

# Effects of Prenatal Alcohol Exposure on the Visual System of Monkeys Measured at Different Stages of Development

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**PURPOSE.** Fetal alcohol spectrum disorder (FASD) is a developmental disease characterized by behavioral problems and physical defects including malformations of the eye and associated optical defects. How these malformations affect retinal functioning is not well known, although animal models have suggested that scotopic vision is particularly deficient. Age is also known to affect scotopic vision. Here, we determined the combined effects of age and fetal alcohol exposure (FAE) on retinal function using full-field electroretinograms (ERGs) in monkeys (*Chlorocebus sabaeus*).

**METHODS.** ERGs were recorded in monkeys aged 3- to 12-years old, at multiple flash intensities under scotopic and photopic conditions, and functions were fit to the amplitudes of the a- and b-waves.

**RESULTS.** We found that both age and alcohol exposure affected ERGs. In photopic ERGs, amplitudes increased with age, and were higher in FAEs than controls, for data related to the OFF- and ON-pathways. In scotopic ERGs, amplitudes were decreased in young FAE compared with age-matched controls but only for the rod-dominated responses, while at brighter flashes, alcohol exposure led to an increase in the amplitude of the a- and b-waves.

**CONCLUSIONS.** The ERGs from the FAE animals closely resembled the data from the older sucrose-control monkeys. This suggests that the FAE monkey retina ages more quickly than the control monkeys. This large sample of nonhuman primates, with carefully monitored ethanol exposure, demonstrates the critical interplay between age and alcohol when assessing the integrity of the retina. We suggest that ERGs might be an important adjunct to diagnosing human FASD.

**Keywords:** alcohol, fetal alcohol spectrum disorder, aging, electroretinography, photopic, scotopic

Even moderate alcohol consumption during pregnancy can negatively affect a developing fetus. Fetal alcohol spectrum disorder (FASD) represents the continuum of behavioral, anatomic, and cognitive effects on the fetus,<sup>1,2</sup> with the most devastating cases being diagnosed with fetal alcohol syndrome (FAS).<sup>1,3</sup> Along with physical defects of the face<sup>4</sup> and neurodevelopmental damage,<sup>5</sup> the structure of the eye is also affected by prenatal alcohol exposure.<sup>6–9</sup> Indeed, children suffering from FASD present a wide range of optical defects and malformations, for example microphthalmia and optic nerve hypoplasia (smaller than normal eyes and optic nerve), strabismus (crossed eyes), amblyopia (lazy eye), nystagmus (involuntary eye movement), persistent hyperplastic primary vitreous, and increased tortuosity of the retinal vessels.<sup>7,8</sup> Along with these ophthalmic malformations, many people suffering from FASD have visual acuity problems.<sup>10</sup>

Full-field electroretinogram (ERG) is a common clinical test used to measure the integrity of the retina as it records changes in the electrical currents across the various cell populations of the retinal mosaic. Previous studies measuring the effects of

FAS on the ERG signal have produced conflicting results. A first study<sup>9</sup> reported no difference in ERG responses in four children with FAS. In contrast, Hug et al.<sup>11</sup> reported that 10 children with FAS had abnormal ERGs. Because it is impossible to control experimental conditions of prenatal ethanol exposure in humans, and these samples tend to be very small, we must capitalize on information from animal models.

Rodents exposed to ethanol during early embryologic development have similar craniofacial malformations to those found in humans.<sup>12,13</sup> These rodent models also have similar deficits as humans with FASD.<sup>14–16</sup> Physical abnormalities in rodents include ocular, cardiovascular, and brain defects.<sup>17</sup> The deficits and malformations vary across studies as a result of the variability in rodent models. The animal's outcome is critically dependent on the time of the fetal alcohol exposure (FAE), how much alcohol is administered or provided, and the administration technique (intravenously or metabolized).<sup>18</sup> In Lantz et al.,<sup>19</sup> scotopic ERGs in FAE mice showed a marked decrease in a- and b-wave amplitude, demonstrating rod-pathway deficiencies. In this study, however, alcohol was injected intravenously



at extremely high levels (blood alcohol level of 411 mg/dL, high enough to cause coma or death), which does not necessarily replicate the conditions of children with FAE. Also, given that mice are nocturnal animals and have a rod-dominated retina (rods are 97% and cones are 3% of all photoreceptors<sup>20</sup>) without a foveal pit, it becomes difficult to extrapolate effects of FAE on retinal integrity from mice to humans.

