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Moderate-Level Prenatal Alcohol Exposure Alters Striatal Dopamine System Function in Rhesus Monkeys

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Background: Moderate prenatal alcohol exposure can cause impairments even in the absence of gross morphological defects associated with fetal alcohol syndrome. The basal ganglia, which include the dopamine-rich striatum, are sensitive to fetal alcohol-induced injury. In this study, we manipulated the timing of moderate-level alcohol exposure and compared the risk of adverse effects on striatal dopamine (DA) system function in rhesus monkeys.

Methods: Thirty-five young adult rhesus monkeys (*Macaca mulatta*) from four groups of females were assessed: (1) an early alcohol-exposed group ($n = 9$), in which mothers voluntarily consumed 0.6 g/kg alcohol solution on gestational days 0 through 50; (2) a middle-to-late gestation alcohol-exposed group ($n = 7$), in which mothers voluntarily consumed 0.6 g/kg alcohol solution on gestational days 50 through 135; (3) a continuous-exposure group ($n = 9$), in which mothers voluntarily consumed 0.6 g/kg alcohol solution on days 0 through 135; and (4) controls ($n = 10$), in which mothers voluntarily consumed an isocaloric control solution on gestational days 0 through 50, 50 through 135, or 0 through 135. We studied striatal DA system function by positron emission tomography in separate scans for trapping of [^{18}F]fallypride and 6-[^{18}F]fluoro-*m*-tyrosine to assess striatal DA D_2 receptor (D_2R) binding and DA synthesis, respectively, via dopadecarboxylase activity.

Results: Moderate-level alcohol exposure during early gestation and continuous exposure throughout gestation (early + middle-to-late exposure) reduced the striatal D_2R binding to DA synthesis ratio, whereas middle-to-late alcohol gestation exposure increased the striatal D_2R binding to DA synthesis ratio. The continuous-exposure group showed the largest effect. Moreover, the D_2R binding/DA synthesis ratio was related to neonatal neurobehavior measures in control monkeys, but these relationships were disrupted in the fetal alcohol-exposed monkeys.

Conclusion: These results suggest that the vulnerability of the DA system to the effects of moderate doses of alcohol during gestation depend on the timing of the alcohol exposure. Early-gestation moderate alcohol exposure resulted in a reduction or blunting of dopaminergic function in adulthood, whereas middle to late exposure (without early exposure) either induced the opposite pattern or heightened dopaminergic function. Continuously exposed monkeys showed the largest effect, suggesting that the sooner women stop drinking, the better it is for the fetus.

Key Words: Dopamine, Rhesus Monkey, Striatum, Fetal Alcohol Exposure, Positron Emission Tomography, Neurobehavior

A growing body of evidence suggests that even moderate-level alcohol consumption during pregnancy can influence the developing fetus and induce a number of functional deficits in affected children (Jacobson and Jacobson, 1999). The extent of fetal alcohol-induced deficits, however, depends on a number of risk factors, including the timing, dose, and duration of alcohol consumed as well as the differential vulnerability among various central nervous system (CNS) regions to alcohol-induced brain injury (Bonthius and West, 1991; Goodlett and Johnson, 1999).

Prenatal alcohol exposure has been found to disrupt several neurotransmitter systems, including dopaminergic (Druse et al., 1990), serotonergic (Tajuddin and Druse, 1999; Sari et al., 2001), glutamatergic (Farr et al., 1988; Kelly et al., 1986), histaminergic (Rawat, 1980), cholinergic (Light et al., 1989), and noradrenergic (Detering et al., 1980). In this project, we focused on the vulnerability of the dopamine (DA) system to fetal alcohol-induced damage based on several lines of experimental research. First, in rodents, fetal alcohol exposure is associated with a reduc-

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We used Rakic's terminology for describing the dates; E (embryologic) is the same as gestation day.

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tion in spontaneous activity in DA neurons, decreased DA uptake and receptor binding sites, decreased DA metabolite homovanillic acid in DA neurons, and decreased D_1 dopamine receptors in the hypothalamus and striatum (Cooper and Rudeen, 1988; Detering et al., 1980; Druse et al., 1990; Rathbun and Druse, 1985; Shen et al., 1999; Zhou et al., 2003). In addition, prenatal alcohol exposure results in smaller cell bodies and reduced dendritic growth in DA neurons (Shetty et al., 1993), changes in DA receptor function (Shen et al., 1995; Wang and Shen, 2002), and DA receptor-mediated behavior (Hannigan and Randall, 1996).

Second, reduced DA neuron activity in the ventral tegmentum induced by prenatal alcohol exposure can be restored to normal by treatment with a DA agonist, methylphenidate, which increases extracellular levels of DA (Choong and Shen, 2004). Third, in both animals and humans, attention, set shifting, executive function, and the frontal-striatal circuitry involved in these functions (known to be dependent on DA) are disrupted by fetal alcohol exposure (Carmichael Olson et al., 1998; Jacobson et al., 1998; Kodituwakku et al., 1995; Mattson et al., 1999; Noland et al., 2003). This also makes DA in the striatum a logical place to look for deficits. Finally, magnetic resonance imaging studies in humans have shown that the basal ganglia, a region rich in DA-ergic neurons, is vulnerable to fetal alcohol exposure (Cummings, 1993; Mattson et al., 1999). Taken together, these findings provided the groundwork for the present study, which investigated whether moderate fetal alcohol exposure during a specific gestation period would adversely affect striatal DA system function.

One of the most interesting questions pertaining to fetal alcohol exposure concerns the timing of alcohol exposure and regional vulnerability in the brain (Maier et al., 1996). Alcohol exposure during a specific sensitive period might have adverse effects related to the region of the brain or cell type undergoing rapid development at that time (Goodlett and Johnson, 1999). A sensitive period during CNS development is a time during which the effects of experience can alter neuronal development and connectivity (Bear, 1995). For example, exposure to teratogens during early gestation, the period of neurogenesis, can induce gross irreversible malformations of the brain. Middle gestation is considered a period during which there is neuroblast proliferation, such that adult numbers of neurons are achieved (Dobbing and Sands, 1970). The latter part of gestation and the first 18 months of life correspond to the brain growth spurt, a period of synaptogenesis during which brain weight and the behavioral development that they subserve proceed very quickly (Dobbing, 1972).

