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Hippocampal Neuron Populations Are Reduced in Vervet Monkeys With Fetal Alcohol Exposure

ABSTRACT: *Prenatal exposure to beverage alcohol is a major cause of mild mental retardation and developmental delay. In nonendangered alcohol-preferring vervet monkeys, we modeled the most common nondysmorphic form of fetal alcohol syndrome disorder with voluntary drinking during the third trimester of pregnancy. Here, we report significant numerical reductions in the principal hippocampal neurons of fetal alcohol-exposed (FAE) offspring, as compared to age-matched, similarly housed conspecifics with isocaloric sucrose exposure. These deficits, particularly marked in CA1 and CA3, are present neonatally and persist through infancy (5 months) and juvenile (2 years) stages. Although the volumes of hippocampal subdivisions in FAE animals are not atypical at birth, by age 2, they are only 65–70% of those estimated in age-matched controls. These data suggest that moderate, naturalistic alcohol consumption during late pregnancy results in a stable loss of hippocampal neurons and a progressive reduction of hippocampal volume.* *© 2015 The Authors. Developmental Psychobiology Published by Wiley Periodicals, Inc.* Dev Psychobiol 57:470–485, 2015.

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INTRODUCTION

The pattern of dysmorphology and neurodevelopmental deficits produced by extreme prenatal exposure to beverage alcohol is relatively rare, but well defined (Jones & Smith, 1973). The latest epidemiological reports (Denny, Tsai, Floyd, & Green, 2005; May et al., 2009) estimate the incidence of the full fetal alcohol syndrome (FAS) at 2–7 cases per 1,000 births. A much broader pattern of abnormalities, characterized by the umbrella term fetal alcohol spectrum disorders (FASD), has been estimated to affect up to 2–5% of live-born infants in North America and Western Europe (Abel & Sokol, 1987; Chudley et al., 2005; May et al., 2009). FASD has been suggested to be the leading cause of preventable neurodevelopmental disorders in the industrialized world (Abel & Sokol, 1991; Clarren & Smith, 1978). In North America, it is estimated that there are up to 40,000 new cases per annum, each with a lifetime cost of up to $2 million (Lupton, Burd, & Harwood, 2004; Popova, Lange, Burd, & Rehm, 2012; Stade et al., 2009). Although many who drink heavily during pregnancy do not have a child with a diagnosis of overt FAS, some studies have reported elevated indicators of childhood psychopathology in children with *any* amount of ethanol exposure during embryogenesis (Sood et al., 2001).

While facial dysmorphology is a hallmark of full FAS, many cases of FASD do not display these features and, thus, are unrecognized or misdiagnosed. Decrements in cognitive and language performance and in academic achievement are common (Brown et al., 1991; Coles et al., 1991; Streissguth, Barr, & Sampson,

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1990) and disruptions in social behavior are pervasive (Kelly, Day, & Streissguth, 2000; Roebuck, Mattson, & Riley, 1999). Many cases are first seen in court during adolescence or young adulthood for offenses often related to their impulsivity and lack of foresight (Fast & Conry, 2004). A pervasive pattern of motor and coordination deficits is not uncommon, and some individuals have clinical or subclinical electroencephalographic abnormalities or psychiatric symptoms (Guerri, Bazinet, & Riley, 2009; Kodituwakka, 2007)

Clinical data (Kodituwakka, 2007; McGee, Fryer, Bjorkquist, Mattson, & Riley, 2008; Riley & McGee, 2005; Uecker & Nadel, 1996), as well as that from rodent models (Berman & Hannigan, 2000; Comeau, Winstanley, & Weinberg, 2014; Elibol-Can et al., 2014; Goodfellow & Lindquist, 2014; Hellemanns et al., 2010; Osborn, Kim, Steiger, & Weinberg, 1998), implicate hippocampal dysfunction as an important determinant of the behavioral components of FASD (e.g., learning [especially social learning], attention, spatial memory, social skills, impulsivity, and fear response). Although some rodent models confirm hippocampal cell loss (Klintsova et al., 2007; Miki et al., 2003), imaging studies in patients with FASD but no facial abnormalities are only able to demonstrate dose-related differences in hippocampal volume (Astley et al., 2009; Autti-Ramo et al., 2002; Guerri et al., 2009; Nardelli, Level, Rasmussen, Andrew, & Beaulieu, 2011; Normal, Crocker, Mattson, & Riley, 2009). Fetal alcohol exposure has also been shown to result in hippocampal deformity restricted to the head and tail regions (Joseph et al., 2014). The hippocampus is not, however, the only ethanol-sensitive structure that might be responsible for the reported cognitive and behavioral deficits. Indeed, in a previous paper (Burke, Palmour, Ervin, & Ptito, 2009a), we reported significantly lower neuronal counts in the prefrontal cortex of 2-year-old alcohol-exposed vervet monkeys, as compared to age-matched sucrose-exposed controls.

Rodent models suggest that the third trimester equivalent is a critical period for deleterious effects of ethanol on the hippocampus (Bonthius, Woodhouse, Bonthius, Taggard, & Lothman, 2001; Elibol-Can et al., 2014; Klintsova et al., 2007; Tran & Kelly, 2003). Because it is difficult to extrapolate rodent neuroanatomical or developmental behavioral findings to man or to control for the stress of artificially administering ethanol to rodent pups during the third-trimester equivalent (Gil-Mohapel, Boehme, Kainer, & Christie, 2010), we developed a nonhuman primate model of FASD using alcohol-preferring vervet monkey dams (*Chlorocebus sabeus* St Kitts) that voluntarily consume moderately high doses (1.6–3.5 g/kg/day) of beverage ethanol. In the studies described here, ethanol exposure was limited to the last half of pregnancy, corresponding to the period of rapid synaptogenesis.

The goal of the present study was to investigate the integrity of the hippocampus following fetal alcohol exposure (FAE) at three postnatal developmental time points (0–30 days, 5–6 months, and 21–24 months). We tested the hypotheses that (1) hippocampal neuronal numbers and volume would be lower in FAE individuals as compared to age-matched controls; and (2) these reductions would be stable across the developmental period examined in this study.