Nonhuman primates are particularly useful animal models because their retina is similar to the human retina. In a handful of studies, the fetal development of nonhuman primates has also been found to be affected by ethanol exposure making them an ideal model for FASD research.<sup>16</sup> Most of the papers investigating effects of FAE on primates have reported deleterious consequences on behavior<sup>21–24</sup> and neurobiological processing deficits.<sup>25</sup> Data obtained in our primate laboratory (in which moderately high levels of alcohol were self-administered during the last trimester of gestation) demonstrated pervasive effects on neuronal anatomy<sup>26–28</sup> including effects on anatomy of the visual system.<sup>29</sup> There have not yet been any studies, to our knowledge, that have examined the effects of FAE on visual function using a primate model. Simultaneous investigations into aging and FAE are particularly important given that both photopic and scotopic ERG responses vary with age.<sup>30–33</sup>

In this study, we used full-field electroretinography to study the effects of fetal alcohol exposure on retinal maturation in vervet (*Chlorocebus sabaues*) monkeys. Using 37 FAE monkeys (whose mothers' alcohol consumption during gestation was carefully monitored), ranging from 3- to 12-years old and 41 age-matched controls, we predicted effects of both FAE and age on ERGs. We found indeed that ERGs of the youngest FAE monkeys were different from their aged-matched controls, and rather resembled the ERGs acquired from older populations.

## MATERIALS AND METHODS

### Animals

All animal procedures were performed in accordance to the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The experimental protocol was also reviewed and approved by the institutional review board of the Behavioral Science Foundation (BSF 1702) that holds a certificate of Good Animal Practice from the Canadian Council on Animal Care (CCAC) and an Office of Laboratory Animal Welfare (OLAW) registration (A5028). None of the animals were killed for this study.

Vervet monkeys (*Chlorocebus sabaues*) have a visual system similar to humans with foveal binocular vision (a high cone density in the center of the retina, decreasing peripherally), trichromatic color vision, and an analogous geniculostriate system.<sup>34,35</sup>

Our FAE model was not intended to have any dysmorphic effects, and was instead meant to model the cognitive and behavioral effects of FAE. As such, alcohol exposure was not given during the period of organogenesis. For this same reason, we avoided binge-alcohol and forced administration (which can cause stress, in and of itself, and could confound the results). In our model, alcohol-preferring dams voluntarily consumed moderate amounts of alcohol or isocaloric sucrose (control) beginning near the end of the second trimester (details in Supplement), thereby targeting the period of synaptogenesis. Further details are available in our previous publications.<sup>27,28,36</sup> Briefly, mothers consumed an average of 2.7 g alcohol/kg body weight (range, 1.89–4.9) or isocaloric

TABLE 1. Summary of Group Characteristics

Age Group, y	Age, y	FAE	Control
Photopic, N = 78			
3–5	3	12	9
	4	0	4
	5	1	1
6–9	6	0	0
	7	8	13
	8	2	3
	9	1	2
10–12	10	1	2
	11	7	5
	12	5	2
Scotopic, N = 43			
3–5	3	14	5
	4	0	5
	5	1	1
6–9	6	5	5
	7	4	3
	8	0	0
	9	0	0
10–12	10	0	0
	11	0	0
	12	0	0

sucrose exposure from about embryonic day 96 (range, 47–149) during a 4-hour period, 4 days per week. Alcohol exposure was discontinued at the time of parturition. All animals lived in social groups throughout, and were trained to enter drinking compartments such that there was no need for anesthesia, gavage, or other stressors.