Some human studies suggest that exposure to alcohol during the latter part of pregnancy, which represents the brain growth spurt, is more strongly associated with developmental problems, including reductions in physical growth, abnormalities in behavior, and deficits in math, reading and spelling (Coles et al., 1991; Cornelius et al., 1999; Day et al., 1999; Richardson et al., 1989). Similarly, rodent studies have found that exposure to alcohol during the postnatal period of high synaptogenesis (second and third trimester equivalent in humans) produces neuronal loss, altered neuronal circuitry, altered gliosis, and apoptotic neurodegeneration in the developing forebrain (Bonthius and West, 1991; Borges and Lewis, 1983; Goodlett et al., 1993, 1998; Guerri, 1987; Melcer et al., 1994; West et al., 1984). Others have found that fetal alcohol exposure during early pregnancy, or neurogenesis, is most damaging to fetal CNS development and function. For example, heavy alcohol exposure during the first trimester human equivalent has been linked to craniofacial anomalies and midline brain abnormalities in both humans and rodents (Graham et al., 1988; Sulik et al., 1981). Likewise, in pig-tailed macaques, even once-weekly drinking during early gestation (wk 0–6 at a dose comparable to six drinks in humans) was sufficient to cause deficits that were as severe as those detected from drinking throughout pregnancy (wk 0–24) (Clarren et al., 1992). However, the links between fetal alcohol exposure during a certain time period of CNS development and the brain abnormalities exhibited by the offspring postnatally are exceedingly complex in most animal models. For example, in rodents, neurons of the locus ceruleus, but not the Purkinje cells of the cerebellum or inferior olive, were reduced in number when exposure occurred during neurogenesis (E11–13) (Maier and West, 2003), even though the cerebellum, which is particularly vulnerable to alcohol-induced cell loss during the brain growth spurt (part of the third trimester equivalent), is vulnerable during early gestation as well. This finding was interpreted as evidence that alcohol exposure during earlier periods (first and second trimester equivalents) might exacerbate cell loss associated with third trimester equivalent brain growth spurt exposure (Goodlett and Eilers, 1997; Goodlett and Lundahl, 1996; Goodlett et al., 1998; Maier et al., 1999; Marcussen et al., 1994; Phillips and Cragg, 1982; Pierce et al., 1989). Also, certain cell types can differ in terms of vulnerability even within the same region. In rodents, pyramidal cells in CA1 of the hippocampus were found to be more vulnerable to alcohol-induced damage than pyramidal cells in field CA3 during the third trimester equivalency period, or brain growth spurt (Bonthius and West, 1991; Bonthius et al., 2001; Miller, 1995; Pierce et al., 1989; Tran and Kelly, 2003; West et al., 1986).

Although moderate alcohol consumption, defined by Dawson et al. (1995) as 7 to 14 drinks per wk, is common during pregnancy in humans (Ebrahim et al., 1998), there are only a few published reports of experimental moderate alcohol exposure in animals. Thus, the extent of regional and temporal vulnerability from moderate-level alcohol exposure is largely unknown. Savage and colleagues (2002) found that in rats, moderate-level alcohol exposure in the less than 30 mg/dl blood alcohol range throughout gestation (comparable to drinking 1 to 1.5 ounces of alcohol

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daily) reduced one-trial acquisition in a spatial learning task. Moreover, this level of exposure produced an enduring dose-dependent impairment of neurochemical mechanisms, such as hippocampal glutamate release, that sustain activity-dependent synaptic neurotransmission in adult offspring (Savage et al., 2002). Moderate fetal alcohol exposure has also been found to induce inhibition of cell-cell adhesion, reduced long-term synaptic potentiation, decreased hippocampal synaptic plasticity and density of *N*-methyl-d-aspartate-sensitive [^{3} H]glutamate binding sites in hippocampal formation, and altered glial development and expression of glial fibrillary acidic protein (Charness et al., 1994; Goodlett et al., 1993; Savage et al., 1991; Swartzwelder et al., 1988; Sutherland et al., 1997). At midgestation, moderate fetal alcohol exposure compromises midline neural tube development, which could delay neurogenesis by reducing brainstem midline structures from which serotonin neurons are derived (Zhou et al., 2001).

We tested the hypothesis that relatively moderate fetal alcohol exposure during particular periods of gestation would alter striatal DA system function in adult rhesus monkeys. The rhesus monkeys in this study are members of a prospective, longitudinal study of the effects of gestational timing of moderate fetal alcohol exposure on neurobehavioral development and brain integrity. We previously reported neonatal neurobehavioral deficits in monkeys exposed to moderate-level alcohol during both early and middle to late gestational periods and that neuromotor and attentional impairments during the first 4 wk of infancy were slightly increased for the monkeys with first trimester equivalency exposure (Schneider et al., 2001b). For the present study, we used positron emission tomography (PET) on these monkeys to investigate the balance of striatal postsynaptic receptor binding (D_2R) and DA synthesis (DA-syn). PET is an important tool in neuroscience research because it uses radiotracers specific for particular neuronal receptor subtypes and enzyme systems. One can quantitatively determine receptor densities and enzyme activities in vivo. Changes in receptor concentration or enzyme activity can provide insights into the underlying state of physiological, biochemical, and pharmacological functions at the molecular level in the living organism (Katsifis and Kassiou, 2004).

The methodology for PET studies of D2R and DA-syn is well established from decades of research into neurodegenerative disease such as Parkinson's. We chose the striatum, which consists of the caudate nucleus and putamen, because striatal circuits can regulate frontal cortex circuits and are involved in shifting attention sets, movement, and spatial working memory, functions known to be affected by fetal alcohol exposure (Herrero et al., 2002). We assessed the ratio of D_2R binding and DA-syn in two separate scans. We assessed both because of the complementary relationship between postsynaptic D_2R density and presynaptic synthesis. Specifically, DA D_2R up- or down-regulation is known to occur in response to lower or higher synaptic DA levels (Donnan et al., 1991). Current imaging agents for presynaptic DA neurons can be classified into groups based on which binding sites are targeted. The three current classes of presynaptic dopamine tracers are (1) metabolic markers such as 6FDOPA, 6-fluoro-*m*-tyrosine (6FMT), and fluoro-α-fluoromethyl-*p*-tyrosine, which target the enzymes in DA biosynthesis; (2) markers of the DA transporter (DAT), like cocaine analogues such as WIN 35428 and methylphenidate; and (3) markers of the vesicular monoamine transporter (VMAT2), such as tetrabenazine. Recent studies have found that levels of DAT protein and DA itself are the most sensitive indicators of DA nerve terminal loss (Wilson et al., 1996). However, no noninvasive means to measure either marker is available. Neuroimaging markers for DAT were found to be more sensitive to neuronal loss than VMAT2 markers. It is not known how the metabolic tracers (6FDOPA, 6FMT, fluoro-α-fluoromethyl-*p*-tyrosine) compare with DAT and VMAT2 markers in terms of sensitivity to neuronal loss, but 6FDOPA uptake constants have been found to be highly correlated with viable DA cells in MPTP-lesioned monkeys and in postmortem human Parkinson's disease brains (Pate et al., 1993; Snow et al., 1993). Precursor tracers, unlike transporter markers, can assess functional DA neuronal activity under normal and perturbed conditions rather than neuronal number. Although the transporter tracers may be better suited to assess DA-ergic degeneration, as in Parkinson's disease, precursor tracers are better suited in the assessment of changes in DA-syn, which may underlie neuropsychiatric disorders and other conditions, such as fetal alcohol exposure, wherein the number of DA neurons is essentially normal but with altered activity.