MATERIALS AND METHODS

Subjects

Healthy female African green monkeys (*Chlorocebus sabeus*) were screened for voluntary alcohol consumption according to previously published methods (Palmour, Ervin, Baker, & Young, 1998). Females that voluntarily and reliably drank at least 3 g alcohol/kg in a 4-hr scheduled period were identified and housed socially (6 per group) with alcohol-avoiding male breeders. All dams were mature females (average age 7.5 ± 1.9 years [range 5–12 years]; body weight $4.35 \pm .5$ kg [range 3.26–5 kg]; number of previous pregnancies 2.22 ± 1.8 [range 0–5]; number of total pregnancies 7.06 ± 2.9 (range 3–14]). All animals were examined clinically a minimum of twice per year and were well-nourished throughout pregnancy and lactation. All dams gained weight during pregnancy, and none of the offspring were underweight at delivery (i.e., they fell within the normal range [.35–.55 kg] at birth). Females were monitored behaviorally and physically for evidence that pregnancy was initiated, and then examined biweekly in order to time gestation.

At about embryonic day 95 (range: 76–114) of the normal 165-day gestational period, shave-marked pregnant females were given access to a drinking bottle containing 44 ml/kg body weight of an 8% w/v solution of alcohol (this provides a maximum of 3.5 g alcohol/kg body weight) or an isocaloric sucrose control mixture (60 ml/kg 5% sucrose in water) on 4 days of the week (M, Tu, Th, F). The 4-days per week administration schedule was selected in order to maintain the health of the dams and reduce the likelihood of repeated withdrawal cycles, while still obtaining an adequate level of ethanol to produce neuronal damage (Burke et al., 2009a; Papia et al., 2010). Drinking occurred in individual compartments of a tunnel attached to the main cage, and involved neither stress nor forced administration. On a drinking morning, each marked animal was gently coaxed into the tunnel and given free choice to two calibrated drinking bottles, one containing tap water and one containing either alcohol or sucrose-water. Both bottles were monitored hourly and remained available for a 4-hr period. At the end of this period, the drinking bottle (ethanol or sucrose) was removed and the quantity of liquid consumed was recorded. Mothers did not continue to drink ethanol or sucrose after the birth of infants. Blood (1 ml,

saphenous vein) was drawn from both alcohol-consuming and sucrose-control dams without anesthesia at the end of the drinking period during weeks 2, 4, 6, and 8 (once each week) for the measurement of blood ethanol level (alcohol dehydrogenase method; Sigma, St Louis, MO). Extreme care was taken not to stress the animals during any of these procedures. Characteristics of exposure and blood ethanol (BEC) levels are summarized in Table 1.

All animals were housed in the laboratories of Behavioural Sciences Foundation, St Kitts, in enriched social environments. The subjects were fed Harlan Teklad 20% protein primate chow (5% body weight per day) and fresh local fruit, with water available ad libitum. Animals enrolled in this study were all fed in their individual compartments on days when alcohol or sucrose was available, and all ate their full daily allotment of calories. Procedures followed the guidelines contained in the US National Research Council Guide for the Care and Use of Laboratory Animals and the Canadian Council on Animal Care Guide to the Care and Use of Experimental Animals. The experimental protocol was reviewed and approved by the McGill University Animal Ethics Committee and the Behavioural Science Foundation

Table 1. Subject Profile

Animal#	Sex	Brain Weight (g)	Alc or Suc Started[a]	Drinking Days	Average Alc (g/kg/day)[b]	Total Alc Exposure	Average BEC	BEC (mM)	Age at Sacrifice
O1808-3-2	f	49.5	87	47	1.46	68.9	61	13.3	30 d
O2898-5	m	51.3	96	39	2.64	103.13	112	24.3	18 d
O3245-3	f	42.4	76	53	2.82	149.5	121	26.3	4 d
O3307-3	f	51.5	94	40	3.17	126.87	132	28.7	35 d
O5011-4	m	49.1	82	42	1.97	82.81	72	15.7	15 d
O5154-2	m	48.3	112	31	2.09	64.82	95	20.7	1 d
		48.7 ± 3.3	91 ± 13	42 ±7	2.36 ± .63	94.36 ± 33.7	98.8 ± 28	21.5 ± 5.5	20 ± 13
O5232-2	m	45.5	107		0	sucrose	0	0	9 d
O6228-1	m	58.3	90		0	sucrose	0	0	27 d
O6332-1	f	47.3	92		0	sucrose	0	0	12 d
O6692-1	m	50.3	84		0	sucrose	0	0	1 d
O6712-1	m	45.8	72		0	sucrose	0	0	4 d
		49.4 ± 5.3	89 ±13						11 ± 10
O3065-8	m	69.2	94	41	2.98	122.13	125	27.2	5 mos
O3066-6	m	58.8	80	48	2.57	123.30	118	25.7	4.5 mos
O3327-4	m	72.6	95	40	2.16	86.37	77	16.7	5 mos
O5330-4	m	70.6	112	32	2.23	71.36	86	18.7	5.2 mos
O5399-1	m	68.6	106	35	2.48	86.75	109	23.7	5.4 mos
		68.0 ± 5.3[c]	97 ± 11	39 ± 5	2.48 ± .29	97.98 ± 20.9	103 ± 18	22.2 ± 4	5 ± .3 mos
N459-1-14-2	m	54.2	78		0	sucrose	0	0	5.6 mos
O2303-2-1-1	m	51.6	91		0	sucrose	0	0	4.9 mos
O2708-3-1	m	50.2	85		0	sucrose	0	0	5.1 mos
O5180-1	m	67.6	108		0	sucrose	0	0	5.1 mos
O9184-4-2	m	53.4	102		0	sucrose	0	0	5 mos
		55.4 ± 6.3[c]	93 ± 10						5.1 ± .2 mos
O3082-3	f	55.2	115	27	1.79	48.3	76	16.5	23 mos
O3066-3	f		95	41	1.66	67.88	67	14.6	23 mos
O3295-2	m	62.1	75	51	2.82	143.88	97	21.1	22 mos
O3307-2	f		111	31	2.64	81.76	105	22.8	19 mos
O3327-1	m	55.4	115	30	2.98	89.4	120	26.1	21 mos
O3327-2	m	55.9	113	31	2.77	85.73	104	22.2	22 mos
O5011-3	m	61.5	77	48	2.81	135.1	118	25.6	19 mos
		58.0 ± 3.5	100 ± 18	38 ± 12	2.5 ± .54	93.15 ± 34.6	98.1 ± 18.6	21.3 ± 4.3	21.3 ± 1.5
O3060-5	m	67.5	116		0	sucrose	0	0	21 mos
O5603-2	m	ND	112		0	sucrose	0	0	22 mos
O4056-3	f	51.1	82		0	sucrose	0	0	24 mos
O5151-1	m	56.1	73		0	sucrose	0	0	19 mos
O6036-1	f	52.1	103		0	sucrose	0	0	24 mos
		56.7 ± 7.5	97.2 ± 18						22 ± 2

[a]Day of gestation alcohol or sucrose treatment started,.
[b]Ethanol (g/kg) ingested by the dam over the entire drinking period.
[c]Brain weights for 5 month animals derived from half-hemisphere weights.