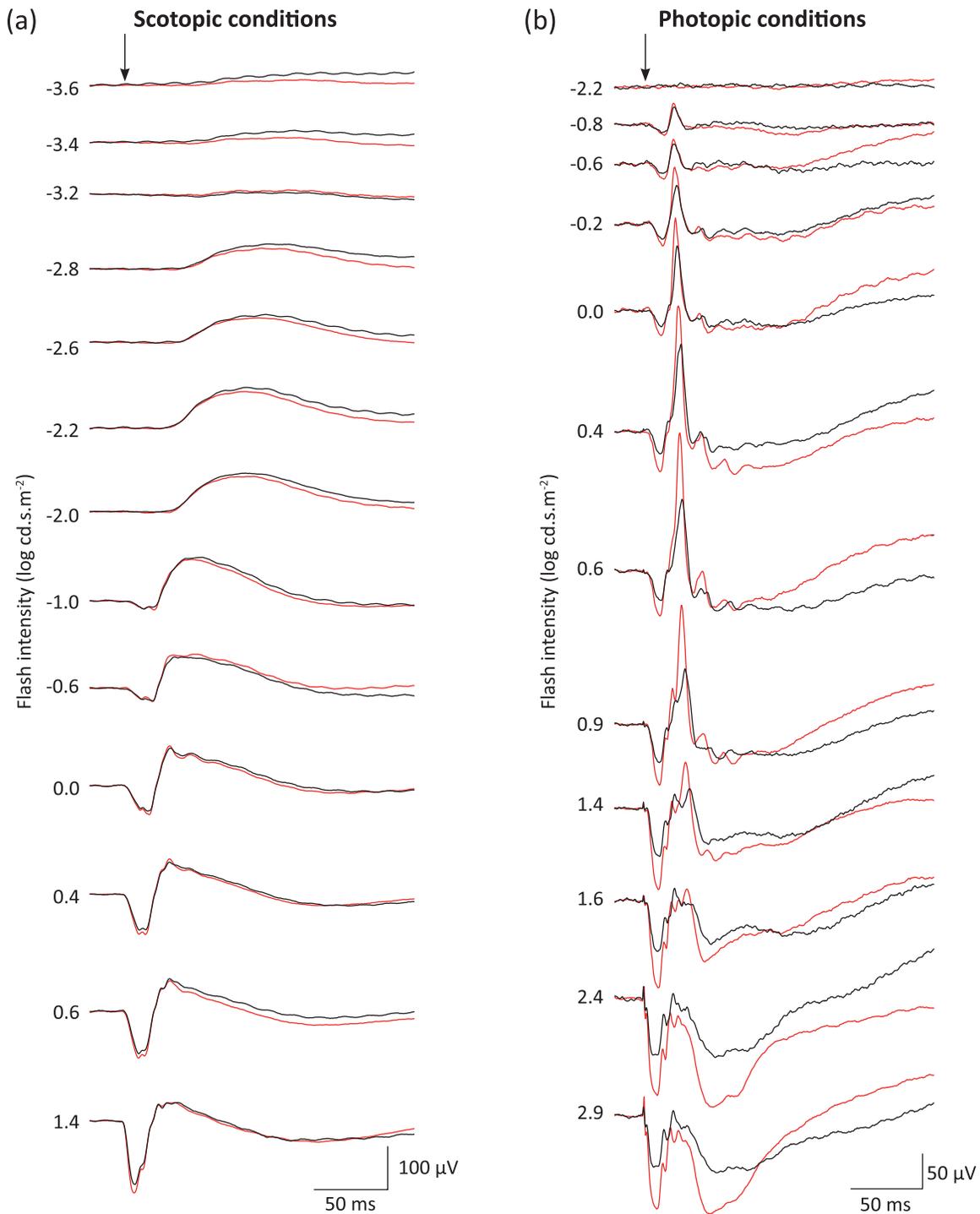
Each animal was tested one time, at one age only, recruitment based on age and rearing conditions (in the FAE or sucrose-control conditions). Sample sizes are summarized in Table 1 and additional details are provided in Supplementary Table S1. For the photopic condition data were obtained from a total of 78 subjects; and for the scotopic condition data were obtained from 43 subjects (some of which were also tested in the photopic condition, for details see Supplementary Table S1). Analysis of the fundus of the eyes showed no striking differences between FAE and controls.

### ERG Recordings

The retinal function of vervets was evaluated using a standardized, noninvasive, painless ERG protocol described earlier.<sup>37</sup> Briefly, animals were sedated, the pupils were fully dilated, and ERG responses were recorded using corneal contact lens electrodes (Jet electrodes; Diagnosys LLC, Lowell, MA, USA). Full-field ERG was performed in dark-adapted (scotopic) and light-adapted (photopic) conditions to differentiate between the rod and cone systems, respectively. For scotopic recordings, animals underwent dark adaptation for 20 to 30 minutes before stimuli were presented at intervals of 5 seconds for  $-3.6$  to  $0.4 \log \text{cd.s.m}^{-2}$  and 15 seconds for  $0.6$  to  $1.4 \log \text{cd.s.m}^{-2}$ . For photopic recordings, stimuli ranging from  $-2.2$  to  $2.9 \log \text{cd.s.m}^{-2}$  were then presented at 2-second intervals, with a steady white background ( $30 \text{ cd.m}^{-2}$  inside the Ganzfeld; additional details are available in Refs. 37–39).