DA is synthesized by conversion of the amino acid l-*p*-tyrosine to l-DOPA by the enzyme tyrosine hydroxylase (see DeJesus, 2003, for details). l-DOPA is then rapidly converted to DA by the enzyme l-aromatic amino acid decarboxylase (AAAD). Although a tyrosine hydroxylase marker would be useful in assessing DA-syn, labeled l-*p*-tyrosine would be ineffective because its primary role is in protein synthesis; thus 2-[^{18}F]fluoro-l-*p*-tyrosine is used as a marker of protein synthesis (Coenen et al., 1989). More than 30 years ago, a fluorine-18-labeled analogue of l-DOPA was reported (Firnau et al., 1973), and 6FDOPA continues to be an important PET tracer today. An important disadvantage of FDOPA, however, is that it is such an excellent analogue of l-DOPA that it undergoes all of the metabolic steps of l-DOPA, including the formation of 3-*O*-methyl-FDOPA in the periphery by the enzyme catechol-*O*-methyltransferase (COMT), which is found in red blood cells, liver and other tissues. Consequently, 3-*O*-methyl-FDOPA enters the brain and distributes widely and uniformly, raising background "noise" in the FDOPA image. Because of this, blood sampling is needed during the PET study to correct for this, and blood sampling is not only experimentally demanding but also reduces the noninvasive nature of PET imaging (DeJesus, 2003).

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Another approach is to use DOPA analogues that are not catechol-*O*-methyltransferase substrates, such as *m*-tyrosine (DeJesus and Mukherjee, 1988). *m*-Tyrosine is the parent of FMT used in this study. FMT is not a tyrosine hydroxylase substrate but rather a potent substrate of AAAD. The main contributor to FMT tracer kinetics, which we assess in this study, is the action of AAAD on FMT. If FMT is not decarboxylated by AAAD, it leaves the cell and clears the CNS, which is what occurs in cells that do not have AAAD. In cells with AAAD, such as monoaminergic neurons, the trapping step involved in PET image formation in FMT studies is decarboxylation. The decarboxylation forms a product that clears the cell very slowly, so that in the PET imaging time frame, clearance is negligible. Thus, the rationale for using FMT in our study as a measure of DA-syn is that FMT tracks the AAAD or DA-syn step.

The tracer used to assess the D_2Rs was [^{18}F]fallypride (FAL), a fluorine-18-labeled raclopride analogue developed by Mukherjee and colleagues (Mukherjee et al., 1997). FAL has a high affinity for DA D_2 receptors and high brain uptake, almost three times higher compared with [^{11}C]raclopride.

A second purpose of our study was to examine the relationship between striatal DA system parameters and neurobehavioral outcomes that were previously measured in these same animals during the neonatal period (Schneider et al., 2001a). Specifically, in our prospective longitudinal study, we conducted repeated observations of neonatal neurobehavioral functions purported to be associated with striatal DA-ergic function. These included measures of attention, neuromotor function, and state control, or emotion regulation, which emerge during early infancy (Schneider and Suomi, 1992; Schneider et al., 200X). It is important to determine whether continuity can be detected between early neurobehavioral measures of attention and neuromotor function and later PET indices of DA-ergic function. If continuity can be found, it supports the importance of early intervention, when the brain shows the most plasticity.

METHODS

Maternal Alcohol Treatments

We identified healthy, adult, female rhesus monkeys within the breeding colony that voluntarily and reliably consumed 0.6 g/kg of a 6% (v/v) alcohol solution sweetened with NutraSweet (300 mg/100 ml; Equal Sweetener, Merisant US, Inc., Chicago, IL). Before breeding, blood samples were obtained 60 min after consumption of 0.6g/kg alcohol, which produced average blood alcohol concentrations of 20 to 50 mg/dl. This dosage is comparable to an average-size woman consuming two drinks daily. Females that consumed alcohol were randomly assigned to the control group or one of three experimental groups (see below), with timing of prenatal alcohol exposure as the independent variable. The alcohol-consuming mothers voluntarily consumed the alcohol solution daily at 1600 hr. Water was available ad libitum, including during the period when the alcohol solution was available. The animals had no chow left by the time of day that the alcohol was introduced. The control mothers consumed a sucrose solution that was designed to be approximately equivolemic and equicaloric (8 g/100 ml water) to the alcohol solution. All females were housed under identical conditions, undisturbed except for necessary routine animal husbandry. These studies were conducted in accordance with the Institutional Animal Care and Use Committee.

Subjects

The offspring subjects in this study were 35 young adult (4–5 yr old; mean, 4.8 yr) rhesus monkeys (*Macaca mulatta*), 19 females and 16 males. Controls consisted of 8 females and 2 males (mean age, 4.15 yr) whose mothers voluntarily consumed an isocaloric control solution on gestational days 0 through 50, 50 through 135, or 0 through 135. Nine monkeys, 4 females and 5 males (mean age, 4.28 yr), were born to female rhesus monkeys that consumed 0.6 g/kg alcohol solution on gestational days 0 through 50. Seven monkeys, 3 females and 4 males (mean age, 4.089 yr), were born to female rhesus monkeys that consumed 0.6 g/kg alcohol solution on gestational days 50 through 135 (second and third trimester equivalent). Nine monkeys, 4 females and 5 males (mean age, 4.16 yr) were born to mothers that consumed 0.6 g/kg alcohol solution on days 0 through 135, a combination of all three trimesters. These monkeys are members of an ongoing longitudinal study that is investigating the effects of moderate-level fetal alcohol exposure, during early or mid to late gestation, on development and neurobehavioral function. The rearing conditions and previous testing of these subjects were described in detail elsewhere (Schneider et al., 2001b). In brief, all infant monkeys were housed with their mothers in individual cages during the first 6 mo of life. They were separated briefly from their mothers weekly and tested for neonatal neurobehavioral function during the first mo of life (see Primate Neonatal Neurobehavioral Assessment {PNNA]). At 6 mo of age, they were separated permanently from their mothers and reared in mixed-sex peer groups consisting of 5 or 6 monkeys from similar prenatal conditions. At the time of this study, they were pair-housed with same-sex peers from similar treatment groups. They were maintained on a diet of Purina Monkey Chow supplemented three times weekly with fresh fruit. All housing conditions were light (8 dark and 16 light) and temperature (21 ± 0.5°C) controlled. When they were 4 to 5 yr old, they were assessed with PET methodology described below.