Animal Care Committee. Both of these institutions hold certificates of Good Animal Practice from the Canadian Council on Animal Care.

FAE and control offspring were examined shortly after birth for signs of facial dysmorphology (Chudley et al., 2005) and neurological impairment (using the Infant Behavioral Assessment Scale of Schneider, Moore, & Becker, 2001). Within 1 month of birth (range 1–30 days), six neonatal FAE and five age-matched sucrose-control subjects (Tab. 1) were sacrificed using approved methods for neuroanatomical evaluation. The remaining animals, both experimental and control, lived in social groups with their mothers, fathers, and other females until the age of 5–6 months of age, then lived for another few weeks in a nursery setting. Thereafter, peer groups of 6–12 animals (each group comprising both FAE and SucCon animals) were formed and moved to playpens (7–12 months of age), and later to large outdoor social pens equipped with swings, jungle gyms, and other learning and foraging opportunities. Animals in the second group (about 5 months of age) were sacrificed at the time they would normally be moved to the nursery, while the final group was sacrificed at about 2 years of age.

Histology

After euthanasia, brains were transcardially perfused with 4% paraformaldehyde in phosphate buffer (pH 7.4), extracted from the skull, stereotaxically blocked into 1 cm slabs in the coronal plane, cyroprotected in 30% buffered sucrose, and frozen at −80 °C until further processing (Burke, Zangenehpour, Boire, & Ptito, 2009b). Parallel series of coronal sections (50 μm) were obtained for each animal with a spacing of 1/6 for the neonatal group and 1/10 for the other groups. One complete series was Nissl-stained with cresyl-violet for volumetric quantification and neuronal counts while other series were banked in antigen preserve (Burke, Zangenehpour, & Ptito, 2009c).

Immunohistochemistry

Matched sections from the hippocampus were processed with anti-calbindin, a putative marker for calcium-binding protein, to aid in the delineation of the hippocampus. Sections were washed five times in PBS to remove residual antigen preserve and underwent antigen retrieval as described above, except that 20 mM citrate buffer (pH 8.5) was used for the initial denaturation and sections were blocked in 5% NDS and 1% bovine serum albumin in PBS containing 0.1% triton, then washed 3× in PBS. Thereafter, sections were incubated overnight at 4 °C in mouse anti-calbindin antibody (Sigma C9848; 1:10,000), then incubated in biotinylated donkey anti-mouse antibody (1:200, Vector) for 1 hr, as described in detail in Burke et al. (2009a). Following another set of PBS washes, the sections were stained with DAB, mounted, dehydrated, cleared in xylenes, and cover-slipped with DPX mounting media.

Stereology

Quantification of hippocampal neuronal population in cresyl violet stained sections was achieved by using design-based stereology with the optical fractionator method (West & Gundersen, 1990). The CA1, CA2, and CA3 subfields of the hippocampus were delineated (Fig. 1) on the basis of cyto- and chemo-architecture (Curtis et al., 2014; Kobayashi & Amaral, 1999) and equidistant sections were examined throughout the entire length of the hippocampus. Topography and superimposed counting frames (disectors) for each subregion were generated through BioQuant® software under 2.5× (topography) and 100× oil immersion (counting) objectives. The Cavalieri estimator (West & Gundersen, 1990) was used to determine the reference volume (V) of CA1, CA2, and CA3. This was achieved by multiplying the total surface of the outlined subregion by the tissue thickness (50 μm) and the spacing between sections.

Estimation of total cell numbers (N) was calculated by the following equation:

$$N = \mathrm{ssf}^{-1} \times \mathrm{asf}^{-1} \times \mathrm{tsf}^{-1} \times \Sigma Q-$$

where ssf is section sampling fraction, asf is area sampling fraction, tsf is the thickness-sampling fraction (the measured thickness of the tissue divided by the disector height), and $\sum Q-$ is the number of cells counted within the disector. Stereological parameters for all groups are presented in Table 2. The surface area of each disector was 2500 μm^2 with a disector height of 10 μm. The volume of the disector was chosen so that an average of 1–2 neurons (Q−) would be counted per counting frame in the control subjects. For this study, a neuron was defined as having a clearly visible nucleolus surrounded by cytoplasm (Joelving, Billeskov, Christensen, West, & Pakkenberg, 2006). Coefficients of error (CE) were calculated for mean number of cells ($\sum Q-$), total number of disectors ($\sum F$), and total number of cells (N) to assess the reliability of measurements (Gundersen & Jensen, 1987; West & Gundersen, 1990).

Statistical Analysis

Analysis of group (treatment, age) differences was performed using the Kruskal–Wallis test, a nonparametric equivalent to a one-way ANOVA, followed by the Mann–Whitney U test, a nonparametric equivalent to a *t*-test robust to small sample sizes. Possible interactions between age and treatment with respect to brain weight and neuronal number were evaluated by two-way ANOVA. Post hoc explorations were based on Fisher's LSD. The coefficient of variation (CV = SD/mean) was calculated for volume and neuronal number. The coefficient of error (CE) for the different measurements was calculated as $\sqrt{meanCE^2}$ (West & Gundersen, 1990).

RESULTS

Here, we report a comparison of the hippocampal neuron numbers and volumes in normal vervets and those exposed to known levels of alcohol in utero. Brains were examined at three different developmental times: neonatal (1–35 days after birth), about 5 months,

