Dow-Edwards¹

¹Program in Neural and Behavioral Sciences and Department of Physiology and Pharmacology, SUNY Health Sciences Center at Brooklyn, Brooklyn, New York

²Department of Clinical Neuroscience, Psychiatry Section, Karolinska Institute, Stockholm, Sweden

Abstract

The objective of this study was to determine if prenatal cocaine affects the levels of prodynorphin and proenkephalin mRNA in male and female adolescent rats. Pregnant dams received cocaine or vehicle from gestational days 8-22 and upon delivery, the pups were fostered. At postnatal days 42-44, pups were killed and brains removed and frozen. Sections of striatum and nucleus accumbens were processed for prodynorphin and proenkephalin mRNA expression. Prenatal cocaine did not affect the expression of proenkephalin mRNA, but males showed higher expression than females. However, prodynorphin mRNA was lower in female rats exposed to cocaine compared to controls. Prenatal cocaine appears to have unique effects on neuropeptides during adolescence.

It is well stated in the pre-clinical literature that prenatal cocaine exposure produces deregulation in the dopaminergic systems [16;19], including impaired dopamine cell firing, dopamine synthesis and uncoupling of D1 receptors from its Gs proteins [6;13;23;24;39]. Behaviorally, animals with prenatal cocaine exposure show increased sensitivity to environmental demands and stressors, problems with inhibitory control, response initiation and information processing speed [14;34], and impaired conditioning and retention [20;21]. Similarly, clinical studies of prenatally cocaine exposed children report school age (7-9 years) deficits in the visual-motor domain and impairments in procedural learning [31;32]. It has been hypothesized that the behavioral abnormalities exhibited by prenatal cocaine exposed individuals are mainly due to a dopaminergic uncoupling and compensatory

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Corresponding Author: Diana L. Dow-Edwards, PhD, 450 Clarkson Ave, Box 29, Brooklyn NY 11209, Tel: 718-270-3987, Fax: 718-270-2241, diana.dow-edwards@downstate.edu.

^aCurrent address: Weill Cornell Medical College, Dept. of Neurology and Neuroscience, Division of Neurobiology 411 E 69th St. Room 410 NY, NY, 10021. ant2013@med.cornell.edu

^bCurrent address: Mount Sinai School of Medicine, Department of Pharmacology and Biological Chemistry, One Gustave L. Levy Place, Box 1215, New York, NY 10029. yasmin.hurd@mssm.edu

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changes in serotonin and norepinephrine as evidenced by pre-clinical research on prenatal cocaine [19].

Cocaine can produce changes in the response to dopamine within medium spiny neurons in the caudate-putamen and nucleus accumbens. These medium spiny neurons differentially contain dynorphin, enkephalin, substance P and GABA [15]. The neuropeptides dynorphin and enkephalin are thought to modulate the behavioral output produced by striatal projections [36]. Our laboratory has previously reported that perinatal cocaine administration [postnatal days (PND) 11-20] decreases the expression of prodynorphin mRNA in the nucleus accumbens when tested during adulthood [12]. However, cocaine administration during adulthood has been reported to increase prodynorphin mRNA expression within the striatum [1;7;22] while prenatal cocaine did not change prodynorphin expression in juvenile rats at PND 21 [9]. Also, prenatal cocaine did not alter proenkephalin or mu opioid receptor activation when assessed during PND 21 or 25 [9;26]. However, to our knowledge, no study has looked at the effects of prenatal cocaine on proenkephalin and prodynorphin mRNA expression during the adolescent period. Alterations in peptide expression within the striatum may depend on the developmental period during which the exposure occurred and the age at which the determination was made. The aim of this study was to examine the expression of prodynorphin and proenkephalin mRNA in the adolescent rat striatum after prenatal exposure to cocaine. Since the effects of prenatal cocaine are also sex dependent [35], we assessed the prenatal cocaine effects during the adolescent period in both sexes.

All procedures were approved by SUNY Institutional Animal Care and Use Committee. Nulliparous Sprague-Dawley rats (Charles River, VAF strain, Charles River Laboratories, Wilmington, MA) in proestrus were placed with males of the same strain at 4:00 PM. The next morning, females were checked for the presence of sperm by vaginal lavage. If present, that day was designated as gestational day 1 (G1). Pregnant dams were individually housed in plastic cages with bedding and assigned to receive either cocaine or vehicle. Dams in the vehicle control group were pair-fed and watered with the cocaine group beginning on G8. All dams except those in the pair-fed group had free access to food and water. The rats were kept under a 12 h light-dark cycle (lights on at 7:00 h) and at a temperature of 20-22 °C. Prenatal cocaine administration was carried out as previously described [11]. Briefly, beginning on G8 and continuing daily until G22 (inclusive) rats received either 60 mg/kg/day cocaine HCl (Research Triangle Institute, Research Triangle Park, NC) or vehicle (sterile water, Baxter, Deerfield, IL) administered by intragastric intubation using a 16 gauge straight feeding needle in a volume corresponding to 5 ml/kg of body weight. We have previously reported that a dose of 60 mg/kg of cocaine by intragastric intubation produces a peak fetal level of the drug of 3.0 µg/ml in plasma at approximately 15 min after maternal administration [10], similar to plasma drug levels seen in human fetuses [3;27]. Each dam in the control group was paired to a dam that belonged to the cocaine group weighing within ± 5 g on G1. The pair-fed dam was only given the amount of food and water consumed by the cocaine-treated rat for the same gestational day. On the day of birth (usually G23) and designated as postnatal day 1 (PND1) all pups were sexed, weighed, culled to 10 pups (5 males, 5 females) and surrogate fostered to non-treated dams delivering within the preceding 48 h. Between PND 41-44 rats were sacrificed with a lethal dose of