PNNA

The PNNA was designed to assess aspects of neurobehavior that show developmental changes during the first

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Table 1. Definition of Items on the Infant Behavioral Assessment Scale

Items	Definitions
Orientation cluster	
Visual orient	Eyes oriented toward plastic Mickey Mouse face held in four positions in infant's periphery (0 = no orient; 1 = direct brief contact; 2 = direct prolonged contact)
Visual follow	Eyes following moving toy in both horizontal and vertical directions (0 = contact, but not following; 1 = starts to follow; 2 = complete following)
Duration of looking	Examiner rating of length of looks on orienting items (0 = brief looks; 1 = 1-sec looks; 2 = 2+ sec looks)
Attention span	Examiner rating of attention span on orienting items (0 = lack of attention on all items; 1 = attentive 25% of the time; 2 = attentive 75% of the time)
Motor maturity cluster	
Head posture prone	Ability to hold head up when infant is held in prone position (0 = flaccid tone with head hanging down; 1 = head lifted but not maintained for 3 sec; 2 = head lifted and maintained for at least 3 sec).
Head posture supine	Ability to hold head up when infant is held in supine position (rated identical to head posture prone)
Response speed	Examiner rating of speed of responding (0 = 25% of responses quick; 1 = 75% of responses quick; 2 = all responses quick)
Coordination	Quality of movement rating (0 = clumsy movement; 1 = adequate movements; 2 = agile movements)
Labyrinthine righting	Realignment of head when body tilted 45 degrees sideways (0 = no righting, head and body in same plane; 1 = head partially rights; 2 = head rights and lines up with the vertical plane)
Activity Cluster	
Motor activity	Examiner rating of amount of motor activity (0 = motion 25% of time; 1 = motion 50% of the time; 2 = continuous motion)
Coordination	Quality of movement rating (0 = clumsy movement; 1 = adequate movements; 2 = agile movements)
Spontaneous crawl	Maturity of locomotor pattern (0 = no reciprocal locomotor pattern; 1 = weak attempt to locomote; 2 = coordinated locomotor pattern)
Passive	Duration of time inactive (0 = no inactivity; 1 = inactive 50% of the time; 2 = inactive 75% or more).
State control cluster	
Irritability	Amount of distress noted (0 = distress minimal during testing; 1 = distress apparent 50% of time; 2 = distress observed continuously)
Consolability	Ease of consoling infant (0 = easy to console by picking infant up; 1 = consoles with difficulty by holding, swaddling, rocking, and/or stroking; 2 = cannot console infant).
Struggle during testing	Amount of squirming noted (0 = 25% of time struggling; 1 = infant squirmed 50% of time; 2 = continuous squirming)
Predominant state	Behavioral state (alert, irritable) of infant during examination (0 = alert, awake, aware; 1 = alert but somewhat agitated; 2 = extremely agitated throughout examination)

mo of life. Items were combined into categories modeled closely on the Brazelton Newborn Behavioral Assessment Scale (Brazelton, 1984), namely, orientation, motor maturity, motor activity, and state control (Schneider and Suomi, 1992; Schneider et al., 1991). The items in each category are defined in Table 1. Orientation items consist of neonatal visual orienting and visual following responses elicited by a visual stimulus (three-dimensional toy with Mickey Mouse face). The proportion of time during which the infant was attentive was rated, as well as the duration of gaze while orienting to the toy. Neuromotor items in the motor maturity category included ratings of muscle tonus, coordination, labyrinthine righting, response speed, and spontaneous motor activity. Temperament ratings in the state control category were based on behaviors observed during administration of the orienting and neuromotor items. Because the goal was to maintain the infant in a quiet, alert state throughout the examination, the infant's temperament was monitored constantly. Temperament items included consolability, irritability, and fearfulness during the testing session.

We administered the 20-min battery of developmental tests four times throughout the first 30 days postpartum. Testing occurred between 1000 and 1200 hr to avoid the possibility of confounding effects with time of day. The test began with the examiner wrapping the infant in a diaper from the waist down, leaving the arms free to move. The orientation items were administered first, followed by the motor maturity, motor activity, and state control items in an invariant sequence. Each item was rated on a scale ranging from 0 to 2, with half-point scores allowed as in human neonatal assessments. Observers were trained to reliability ($r > 0.90$). and they were blinded to the infants' pregnancy conditions. After testing, the four composite scores were computed for each infant (Schneider et al., 1991).

PET Procedure

All procedures followed an overnight fast. Monkeys are typically fed chow at 0600 each day. On the day before PET scans, all monkeys were supplemented with half rations at 1200 and food and water deprived at 1630. On the morning of the PET scan, monkeys were lightly sedated with ketamine (15 mg/kg), and an intravenous catheter was inserted to allow for the placement of PE tubing (0.76-mm internal diameter and 1.22-mm outer diameter) into the saphenous vein. Monkeys were then intubated and transported to the PET facility, and on arrival, isoflurane anesthesia was initiated at 3 to 5% and maintained at 1.25 to 1.5% throughout the duration of the procedure (2 hr). Although ketamine is known to alter endogenous DA levels, these levels only minimally affect the data with [^{18} F]FAL on DA D2 receptor binding. Moreover, the time from the last ketamine injection to the start of the scan was typically longer

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than 60 min, further reducing any possible effects. A fixed laser line reference was used to align each anesthetized animal in the PET scanner for horizontal slice imaging parallel to the orbital-meatus line at the center of the seven-slice, 5.5-cm field of view in the ECAT 933 scanner.