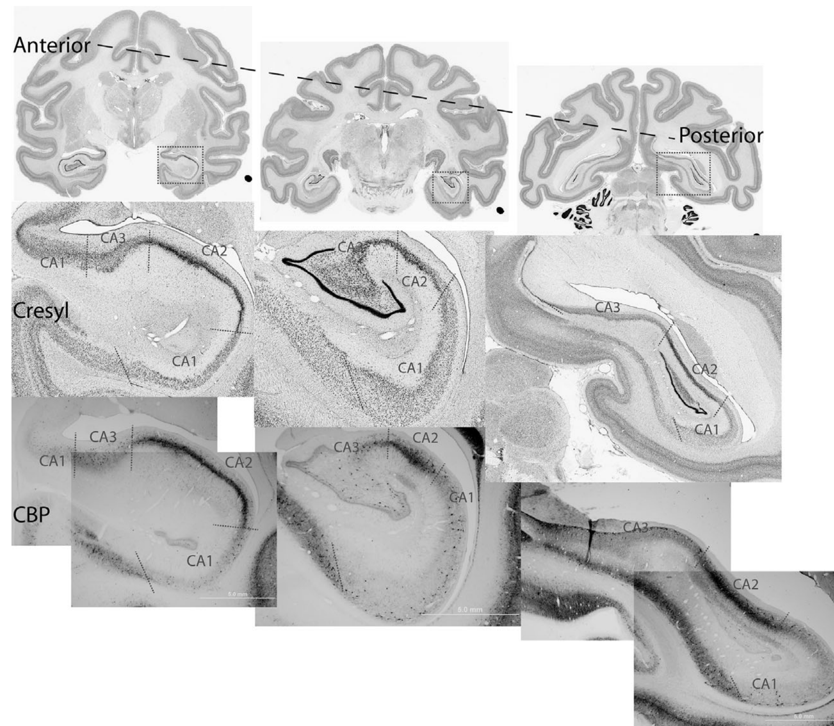

FIGURE 1 Delineation of Hippocampal Regions. A combined cytoarchitectural and chemoarchitectural approach was used to delineate the hippocampus and the regions of the Ammon's Horn. Nissl-stained sections through the entire anterior–posterior extent of the hippocampus were used for cytoarchitectural delineation, while matched sections were immunostained for calbindin, a putative calcium-binding protein (CBP) for chemoarchitectural delineation. Scale bar = 5 mm.

and about 2 years of age. These time points approximate early infancy, early childhood, and a juvenile period of complete autonomy that is well before the onset of adolescence.

Table 1 summarizes the characteristics of the FAE animals and their age-matched controls. Across the whole sample, control and FAE groups were well matched on age and duration of fetal exposure to ethanol or sucrose, respectively. Within age groups, the duration of alcohol versus sucrose exposure was not different, but neonates had on average 1 week longer exposure (4 drinking days) than did 2-year-old animals. Maternal blood ethanol levels were well correlated ($r = .93$, 95%CI .83–.97) with average daily ethanol exposure in all age groups. There was no difference in body weight between FAE animals and sucrose controls at any of the three ages (neonates: FAE .38 ± .07 kg, Suc .37 ± .08 kg; 5 months: FAE 1.0 ± .08 kg, Suc .99 ± .11 kg; 2 years: FAE 2.0 ± .16 kg, Suc 2.04 ± .13 kg). With respect to brain weight, there was an interaction ($F_{2,24} = 4.48$, $p = .022$) between age and treatment (Fig. 2, top panel), as well as a main effect of age ($F_{2,24} = 14.88$, $p < .0001$). The interaction effect was disproportionately due to the 5-month time point, as illustrated by unfixed representative FAE and Suc brains at this time point (Fig. 2, bottom panel). None of the animals had evidence of physical or facial malformation (using measures defined by Chudley et al., 2005), or overt neurological impairment (as measured by the Infant Behavioral Assessment Scale [Schneider et al., 2001]). The details and outcome of this testing are described in greater detail elsewhere (Beierschmitt, Huggins, Burke, Palmour, & Ervin, 2008 and in preparation). Normal ventricular sizes in all animals allowed us to rule out hydrocephalus as an explanation for the larger brain size in 5-month-old FAE animals.

As compared to sucrose-treated controls, FAE animals at every age had fewer neurons in Ammon's Horn (Tab. 2). Neuron numbers in the major hippocampal subregions (Fig. 3) differed significantly ($p < .0001$) between FAE animals and sucrose controls ($F = 6.58$, 87.8, and 55.82, respectively). There was significant age x treatment interactions for all regions (CA1: 3.89, $p = .033$; CA2: 4.56, $p = .02$; CA3: 6.85, $p = .004$), as well as a main effect of age in the CA2 region ($F = 5.21$, $p = .012$). Post hoc analysis (Fisher's LSD)

Table 2. Stereological 1 Parameters

Subregion	Section Sampling Fraction	Average Number Sections	Dissector Volume mm^3 (x*y*z)	Average Tissue Thickness	Mean SF	Average xy Step	Mean	Mean V^{ref} (mm^3)	Mean N (In Millions)	Mean CE (N)*
Infant										
Control										
CA1	1/18	12.4	25,000	16.49	226	562.8	452.4	57.2	1.501	.984
CA2	1/18	12.4	25,000	16.55	202	288.6	302.8	15.91	.289	.104
CA3	1/18	12.6	25,000	15.85	201	428	504.4	33.26	1.030	.089
FAE										
CA1	1/18	13.17	25,000	14.56	221	524	277.17	49.75	.726	.081
CA2	1/18	13.0	25,000	14.49	205	271	179.0	13.33	.141	.095
CA3	1/18	13.17	25,000	14.45	198	447	290.0	36.25	.589	.082
5 month										
Control										
CA1	1/20	12.6	25,000	14.68	227	648	253.0	94.39	1.239	.077
CA2	1/20	12.6	25,000	14.53	210	363	207.4	10.67	.314	.084
CA3	1/20	12.6	25,000	14.08	210	510	311.4	21.14	.901	.063
FAE										
CA1	1/20	14.25	25,000	13.43	217	618	193.4	89.46	.856	.089
CA2	1/20	14.25	25,000	13.48	215	358	156.6	17.63	.232	.084
CA3	1/20	14.25	25,000	13.41	204	523	243.8	35.34	.776	.077
2 year										
Control										
CA1	1/20	1.4	25,000	15.81	104	1026	118.0	107.30	1.528	.089
CA2	1/20	13.4	25,000	15.94	108	479	107.6	23.74	.324	.126
CA3	1/20	13.6	25,000	15.57	110	756	170.8	59.01	1.095	.099
FAE										
CA1	1/20	12.2	25,000	17.69	105	783	58.8	70.32	.572	.111
CA2	1/20	12.2	25,000	18.42	98	372	52.4	16.30	.120	.138
CA3	1/20	12.4	25,000	17.35	100	596	85.0	40.58	.746	.150