### Analysis

Raw ERG recordings were averaged across both eyes because ERGs do not vary considerably across eyes.<sup>40</sup> The lack of difference of the ERG curves was confirmed by visual

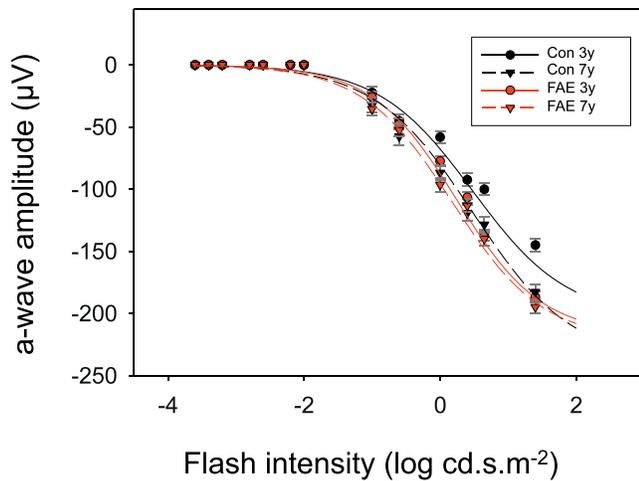


**FIGURE 1.** Average raw ERG (all animals tested) is plotted at each stimulus flash intensity in *red* for FAE subjects, and for controls in *black*. Raw ERG recordings obtained in scotopic (a) and photopic (b) conditions averaged across all monkeys in FAE and sucrose-control conditions. Scale is given by the *inserts*. *Arrow* indicates the onset of the flash.

inspection of the data collected here. When the curve for low light intensities (less than  $-2 \log \text{cd.s.m}^{-2}$ ) did not return to baseline 350 ms after the stimulus, the amplitudes of the a- and b-waves were corrected for the baseline shift. Amplitude of 0 was given at stimulus intensities when there was no a-wave or b-wave.

The amplitude of the a-wave was measured from the baseline to the most negative trough, while the amplitude of

the b-wave was measured from the trough of the a-wave to the most positive peak. Raw retinal response diagrams (see Fig. 1) were drawn using Adobe Illustrator and processed in Adobe InDesign (software version CS5; Adobe Systems Canada, Ottawa, ON, Canada). Different parts of the ERG trace, provide information about the functioning of specific cell populations including photoreceptors, bipolar (ON and OFF), and Müller cells.<sup>41</sup>



**FIGURE 2.** A-wave amplitude in scotopic conditions. The amplitudes of the a-wave were fit with a three-parameter sigmoid for control in *black* and FAE in *red* for both 3- to 5-year-old monkeys (*solid lines*) and 6- to 7-year-old monkeys (*dashed lines*).

The b-waves from the photopic flash sessions were fit with the sum of an unnormalized Gaussian curve and a logistic growth function from which we can estimate the contribution of ON and OFF retinal pathways to the amplitude of the photopic hill.<sup>42,43</sup> From this curve fitting, the following variable can be extracted:  $L_{max}$  is the maximal asymptotic logistic growth,  $G_{height}$  is the maximal Gaussian amplitude and  $G_{peak}$  is the flash luminance at which  $G_{height}$  occurs (these variables are sometimes referred to as  $V_{bmax}$ ,  $G_b$ , and  $R$ , respectively).

Rod function was assessed by fitting the Naka-Rushton function,<sup>44</sup> a logistic function, to the amplitudes of the scotopic b-waves, using Matlab (2013a; Mathworks, Natick, MA, USA). In order to isolate the first limb of the luminance response function, because only this segment can be fit with a single function, we fit the function with only the values that corresponded to the first limb (less than  $-0.5 \log \text{cd.s.m}^{-2}$ ).<sup>45</sup> From the Naka-Rushton nonlinear analysis, the following variables can be extracted:  $R_{max}$  is the asymptotic b-wave

amplitude,  $k$  is the flash illumination at which the b-wave amplitude is half of its asymptotic value, and  $n$  is the slope of the function at the half amplitude. Amplitudes of the a-wave were fit with three- or four-parameter sigmoids, respectively.

To assess the statistical differences of these parameters between groups, we used a 3 (age)  $\times$  2 (alcohol intake) univariate ANOVA to determine main effects of maturation and alcohol exposure, or any interaction effects between the two factors on parameters extracted from the functions fit to the data. For scotopic data, there were only two levels for the age parameter. When appropriate, we also use this ANOVA to test effects of these factors at individual flashes (with “flashes” as an additional repeated measures factor). Significant interaction effects or main effects of age were followed-up, when appropriate, with post hoc pairwise comparisons (with Bonferroni corrections). Mean values ( $\bar{x}$ ) are presented with the standard error of the mean (SEM).