Striatal DA-syn was assessed with the use of FMT as a PET tracer. The striatum to occipital cortex uptake ratio for FMT is about twice that for 6FDOPA, and it is not confounded by nonspecific tracer metabolite uptake in the brain. The tracer used to assess DA D_2Rs was [^{18}F]FAL, a fluorine-18-labeled raclopride analogue developed by Mukherjee and colleagues (1997). Radiotracers were administered as an intravenous bolus. Identical image acquisition protocols were used for the FAL and FMT tracers. Tracer injection of 5 mCi in 1 to 5 ml normal sterile saline was followed by a dynamic sequence of images over 90 min, including a total of 13 frames with duration increasing from 2 to 10 min. At the end of scanning, the PE tubing was removed, and the animals were extubated and allowed to awaken, returned to their transport cages, and immediately transported to the animal care facility, where they were housed in a designated room until the radioactivity decayed to background levels.

PET images were reconstructed from the raw data using the ordered subset estimation method (Hudson and Larkin, 1994). Standard regions of interest were placed on the occipital cortex (an area known to contain little significant D_2 DA-ergic innervations) to produce reference-region time-activity curves for use as input functions in graphical analysis. Other regions of interest were placed to cover both the left and right caudate and putamen in the basal ganglia. Time-activity data for these regions of interest were analyzed with the graphical method of Logan et al. (1996). Logan et al. (2000) developed linear models that have become a standard calculation method for receptor-ligand studies. Logan plots are used to calculate the distribution volume (DV) of ligand tracers that have reversible binding kinetics. The DV is a linear function of receptor availability and is widely used as a model parameter in imaging studies. The distribution volume ratio (DVR) is the ratio of the DV in a receptor region to the DV in a nonreceptor-containing region. In our study, this is the ratio between the striatum and the occipital cortex. Ratios of tracer total distribution volumes (DVRs) to those in the reference region were evaluated as described below. Our method assumes that the unbound components of the tracers are the same in the target regions (e.g., striatum) as in the reference region (occipital cortex). The DVR values can then be interpreted directly in terms of FAL binding potential: $B_{max}/K_d = DVR-1$, where B_{max} is the mass-specific concentration of available receptors and K_d, the receptor-ligand dissociation constant in that voxel. The DVR can be extracted from dynamic imaging data by Equation 1.

In this case, the striatum activity concentration at time t is given by $STR(t)$, with the occipital cortex concentration as $OC(t)$. The parameter k_2 is the efflux rate constant for wash-out of the compound from the nonreceptor-containing regions. For this analysis, we assumed $k_2 = 0.163\ min^{-1}$, the value for [^{11}C]raclopride (Logan et al., 1996). With the relatively large STR/OC ratios achieved with FAL and FMT, the k_2 term is a relatively small correction, and variations of a factor of 2 in k_2 from our assumed value result in a less than 7% change in the resulting DVR.

Statistical Analysis

Treatment effects were analyzed by a 2 (early alcohol or not) × 2 (late alcohol or not) × 2 (sex) ANCOVA for prenatal condition on PET DA data, followed by Fisher-Hayter post hoc tests to determine which groups differed significantly from each other. The D_2R binding/DA-syn ratio was used as an index of DA-ergic system function, given the well-established compensatory relationship between synaptic concentration and receptor density. A relatively high ratio would be indicative of high receptor binding in relation to a low synaptic concentration of DA. A low ratio would be indicative of low receptor binding in relation to high presynaptic DA-syn. To determine whether neonatal neurobehavioral scores were related to the D_2R to DA-syn ratio, correlations were calculated overall and within treatment conditions. Finally, to explore whether the balance between D_2R binding and DA-syn was altered by the prenatal treatments, correlations between FAL DVR and FMT DVR were conducted within treatment groups.

RESULTS

PET

Because findings in humans have indicated that the incidence of fetal alcohol effects are increased in women older than 30 yr (Jacobson et al., 1996), we examined the maternal gestational variables of maternal prepregnancy weight, maternal age, weight gain, and length of gestation period to determine whether these variables were related to the PET DA measures. Maternal age was the only maternal variable that was significantly related to D_2R binding to DA-syn ratio, $r = -0.42$, $p < 0.01$, and it was also marginally related to D_2R binding, $r = -0.32$, and DA-syn, $r = -0.32$, both $p < 0.06$. On the basis of these findings, we included maternal age as a covariate in the main analyses. There were no significant differences in maternal age across treatment groups (Schneider et al., 2001a).

As shown in Figure 1a, monkeys in the group exposed to alcohol during middle to late gestation (only) showed the lowest FMT uptake, reflecting relatively low DA-syn, whereas the monkeys exposed continuously throughout gestation (early + middle to late exposure) showed the highest presynaptic DA-syn [early × late interaction, $F(1, 26) = 4.78$, $p < 0.04$]. For striatal DA D_2R binding poten-

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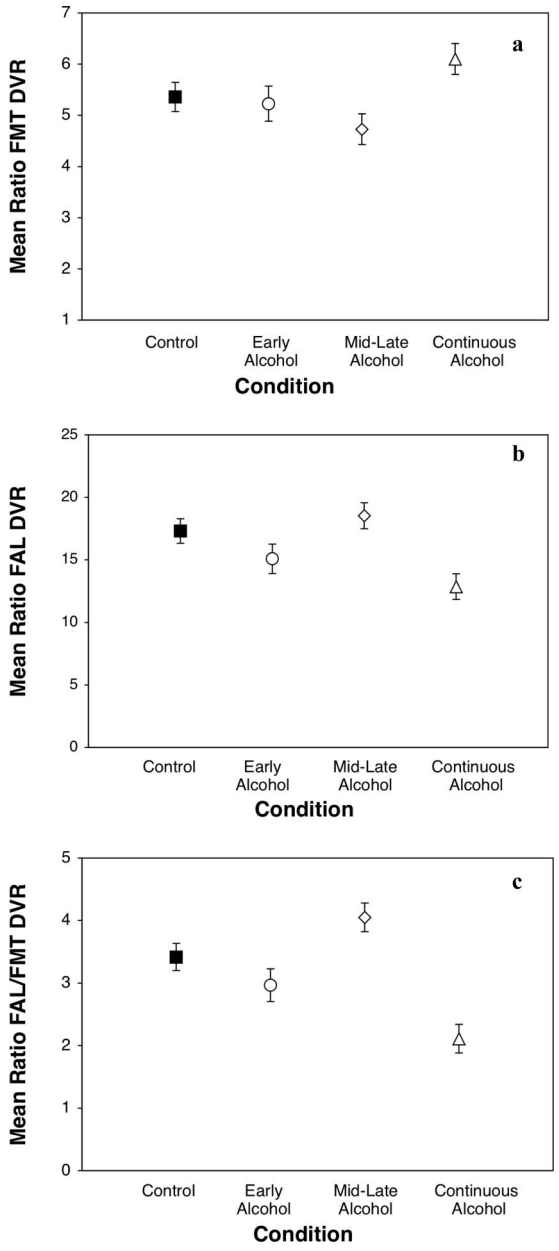