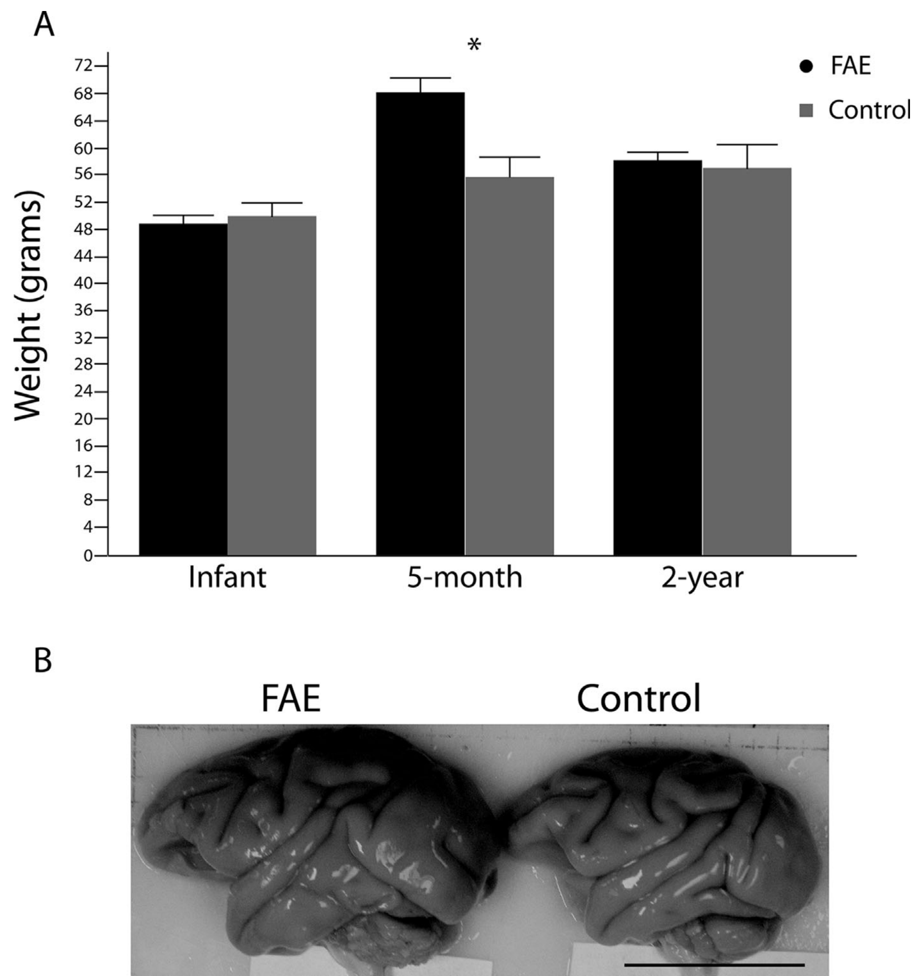

FIGURE 2 Brain Size and Age. There was a significant interaction between age and treatment (alcohol, sucrose) with respect to brain size (A). In control subjects, the brain progressively grew in weight and length from birth to 2-years, as is also seen in the volumetric estimates of the hippocampus and its regions (Fig. 3). FAE subjects did not follow the same progression with 5 month FAE subjects displaying significantly heavier and longer brains than age-matched controls (B), and also heavier and longer brains than neonatal and 2-year-old FAE animals. Scale bar = 1.2 cm.

revealed that treatment was a significant ($p < .0001$) driver for each region. For CA2, there were significant post hoc age differences between neonatal and 5-month-old animals ($p = .0025$), and between 5-month and 2-year-old animals ($p = .0156$). In FAE animals, there were significant neuronal reductions in all three CA regions at each developmental age (statistical details: Fig. 3 legend). The estimation of neurons produced a BCV^2/CV^2 ratio of greater than .85 for each time point for both treatment groups, indicating a low sampling error and a precise estimate of the hippocampal neuronal populations. There was no evidence of gliosis in any of the brains examined in this study.

Fetal ethanol exposure had differential effects on the volumes of subregions of Ammon's horn (Fig. 3). In CA1, there was a main effect of age ($F = 16.5$, $p < .0001$) and a more modest effect of treatment ($F = 6.3$, $p = .018$). A similar pattern, with a main effect of age ($F = 15.54$, $p < .0001$) and an interaction between age and treatment ($F = 5.2$, $p = .012$), characterized the volumetric development of CA3. Although the volumes of CA2 were lower in neonatal and 2-year-old FAE animals, the differences were not significant. There was, however, a main effect of age ($F = 26.98$, $p < .0001$). Between the neonatal period and 2 years of age, the principal regions of Ammon's