sodium pentobarbital (60 mg). The brain was removed and frozen in 2-methylbutane at -20°C for 5 min, coated with matrix and stored at -70°C . Female rats were subjected to vaginal smears for determination of the stage of estrous cycle. Brains were sectioned coronally at 20 mm. We chose PND 45 rather than an earlier age (PND 30 or 35) because at this age females have reached sexual maturity (vaginal opening) and this age is close to the onset of puberty (preputial separation) for males [25].

For the *in situ* hybridization procedure, at least 2 sections from each subject in each group were simultaneously processed through all conditions. Prior to hybridization, the tissue-mounted slides were warmed to room temperature and fixed with 4% paraformaldehyde, dehydrated in ethanol washes, delipidated in chloroform, rinsed in ethanol, and air-dried. All solutions were made with autoclaved 0.1% diethylpyrocarbonate-treated water and racks and staining dishes were also treated with this solution to inactivate RNases. Antisense (complementary to target mRNA) riboprobes (single-stranded RNA molecules synthesized from a cloned DNA or PCR-derived fragments, with attached promoter sequences), were used. The prodynorphin mRNA probe was complementary to a 534 KB fragment of the rat prodynorphin cDNA (bp 349-883 GeneBank accession number NM_019374). The proenkephalin mRNA probe was complementary to a 333 kb fragment of the rat proenkephalin cDNA (bp 291-624 GeneBank accession number Y07503). The riboprobes were transcribed and radiolabelled by the addition of RNA polymerase enzymes (such as SP6 or T3) with [^{35}S] uridine 5' -[α -thio] triphosphate (1000-1500 Ci/mmol; New England Nuclear, Boston, MA, USA). Brain sections were hybridized overnight with radiolabeled probes (4×10^6 cpm/slide) in a humidified chamber at 55° - 65°C (depending on the stringency requirements) under appropriate hybridization buffer conditions. The brain sections were carried through post-hybridization washes, dried, and apposed to Hyperfilm b-Max (Amersham, Buckinghamshire, UK) with 14 C standards (calibrated against ^{35}S -impregnated brain paste standards) of known radioactivity. Control experiments were carried out using sense RNA probes to validate the specificity of the antisense hybridization signal. The films were developed after various time periods depending on the abundance and expression of the specific marker in the tissue. Once developed, the films were analyzed using the MCID-M5 system according to the atlas [29] for the caudate-putamen and nucleus accumbens at the levels of Bregma 0.2, 1.2, 1.6 and 2.2. Data were analyzed using a Mixed Linear Model with region, prenatal treatment and sex as fixed factors and controlling for litter as a random factor in the model. Simple effects post hoc analysis was carried out. The dependent variables were log transformed to ensure normal symmetrical distribution of the variables.

For both males and females we observed a higher amount of prodynorphin mRNA expression in the nucleus accumbens compared to the dorsal striatum as previously shown [8;12;22] (see Fig. 1). A significant main effect for brain region was observed ($F_{(10,30)} = 312$, $p < 0.0001$), but no interaction effects involving the different regions were significant. There was a significant sex by prenatal treatment interaction for prodynorphin mRNA expression ($F_{(1,28)} = 9.25$ $p = 0.005$). Post hoc analyses revealed that there was a significant prenatal cocaine effect among female rats ($F_{(1,17)} = 6.21$, $p = 0.024$) but not males ($F_{(1,15)} = 0.15$, $p > 0.05$). Prenatally cocaine exposed females showed lower levels of prodynorphin

mRNA (collapsed for regions) than prenatal control females (Fig.3A). Interestingly, we also observed a significant sex difference among prenatal control subjects ($F_{(1, 45)} = 5.89$, $p = 0.019$) but this sex difference was not observed between prenatal cocaine treated animals ($F_{(1,46)} = 2.24$, $p = 0.141$). Females in the prenatal control group showed higher levels of prodynorphin mRNA compared to prenatal control males.