Figure 1. Comparisons of DA-ergic parameters measured with PET in control, middle to late, early, and continuous alcohol-exposure groups. Group means and standard errors of the means are shown. DVR for 6 FMT uptake, indicating presynaptic DA synthesis (a), DVR for FAL binding on postsynaptic D_2 receptors (b), and DVR ratio for these measures (c).

tial (indexed by FAL uptake), there was a significant main effect of early versus no early alcohol, showing that monkeys that were exposed in early gestation (i.e., the early-only and continuous-gestation exposed animals) showed reduced DA D_2R binding relative to monkeys not exposed to early alcohol [i.e., controls and middle to late only exposed) main effect of early alcohol or not, $F(1, 26) = 12.21$, $p < 0.002$] (Fig. 1b). Finally, for the D_2R binding-DA-syn ratio, those exposed to alcohol during middle to late gestation showed an increased D_2R binding to DA-syn ratio, whereas early- and continuously exposed animals showed effects in the opposite direction, or a decreased

Table 2. Relationships Among the Four Neonatal Neurobehavior Variables in the PNNA and PET Indices of Striatal DA Function

	Orientation	Motor maturity	Motor activity	State control
Motor maturity	0.54‡	—	—	—
Motor activity	−0.07	0.40†	—	—
State control	−0.41†	−0.04	0.54‡	—
FAL/FMT Ratio	0.44‡	0.06	−0.19	−0.23

The FAL-FMT ratio refers to the DA D2R binding to DA synthesis ratio, $n = 35$.*$p < .05$, †$p < .02$, ‡$p < .01$.

D_2R binding to DA-syn ratio (Fig. 1c). The 2 (sex of animal) × 2 (early alcohol exposure) × 2 (middle to late alcohol exposure) ANCOVA on DA D_2R binding to DA-syn ratio with maternal age as a covariate showed a significant main effect of early alcohol exposure, $F(1, 26) = 22.82$, $p < 0.001$, and a significant interaction of early × middle to late alcohol, $F(1, 26) = 8.10$, $p < 0.01$. Post hoc tests with the Fisher-Hayter method (Keppel and Wickens, 2004) showed that continuously alcohol-exposed monkeys differed significantly from controls ($p < 0.05$) for FAL and FAL/FMT. Continuously and middle-to-late-exposed monkeys were significantly different from each other for FMT ($p < 0.05$), FAL ($p < 0.05$), and FAL/FMT ($p < 0.01$). The remaining post hoc comparisons were nonsignificant. A marginally significant difference was found between the controls and middle-to-late-alcohol-exposed monkeys for FAL/FMT. Overall, the continuous-exposure condition showed the largest deviation from controls.

Relationship Between PET DA Measures and Neonatal Neurobehavioral Measures

We examined the relationships among the PET indices of striatal DA function and the four neonatal neurobehavior variables (orientation, motor maturity, motor activity, and state control) in the PNNA (Schneider and Suomi, 1992; Schneider et al., 200X) (see Table 1 for definitions). We averaged four weekly observations during the neonatal period (postnatal days 2, 8, 15, and 22) to create a single score for each of the four neurobehavior variables of orientation, motor maturity, motor activity, and state control. When all four groups were included, the D_2R binding to DA-syn ratio was significantly related to orientation, $r = 0.44$, $p < 0.01$, but not to the other three neonatal neurobehavior variables (see Table 2). Because maternal age was used as a covariate in the main analyses of the PET variables, we also tested the relationship between neonatal orientation and D_2R binding-DA-syn ratio with maternal age as a covariate, and the relationship remained significant, partial $r = 0.36$, $F(1, 32) = 6.19$, $p < 0.05$.

An important question that arises is whether the treatment conditions could alter the relationship between neonatal neurobehavior measures and PET measures of DA-ergic function. Table 3 shows the correlations of the neonatal neurobehavioral measures and PET variables within the treatment groups. Examination of the correlations for each treatment group shows that prenatal alcohol

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Table 3. Correlations of Neonatal Neurobehavioral Measures and PET Measures of DA Function (FAL-FMT Ratio) Within Treatment Groups

	FAL/FMT			
Neonatal measure	Controls (n = 10)	Early gestation (n = 9)	Middle to late gestation (n = 7)	Continuous (n = 10)
Orientation	0.59*	0.19	0.34	0.29
Motor maturity	0.60*[a,b]	−0.08	−0.66†[a]	−0.50[b]
Motor activity	−0.05	−0.39	−0.41	−0.43
State control	0.59*	−0.07	−0.75*	−0.1

Note the FAL to FMT ratio refers to the D2R binding to DA synthesis ratio, n = 35.* $p < 0.05$, †$p < 0.10$. Groups that share subscripts differ significantly from each other at $p < 0.02$.

treatment disrupted the relationship between the neurobehavioral measures of orientation, motor maturity, and state control and PET measures of DA-ergic function. The control group showed significant relationships between neonatal neurobehavior and DA-ergic function for orientation (r = 0.59), motor maturity (r = 0.60), and state control (r = −0.59), all $p \leq 0.05$. For the early and continuously exposed monkeys, correlations between orientation, motor maturity, motor activity and state control scores and D_2R binding-DA-syn ratio were not significant. For middle-to-late exposure, however, the correlation between state control and the PET ratio measure was significant ($r = -0.75$, $p < 0.05$), and for motor maturity, the correlation was in the opposite direction of controls and marginally significantly different from zero, r −0.66, $p < 0.08$. For motor activity, there were no significant correlations between neurobehavior measures and PET measures, either for the overall group or for individual treatment groups. When correlations of neonatal behaviors and PET measures were compared between treatment groups, controls differed from the middle-to-late-exposed and continuous-exposure groups for motor maturity (all $p < 0.02$), whereas all other pairwise comparisons were nonsignificant.

Prenatal alcohol exposure also disrupted the relationship between DA D_2R binding (FAL binding potential) and DAsyn (FMT binding potential). Controls showed a marginally significant relationship of D_2R binding to DA-syn ($r = -0.52$, $p = 0.10$), with a trend for decreased DA-syn to be associated with compensatory increased or up-regulated D_2R density, as expected. The three alcohol-treated groups, on the other hand, showed no relationship between DA D_2R binding and DA-syn ($r = -0.08$, 0.36, and 0.07 for early, middle to late, and continuous exposure, respectively). The correlation for the middle-to-late exposure group differed significantly from that of the control group.