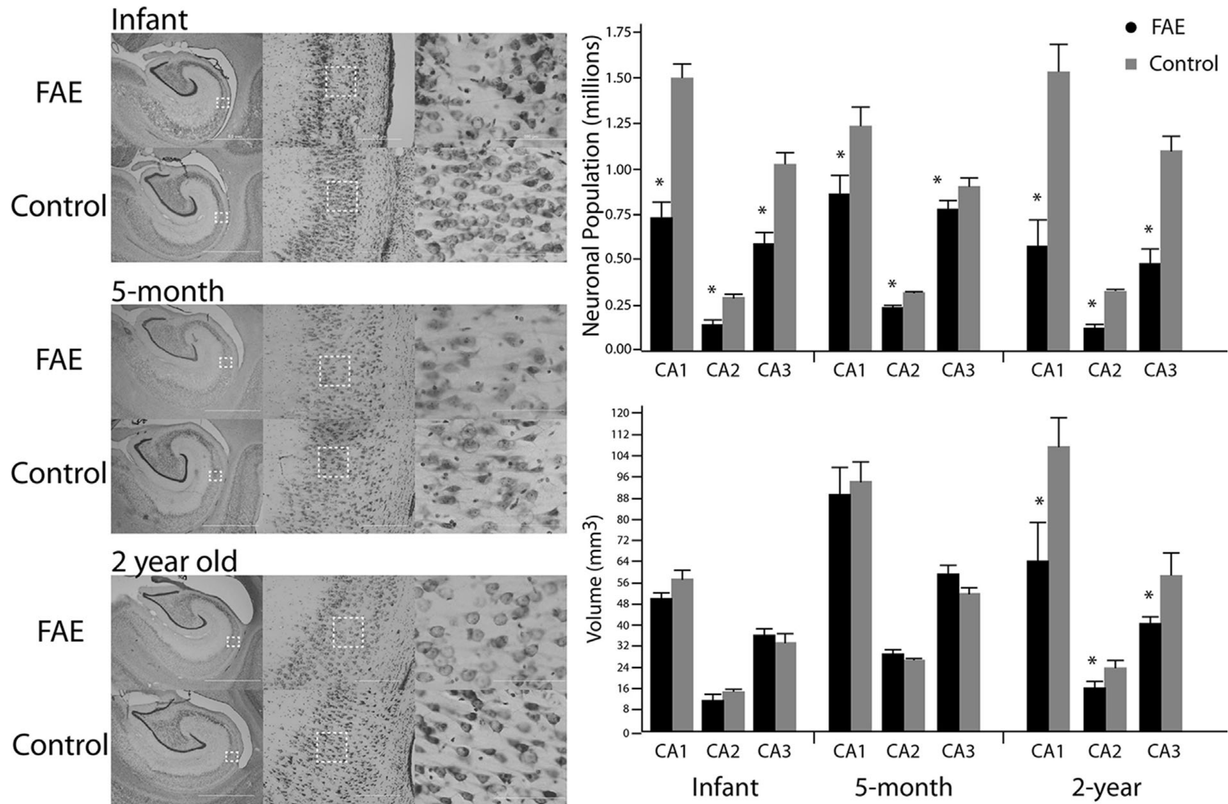

FIGURE 3 Representative Histological Sections. The pyramidal cell layer in Ammon's Horn of neonatal FAE subjects is visibly thinner than that of controls and more sparsely populated than that of controls at each time point (left panels). As shown in the top right panel, there were significant neuronal reductions in the CA1 region at infancy (FAE: $.726'10^6$, $CV = .293$; Con: $1.501'10^6$, $CV = .110$), at 5 months (FAE: $.856'10^6$, $CV = .273$; Con: $1.240'10^6$, $CV = .178$), and at 2 years of age (FAE: $.572'10^6$, $CV = .573$; Con: $1.528'10^6$, $CV = .241$). The CA2 region of FAE subjects also displayed a significant neuronal reduction at infancy (FAE: $.141'10^6$, $CV = .470$; Con: $.289'10^6$, $CV = .140$), 5 months (FAE: $.232'10^6$, $CV = .149$; Con: $.315'10^6$, $CV = .083$), and at 2 years of age (FAE: $.120'10^6$, $CV = .400$; Con: $.324'10^6$, $CV = .093$). Likewise, the CA3 region of FAE subjects displayed significant neuronal reductions at infancy (FAE: $.589'10^6$, $CV = .247$; Con: $1.03'10^6$, $CV = .127$), 5 months (FAE: $.776'10^6$, $CV = .144$; Con: $.901'10^6$, $CV = .121$), and at 2 years of age (FAE: $.476'10^6$, $CV = .373$; Con: $1.095'10^6$, $CV = .181$). There were also significant volume reductions in CA1-CA3 in 2-year-old FAE subjects as compared to controls (bottom right panel). The estimation of neurons produced a BCV^2/CV^2 ratio of greater than .85 for each time point for both groups, indicating a low sampling error and a precise estimate of the hippocampal neuronal population. $^*p < .05$, FAE versus Con.

horn nearly double in size in sucrose control animals (CA1: 87.7%; CA3: 77.4%). In animals fetally exposed to an average of 2.45 g/kg ethanol on 4 days of the week, these regions grew only 42.7% and 12.1%, respectively.

DISCUSSION

The naturalistic model of maternal ethanol consumption reported here produces blood alcohol levels similar to those found in women after 3–5 standard drinks (Ebrahim et al., 1998); this level is frequently encountered in people drinking in a social context (Riley & McGee, 2005; Riley, Infante, & Warren, 2011). In this paper, we report that monkeys with third trimester ethanol exposure consistently have lower neuron counts (as compared to age-matched sucrose controls) in all three regions of Ammon's Horn and at all three developmental ages investigated. Second, because the volume of Ammon's horn did not expand normally in FAE animals, by 2 years of age, the hippocampus of

the FAE subjects was significantly smaller than that of the controls. Conspecifics exposed to similar quantities of ethanol prenatally display alterations of social behavior and deficits in acquiring cognitive tasks (Palmour et al., unpublished) and rhesus macaques exposed to somewhat lower concentrations of ethanol during gestation exhibit elevated acoustic startle responses and reduced habituation to stress (Schneider, Moore, & Kraemer, 2004; Schneider et al., 2008, 2013).

Similar behavioral effects have been reported for rodent models (Abel & Berman, 1994; Berman & Hannigan, 2000; Goodfellow & Lindquist, 2014; Lugo, Marino, Crosine, & Kelly, 2003), but neuronal deficits are strongly dependent upon dose and timing of exposure (Gil-Mohapel et al., 2010). Although an early study (Barnes & Walker, 1981) reported that 2nd trimester ethanol exposure reduced hippocampal pyramidal cells by 20%, subsequent studies (Bonthius et al., 2001; Maier & West, 2001; Miller, 1995; Wigal & Amsel, 1990;) found little or no loss of neurons except in CA1. If alcohol exposure includes or is restricted to the 3rd trimester equivalent (postnatal in rodents), consistent reductions in CA1 and sometime CA3 neurons have been reported (Livy, Miller, Maier, & West, 2003; Miki, Harris, Wilce, Takeuchi, & Bedi, 2004; Tran & Kelly, 2003; Wigal & Amsel, 1990). The data we report here are congruent with these findings, except that BECs measured in rodents are typically two to threefold those reported here for monkeys.

Other differences between the current study and studies of rodent models may also contribute to the extensive neuronal loss in the monkey. Despite the fact that ethanol exposure was restricted to 4 days per week in this study, the duration of exposure is still considerably longer in real time than would ever be the case for a rodent (Clancy, Darlington, & Finlay, 2001). In the present study, ethanol was consumed for an average of 5.8 ± 1.01 weeks as compared to ethanol administration ranging from 4 to 21 days in the rat. Thus, monkeys were exposed to a higher total ethanol exposure because of the protracted gestational period. Another methodological difference was the use of voluntary ethanol consumption in a compartment adjacent to the home cage. Consequently, animals were not subject to the stress caused by handling, injection, or gavage (Miki et al., 2004; Miller, 1995; Tran & Kelly, 2003), nor did they experience the binge-like peaks in BEC characteristic of some rodent models. It should be noted, however, that in the single nonhuman primate study examining the interaction between prenatal ethanol exposure and prenatal stress (Schneider et al., 2004), offspring exposed to both of these adverse conditions were more impaired than those experiencing either one alone.