For proenkephalin, we observed similar expression in both the caudate-putamen and nucleus accumbens (Fig. 2). Unlike prodynorphin mRNA, we did not find main effects of prenatal treatment or sex by prenatal treatment interactions. There was a significant main effect of sex when the data were collapsed for region and prenatal treatment ($F_{(1, 32)} = 6.37$, $p = 0.017$). In general, males in both prenatal treatment groups showed higher levels of proenkephalin mRNA expression compared to the females in both prenatal treatment groups (Fig. 3B).

Our data indicate that prenatal exposure during early brain developmental periods has long-lasting effects in dopaminergic striatal circuits, more so in females than in males. Previous work from our laboratory has shown that prenatal exposure to cocaine can decrease the locomotor activity of adolescent female rats challenged with methylphenidate (a dopamine reuptake inhibitor with a similar mechanism of action as cocaine) when compared to prenatal control females [38]. The observations of decreased prodynorphin mRNA in females with prenatal cocaine exposure parallels the behavioral results observed in the same group of animals. It is well documented that while acute cocaine increases prodynorphin mRNA, withdrawal from cocaine can result in a significant reduction of prodynorphin mRNA and D1 dopamine receptors [37]. This suggests that decreased prodynorphin mRNA levels in striatal neurons might result from decreased dopaminergic activity in females exposed to prenatal cocaine.

Our data suggest that striatonigral projections, which contain mostly prodynorphin mRNA [36] are more sensitive to the deleterious effects of cocaine exposure in females during the prenatal period compared to males. It is well documented that prenatal cocaine produces uncoupling of D1 receptor from its second messenger [13;39]. Also, dopamine D1 receptors have been colocalized in the same neurons as the prodynorphin mRNA containing neurons in the nucleus accumbens [18]. The decrease in prodynorphin mRNA expression in female rats could be due to a deficient dopaminergic D1 activity. Another possibility is that differences between males and females observed in this study might be in part due to inherent sex differences in striatal development during the time at which cocaine exposure occurred. Males show a maturational delay in mesencephalic neurons compared to females [5]. Rats from embryonic days 16-21 showed sex-specific differences in the density of GABAergic and tyrosine hydroxylase immuno-reactive cells in the prenatal striatum [28]. There is also increased dopamine reuptake for males in utero compared to females [30]. Thus developing dopaminergic neurons of female rats mature before those of males. Consequently a similar prenatal cocaine exposure time in males and females will result in a longer exposure for striatal cells in females since the maturation is earlier than that in the males. This might render females more susceptible to the neurotoxic effects of cocaine. In addition, we observed in this study that control animals show sex differences in the levels of prodynorphin mRNA expression. Some effects of prenatal cocaine exposure on striatal

dopaminergic activity are exclusively observed in cycling female rats and not in males [17]. It is suggested that this effect can result from a lack of regulation of striatal dopamine neurons to hormonal (estrogen, progesterone, and others) fluctuations in prenatal cocaine exposed female rats as compared to non-exposed females.

However, our results contrast with previous results from our laboratory showing that *perinatal* cocaine exposure produced a decrease in prodynorphin mRNA in adult males [12], which we did not observe in the current experiment for males exposed to prenatal cocaine. The most reasonable explanation for these results is the difference in period of brain development at which cocaine was administered. The development of the striatum during the prenatal period of rats is characterized by neurogenesis, whereas pruning and remodeling occurs during the perinatal period [4]. Sex differences in relation to the functional development of, e.g., dopamine neurons, dynorphin and D1 receptors, needs to be explored in order to assess the underlying mechanisms for increased vulnerability of the females to prenatal cocaine exposure. Sex differences have been shown in adolescent animals relative to dopamine receptor density [2], however, sex differences for the effects of peptides need to be further explored.

In contrast to prodynorphin mRNA, prenatal cocaine exposure produced no effect in proenkephalin mRNA expression for either sex. Our results agree with several studies that have shown that prenatal cocaine exposure produces no changes in proenkephalin mRNA expression [9;26;33]. In conclusion, our study carried out in adolescent animals continues to emphasize a strong role of the prodynorphin mRNA striatal system in response to prenatal cocaine exposure, and reveal a significant impact of sex.

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Figure 1. Prodynorphin mRNA expression (dpm/mg) in adolescent males (A) and females (B) exposed to prenatal cocaine. For this and subsequent figures, numbers for the caudate-putamen and nucleus accumbens correspond to distance from Bregma based on Paxinos and Watson (2004). Here and in all other figures, bars represent mean $\pm$ SEM. N's are 12 males and 10 females from 6 cocaine-treated litters and 12 males and 11 females from 6 control litters..

Figure 2. Proenkephalin mRNA expression (dpm/mg) in adolescent males (A) and females (B) exposed to prenatal cocaine. N's are as for figure 1.

Figure 3.

Prodynorphin (A) and proenkephalin (B) mRNA expression levels collapsed for all regions analyzed. * represents significantly lower than same sex control; ^ represents significantly higher than all other groups; # represents both groups of males significantly different than both groups of females.