DISCUSSION

This study adds to the growing body of evidence that suggests that fetal alcohol exposure, even at moderate levels, is associated with both behavioral disturbances and compromised brain function. The principal findings of this study were the following: 1) Continuous fetal alcohol exposure produced the largest effect relative to the control condition. Fetal alcohol-exposed monkeys from three different gestational alcohol exposures (early, middle to late, and continuous or both early + middle to late exposure) showed disrupted striatal DA system function in slightly different ways. Moderate-dose alcohol exposure occurring during early gestation, a period that is comparable to the first trimester in humans, or during both early and middle to late gestation reduced the ratio of D_2R binding to DA-syn. Middle-to-late gestation exposure, by itself, without early exposure, induced effects in the opposite direction relative to the early- and continuously exposed monkeys. 2) Overall, there was a positive relationship between striatal D_2R binding-DA-syn ratios and scores on the neonatal orientation score. Orientation, motor maturity, and state control neonatal scores were correlated with the PET ratio scores in the control monkeys. This was not surprising, because these functions are known to be dependent on DA. These relationships were disrupted in the fetal alcohol-exposed monkeys. (3) The expected complementary relationship between measures of DA-syn and D_2R binding availability was observed in the control group but was disrupted in the three fetal alcohol-exposed groups.

The first finding was that fetal alcohol exposure impacted DA system function in different ways, depending on the timing and onset of exposure. Fetal alcohol exposure throughout pregnancy (continuous exposure) showed the largest effect. This may be a consequence of more total alcohol exposure owing to a longer duration of exposure time. The implications appear to be that the sooner pregnant women quit drinking alcohol, the better it is for the fetus. Early gestation and continuous (both early and middle to late) exposures yielded a reduction in the D_2R binding to DA-syn ratio, whereas middle to late exposure (without early exposure) increased the D_2R binding to DA-syn ratio. The most likely mechanism underlying the differing alterations in striatal DA system function found in early-gestation-exposed versus middle-to-late-exposed monkeys is interference with neurodevelopmental processes that vary systematically with time during gestation. Rakic (1988) described three broad phases of brain development in rhesus monkeys: generation of neurons, neuronal migration, and synaptogenesis. Rakic suggested that, in both humans and monkeys, cortical neurons are generated near the surface of the cerebral ventricle during early gestation (0 through E40[1]). After their last division, postmitotic cells produced within the proliferation zones migrate along radial glial fascicles and enter the developing cortical plate, forming ontogenetic columns (E40–E70/100) (Rakic, 1995). By day E112, the developing cortex has its full complement of neurons. The phase of rapid synaptogenesis, which occurs synchronously in the somatosensory, motor, and association areas (Zecevic and Rakic, 1991), begins at day E112 and continues to the third mo postnatally (Bourgeois and Rakic, 1993).

Data from the present study advance our understanding of fetal alcohol effects on striatal DA system function by

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demonstrating that moderate-level alcohol exposure during middle to late gestation (E50–E135), approximating the period of neuronal migration (E40–E70/100) and early synaptogenesis (E112–third mo postnatally), differs from fetal alcohol exposure during early gestation (0–E50), approximating neurogenesis (0–E40). Interestingly, studies have found that neuronal cell migration is highly sensitive to various perturbations, including toxins, viruses, and genetic mutations (Rakic, 1988). Abnormal neuronal migration is considered a significant cause of gross and subtle abnormalities in synaptic circuits (Barth, 1987; Caviness et al., 1989; Rakic, 1988), including developmental dyslexia (Galaburda et al., 1989) and schizophrenia (Kotrla et al., 1997). Correct cell migration allows communication between early- and later-forming neurons at the critical developmental stages before making their synaptic connections (Rakic, 1985). Proper neuronal migration is necessary for appropriate neuron position, which then affects brain morphology and function.

Moreover, the effects observed on striatal DA system function in the continuous alcohol-exposed group suggest that if the nervous system has already been exposed to alcohol during early gestation, then middle to late exposure yields different effects on the DA system relative to middle to late gestation exposure alone. In other words, the effects of middle to late alcohol exposure may depend on developmental events that occur during early gestation, and in the case of fetal alcohol exposure, the developmental trajectory of nervous system development has been altered in some way by early exposure, so that it now responds to a later perturbation (middle to late gestation alcohol exposure) in a different way. Findings such as these are not unprecedented. Others have reported that some areas of the brain may be differentially affected by longer durations than shorter exposures to alcohol (Livy et al., 2001; Sulik et al., 1981). Livy and colleagues (2001) found that in rats, alcohol exposure during E14 and E15 resulted in lower numbers of cells in the ventral lateral nucleus of the thalamus, but that alcohol exposure during E11 to E20 did not have this effect. They speculated that the timing of alcohol exposure *initiation* may be an important influence on the effects of toxic exposures. Moreover, Clarren et al. (1990) reported that in a high alcohol exposure condition, offspring striatal DA was negatively correlated with maternal blood alcohol concentration, but that in a moderate alcohol condition, the relationship between maternal blood alcohol concentration and later offspring striatal DA was positive.

Either an increase or a decrease in the D_2R to DA-syn ratio can have repercussions on normal functioning. It has been shown that there is a critical range of DA activity for optimal functioning (Arnsten, 1997). DA is an important neurotransmitter that modulates the activity of many brain regions, promoting both excitatory and inhibitory signals. In particular, DA is a critical regulator of frontal-striatal function, which is identified as a region involved in the modulation of complex cognitive functions, such as attention and executive function, as well as movement and affect. DA is thought to underlie the behavioral response to important or salient events, whether aversive or appetitive (Berridge and Robinson, 1998; Redgrave et al., 1999). Disrupted DA-ergic transmission causes motor abnormalities, such as parkinsonism, and neuropsychiatric disorders, such as schizophrenia and drug addiction (Bergman et al., 1998; Berke and Hyman, 2000).