A potential limitation of this model is that the choice of animals for the study introduces an intentional bias and, thus, restricts the generalizability of the results. With respect to dams, all animals were voluntary consumers of reasonably high quantities of beverage alcohol. On the positive side, this allowed us to provide a significant prenatal exposure to beverage ethanol without unduly stressing the dams with repeated anesthesia and gavage. On the negative side, it restricts the findings to alcohol-preferring animals, but this is also the case in the clinical situation: children with fetal alcohol damage are not born to abstinent women. We intentionally selected alcohol-avoiding sires because there is substantial evidence that, in the clinical situation, some of the behaviors observed in children with fetal ethanol exposure could easily be attributed to paternal transmission of poor impulse control and symptoms related to personality disorders. The goal was to remove a potential confounding variable, but again this limits the findings to a specific pattern of breeding.

Another potential limitation of this paradigm is the lack of strict nutritional control between groups. All dams gained weight during pregnancy, and all alcohol-exposed infants were of normal birth weight. Thus, protein–calorie malnutrition is unlikely to be the source of the neuronal paucity reported here. Effects on fetal micronutrient levels in the brains of FAE offspring are both more plausible and less amenable to control (Shankar, Ronis, & Badger, 2007; Weinberg, 1984). It is known (review: Feltes, de Faria Poloni, Nunes, & Bonatto, 2014) that ethanol impairs intestinal absorption and metabolism of particular vitamins and other micronutrients and that normally sufficient levels of zinc and iron (Keen et al., 2010; Rufer et al., 2012) may be inadequate for neural development under conditions of maternal ethanol consumption. Of the principal vitamins, folic acid, a micronutrient critical to fetal brain development (Christensen & Rosenblatt, 1995), and nicotinamide (Ieraci & Herrera, 2006) are selectively depleted by chronic ethanol exposure, while conversion of vitamin A to retinoic acid (Keir, 1991; Zachman & Grummer, 1998) is competitively antagonized by ethanol. Supplementation with each of these micronutrients, as well as vitamins C and E, produced modest improvement in either the neural and/or behavioral effects of fetal ethanol exposure (e.g., Hewitt et al., 2011; Ieraci & Herrera, 2006; Marino Aksenov, & Kelly, 2004; Naseer et al., 2011; Wang et al., 2009), but the mechanisms of damage and amelioration have not been persuasively identified. Using a systems approach and transcriptome data, Feltes et al. (2014) have shown that genes related to retinoic acid, the niacin component of nicotinamide, and folate metabo-

lism were underexpressed in mice exposed to ethanol at 14–16 days of gestation. Another relevant focus of contemporary study is the epigenome: among other effects, ethanol enhances global DNA hypermethylation in frontal cortex and hippocampus (Muralidharan, Sarmah, Zhou, & Marrs, 2013; Zhou et al., 2011). Choline supplementation (Monk, Leslie, & Thomas, 2012; Otero, Thomas, Saski, Xia, & Kelly, 2012) ameliorates behavioral anomalies but does not influence cell counts or morphological effects in these areas. The extent to which a cocktail of these micronutrients might abrogate or reverse multiple phenotypic sequellae of FAE has not been reported, and certainly merits testing in a nonhuman primate model.

The variation in age at sacrifice within an individual time point (neonatal, 5 months, 2 years) is a consequence of the fact that monkey infants are born singly, not as litters. Sacrifices were scheduled to occur for each time point within a period of a few days, and animals closest to the target time were selected for sacrifice. Similarly, it was not possible to obtain an equivalence of males and females with the relatively small breeding populations for these studies. In a larger sample of 2-year-old animals with a better balance of males and females (in preparation), there were no statistical differences between males and females with respect to cell counts in hippocampal regions, and this observation has also been made repeatedly in rodent studies of prenatal and early postnatal exposure to beverage alcohol (Tran & Kelly, 2003). However, this does not preclude gender differences in hippocampal functioning (Sickmann et al., 2014) or stem cell differentiation (Uban et al., 2010), or behavior (Hellemanns et al., 2010).

It is difficult to relate these findings explicitly to the consequences of fetal ethanol exposure in man because there are no published post-mortem stereological studies. The most recent imaging studies show volumetric reductions in the hippocampus that vary as a function of diagnostic severity (Astley et al., 2009) and are stable across developmental stages (Nardelli et al., 2011). Presumably, this volumetric loss is related, as least in part, to cell loss but there is not yet a direct confirmation of this hypothesis.

Stereological studies of the primate hippocampus are generally restricted to sections with a visible dentate gyrus (Amaral & Lavenex, 2007; Jabes, Lavenex, Amaral, & Lavenex, 2011). This may facilitate reproducibility between subjects, but it also underestimates the total neuronal population of Ammon's horn. In the current study, we combined cytoarchitectural features of our own tissue with reported chemoarchitectural differences (Curtis et al., 2014) to facilitate delineation of the CA fields across the extent of the hippocampus. Despite some differences in defining range, the neuronal numbers estimated for the control subjects are of the same order of magnitude as those previously reported in normal nonhuman primates (Jabes et al., 2011). In control brains, there is minimal variation in neuronal estimates and error coefficients across age groups despite the doubling of the number of optical dissectors sampled between the newborn and 5-month time points. None of this is surprising given that neurogenesis in the CA1-3 fields in the nonhuman primate ends by embryonic Day 80 (Nowakowski & Rakic, 1981; Rakic & Nowakowski, 1981), a time that is similar to the developmental sequence in humans (Seress, Abraham, Tornockzky, & Kostolanyi, 2001).

By contrast, in monkeys with ethanol exposure during the third trimester, neuron numbers in CA regions of the hippocampus were not stable across age groups and coefficients of variation were also higher in these animals. Neonatal FAE animals have nearly 50% fewer hippocampal neurons (49% lowr in CA1, CA2; 57% lower in CA3) than do age-matched sucrose controls, but at 5 months of age, the mean number of neurons (Tab. 2) was higher in all CA regions for FAE animals, and the differential between FAE and Con had decreased (CA1, 69.6%; CA2, 73.9%; CA3, 86.1%. Yet by 2 years of age in FAE vervets, there was fewer neurons in CA1 and CA2, in absolute numbers as compared to FAE animals at both earlier time points and proportionally as compared to SucCon animals. The molecular basis of this variation is unknown, but the observation suggests that the 5-month time point might be a window of opportunity for therapeutic intervention.