DA–mediated striatal function is regulated by finely modulated interneurons via certain DA receptor subtypes. In other words, DA affects function according to the receptors involved as well as some other factors. There are two families of receptors, D_1–like and D_2–like. The striatum, a region of high density of D_2Rs, has been suggested to have a gating function, in that the basal ganglia thalamocortical circuits are thought to underlie inhibitory control, defined as the ability to suppress competing attentional and behavioral responses (Casey, 2001). If D_2R density is too low or reduced, DA function may be blunted or the DA signal may be blunted, which could result in reduced attention, compromised motor function, and disrupted executive function. For example, DA D_2R-deficient mice require significantly more trials than do wild mice to alter their responses during reversal trials, which requires recognizing an unexpected consequence and altering strategies accordingly. Carlsson et al. (2001) suggest that DA D_2R stimulation in the striatum serves to "brake" or diminish excitatory corticostriatal signaling so that goal-directed behavior is guided by signaling the organism about changes in contingencies. Reduced striatal D_2R availability has been found in detoxified chronic cocaine-dependent subjects (Martinez et al., 2004), nonviolent alcoholics (Hietala et al., 1994), patients with obsessive-compulsive disorders (Denys et al., 2004), and patients with severe Alzheimer's disease who also show significant behavioral and psychological problems (Tanaka et al., 2003). Volkow et al. (2004) suggest that decreased striatal D_2R availability might reduce sensitivity to nondrug-related stimuli, such as hedonic positive stimuli, which could increase the compulsive drive to take drugs.

If D_2R density is too high, DA function may be more responsive or supersensitive, which could result in heightened sensitivity to novel stimuli and/or unfamiliar situations, extraordinary sensitivity to environmental stimulation, and abnormal sensory responses. Higher striatal D_2R density has been found in unmedicated patients with schizophrenia compared with normal controls (Laruelle, 1998) and in adolescents with a history of attention-deficit hyperactivity disorder who had low neonatal cerebral blood flow (suggesting deficient oxygen and/or metabolites) at preterm birth (Lou et al., 2004).

A second finding in our study was that there was a positive relationship between the striatal D_2R binding to DA-syn ratio and scores on the neonatal orientation score, overall. This suggests that early orientation or attention to a novel stimulus is linked with DA-ergic function and that this relationship emerges early in postnatal life. This find-

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ing is consistent with the notion that higher D_2R availability in the striatum is associated with heightened sensitivity to novel stimuli. This early life behavioral finding can have consequences for later cognitive functioning. In a different experiment involving prenatal alcohol exposure alone or in conjunction with prenatal stress, we found that neonatal orientation and motor maturity predicted performance on a learning task during adolescence (Schneider et al., 2001b). Moreover, we found that measures of D_2R binding to DA-syn were correlated with performance and behavior in a learning task (Schneider et al., 2001b). In the present study, we also found that the D_2R binding to DA-syn ratio was correlated with orientation, motor maturity, and state control, but not motor activity, in the control monkeys. These functions are known to be dependent on DA. These relationships, however, were disrupted in the fetal alcohol-exposed monkeys. Two possible mechanisms underlying this finding are altered migration of the DA system and altered neural pathways connecting the striatum to brain areas that control these behaviors. This disruption could reflect the adverse effects of fetal alcohol exposure on striatal DA system development per se, as well as on other neurotransmitter systems, given the complex interaction between DA and other neurotransmitters that are also known to be disrupted by fetal alcohol exposure. Because neurotransmitter systems are interactive, up-regulation of one type of DA receptor might be associated with down-regulation of another DA receptor subtype, and the balance of neurotransmitter receptor density to synthesis can have important functional consequences.

A third finding of this study was that the complementary natures of postsynaptic D_2R binding and presynaptic synthesis, although evident in control monkeys, were disrupted in fetal alcohol-exposed monkeys. It is well recognized that DA D_{2Rs} are up- or down-regulated in response to lower or higher synaptic concentrations of DA (Donnan et al., 1991), and our finding in control monkeys is consistent with this notion. That the relationship was disrupted by fetal alcohol exposure is interesting. It could be that other neurotransmitters, perhaps those for γ-aminobutyric acids, are involved. For example, it is known that γ-aminobutyric acid modulates DA activity in the brain, and changes in D_2R density can result from changes in striatal γ-aminobutyric acidergic neurons. Fetal alcohol-induced disruptions in γ-aminobutyric acidergic neurons could thus disrupt the normal feedback system mediating the relationship between DA synthesis and D_2R density.

In summary, the results of this study show a long-lasting impact of prenatal alcohol exposure on adult striatal D_2R to DA-syn ratio, with the timing of the alcohol exposure playing an important role and producing effects in opposite directions. For the early- and continuously exposed groups, the mean DA D_2R-DA-synthesis ratio score was reduced, suggesting a blunting of DA-ergic tone or DA signal. For the middle-to-late-exposed group, the mean was in the opposite direction relative to the early- and continuously alcohol exposed groups, suggesting heightened sensitivity or responsivity of the DA system. These results show that fetal alcohol exposure can result in either increases or decreases in DA-ergic tone. Therefore, one should avoid oversimplified notions of fetal alcohol exposure effects on DA system function. Our results together with those of Livy et al. (2001) and Clarren et al. (1990) suggest that it might be appropriate to consider these subgroups separately. Moreover, the differences between these groups in terms of heightened versus blunting of striatal DA-ergic tone might contribute significantly to intersubject variability in cognitive function, emotion regulation, behavioral control, response to stimulant drugs, and risk for predisposition to drug and/or alcohol abuse.

A limitation of this study is that the PET measurements of DA synthesis and D_2R binding availability were limited to striatal brain regions and one receptor type (D_2). Sites other than the striatum as well as D_1-type receptor functioning are undoubtedly important and relevant to fetal alcohol exposure. Further studies are needed to help us understand the relationship between blunted versus heightened DA-ergic function in the striatum and other regions and predisposition to cognitive deficits, stress-related disorders, and even vulnerability to drug or alcohol abuse (Christian et al., 2000; Volkow et al., 2004). Future studies should consider other neurotransmitter systems, perhaps γ-aminobutyric acid, that represent important aspects of the functional regulation in the frontal-striatal circuitry. Another limitation is that monkeys were preselected such that they would voluntarily consume alcohol. Whether this involves a genetic taste preference or susceptibility to prenatal alcohol effects is unknown. Future studies could include a second control group consisting of nonvoluntary alcohol consumers, akin to human abstainers. Experiments with nonhuman primates, which permit in vivo PET imaging of brain regions with control of the dose and timing of fetal alcohol exposure, along with the use of behavioral measures that are relevant to humans, are important to further our understanding of the temporal and regional vulnerabilities of fetal alcohol-induced brain alterations (Christian et al., 1998; Dobbing and Sands, 1979).

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