The failure to flourish in the FAE brain is reflected in other measures as well. In the normal control animals, there was little change in cell count between birth and age 2, but there was a near doubling in the volume of CA1 and volumetric increases in other regions as well. This no doubt reflects the rich arborization of dendrites and synaptogenesis, as well as the axonal myelination that occurs throughout the first year of life in the nonhuman primate (Lavenex, Banta Lavenex, & Amaral, 2007; Seress & Riback, 1995a, 1995b). In FAE animals, a similar volumetric increase is seen at 5 months, but by 2 years, volumetric involution is marked. Although fine-grained studies of axonal and dendritic elaboration in the FAE vervets remain to be done, these observations are consistent with rodent data indicating a low density and high proportion of stubby spines on CA1 pyramidal neurons after early postnatal ethanol exposure (Gonzalez-Burgos et al., 2006) and reductions of dendritic length and branching (Rice et al., 2012). Similar findings have been reported in neuronal cell cultures (Romero et al.,

2013; VanDemark, Guizzetti, Giordano, & Costa, 2009). These results are also broadly consistent with recent imaging studies indicating reduced connectivity in clinical FASD (Dodge et al., 2009; Roussotte et al., 2012; Santhanam, Li, Hu, Lynch, & Coles, 2009). Animals sacrificed in the neonatal period and those sacrificed at 5 months of age lived only in their natal cages, while those sacrificed at 2 years of age had multiple housing experiences. In the vervet, mother–infant interactions consume the majority of time up until about 3 months of age, and thereafter, peer interactions are increasingly important. At about 6 months of age, all infants born into our colony (both with and without fetal ethanol exposure) are moved to a nursery setting for a short period of vaccination and other medical workups. They are moved thereafter to small groups of six, then a few months later to larger outdoor peer groups. During all of these periods, behavioral enrichments in the form of swings, jungle gyms, and foraging opportunities are available. The purpose of these housing transitions is to ensure good health and to develop constructive and independent behavioral interactions with peers. There is ample data in rodents suggesting that physical activity and enriched environments promote acquisition and performance of hippocampally driven behaviors, and a solid body of research shows that some effects of FAE in rodents can be ameliorated by voluntary exercise (Boehme et al., 2011; Redila et al., 2006). In vervet monkeys exposed prenatally to doses of ethanol in excess of 2 g/kg of the mother's body weight per drinking day, hippocampal neuron numbers were lower at 2 years of age, as compared to neonatal or 5 month FAE cohorts. This differential is not simply a contrast between FAE and control animals at 2 years of age, but instead represents a progressive age-dependent reduction in neuron numbers in FAE animals. If the increased peer-group exposure to activity and social interactions operated in vervets as it does in rats, one would have expected the opposite result.

The increased size of FAE brains at 5 months of age remains puzzling. As noted before, body weight did not differ between FAE and Suc animals at this time point, and there was no evidence of hydrocephalus or ventricular enlargement upon gross examination or during sectioning of relevant sections of the brain. As this is a global change, it is unlikely that the neuron counts or volumes of any single region can account for the observation. Neuron counts in Ammon's horn were transiently (but not always significantly) higher in FAE animals at 5 months of age than in infancy (Tab. 2 and Fig. 3), but this increase is not sufficient to explain the weight differential. In both human and nonhuman primates, the period of early infancy is marked by a reduction in the density of neurons in the hippocampus and frontal cortex (Conel, 1955; Rakic, Bourgoeis, Eckenhoff, Zecevic, & Goldman-Rakic, 1986), but this is due to growth in volumes and neuronal processes rather than a decrease in numbers of neurons. As shown in Figure 3, the volumes of CA regions do not differ between FAE and Suc animals at the 5-month time period, so this is also not a satisfactory explanation. In the present study, we did not evaluate the extent of postnatal neurogenesis in either group of subjects, and it is conceivable that these data might be informative. It is also possible that there is a delayed brain growth spurt in the FAE animals as a response to the termination of ethanol exposure at the time of birth.

Lower than normal brain volumes have been well documented in fetal alcohol exposed children (Meintjes et al., 2014), but these studies are typically performed after the age of 5 years (Lebel et al., 2012; Meintjes et al., 2014; Rajaprokash, Chakravarty, Lerch, & Rovet, 2014; Treit et al., 2013). Instead, the enlarged brain of FAE subjects at 5 months resembles a neuroanatomical feature of fragile X syndrome (FXS), where brain overgrowth has been reported between the ages of 18 and 42 months (Hazlett et al., 2012; Wassink, Hazlett, Davis, Reiss, & Piven, 2014). Enlarged brains within the first 2 years of life are also reported as a biological correlate in autism (Hazlett et al., 2012; Lainhart, 1997). Phenotypic characteristics of FASD, FXS, and autism suggest a commonality of frontolimbic dysregulation leading to social deficits, abnormal responses to sensory stimuli, and deficits in language performance (Abkarian, 1992; Hazlett et al., 2012; Streissguth et al., 1990). Serotonin levels have also been implicated in brain overgrowth in both FSX and autism (Wassink et al., 2014) and may contribute to the pathophysiology of FAE (Schneider et al., 2011).

The brain overgrowth reported here may be related to an attempted compensatory mechanism. The observed alterations may be a reflection of quite specific interference with pathways of neurodevelopmental gene expression and regulation (Alfonso-Loeches & Guerri, 2011; Aronne, Evrard, Mirochnic, & Brusco, 2008; Camarillo & Miranda, 2008; Galindo, Azmudio, & Valenzuela, 2005; Guerri, 2002; LeMagueresse & Monyer, 2013; Perkins, Lehmann, Lawrence, & Kelly, 2013; Singh, Gupta, Jiang, Younus, & Ramzan, 2009). The subsequent smaller brain size at 2 years of age further suggests that the 5 month advance was not sustained. This pattern is similar to MRI data showing that patients with FASD have a steeper hippocampal volumetric decrease and fewer age-related increases, as compared to control subjects (Treit et al., 2013). These findings contrast with the common impression that alcohol leads to indiscriminate

cell damage via oxidative stress or apoptosis (Ikonimidou et al., 2000; Olney et al., 2002). This latter mechanism clearly comes into play with acute, high to very high-dose ("binge-like") alcohol intake, but is unlikely to account for the damage that occurs with moderately high levels of intake and with damage that continues long past the duration of ethanol exposure.

Although it is likely that there are neuronal anomalies in many other, as yet unexamined, vervet brain regions, the anatomical changes in hippocampus, and frontal cortex (Burke et al., 2009a) are consistent with the behavioral pathologies most often seen in human cases. That such impairments become relatively worse over time is also consistent with the human experience. The clear parallels with clinical FASD mandate further exploration of both behavioral and neuroanatomical studies in this primate model.

NOTES

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