## RESULTS

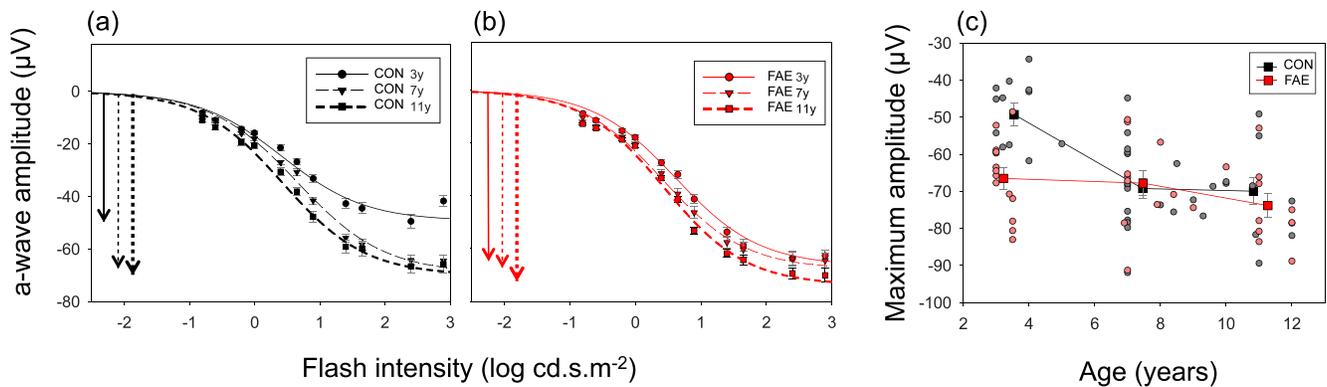
### A-Wave Amplitude: Scotopic Condition (Dark Adaptation)

In the scotopic lighting, the a-wave is not present at the lowest flash intensities (see Fig. 2). The amplitude of the a-wave, measured at the six brightest scotopic flash intensities, was fit with a three-parameter sigmoid representing  $a$  (the peak amplitude),  $x_0$  (the flash intensity at the inflection point of the sigmoid), and  $b$  (the slope at the inflection point). None of the three parameters were significantly affected by age or alcohol intake (see means in Table 2).

To assess individual points, and for comparison with previous publications, we ran a repeated measures ANOVA at the six brightest scotopic flashes. The repeated measures in the model were: flash intensity (6 levels)  $\times$  eye (2 levels), and the between-subject effects were alcohol intake (2 levels)  $\times$  age (2 levels). A full factorial model was used to assess all main effects and interaction effects. The main effect of eye, as well as the interaction effects of eye with other factors, were not significant in the model and are therefore not presented here. There was a significant main effect of age ( $F_{1,39} = 4.12$ ,  $P = 0.049$ , partial  $\eta^2 = 0.10$ ) where amplitudes were on average

**TABLE 2.** Means of a-Wave Amplitude Parameter Fits

Parameter, Units	Group	Age, y	Scotopic		Photopic		
			Mean ( $\bar{x}$ )	SEM	Mean ( $\bar{x}$ )	SEM	
Maximum amplitude of fit function, $\mu\text{V}$	FAE	3-5	216.7	15.1	-66.51	3.01	
		6-9	221.9	19.5	-67.80	3.27	
		10-12	-	-	-73.94	3.27	
	Control	3-5	203.2	17.7	-49.29	3.01	
		6-9	238.2	20.7	-69.23	2.56	
		10-12	-	-	-70.21	3.43	
	Inflection point of fit function, $\log \text{cd.s.m}^{-2}$	FAE	3-5	0.24	0.12	2.18	0.52
			6-9	0.18	0.16	0.20	0.57
			10-12	-	-	0.33	0.57
Control		3-5	0.48	0.14	0.17	0.52	
		6-9	0.48	0.17	2.61	0.44	
		10-12	-	-	0.49	0.60	
Slope at inflection point, $\log \text{cd.s.m}^{-2}$		FAE	3-5	-0.62	0.03	6.40	0.27
			6-9	-0.66	0.04	6.32	0.29
			10-12	-	-	6.08	0.29
	Control	3-5	-0.69	0.04	6.43	0.27	
		6-9	-0.72	0.04	6.65	0.23	
		10-12	-	-	6.52	0.30	



**FIGURE 3.** A-wave amplitude in photopic conditions. The amplitudes of the a-wave were fit with a three-parameter sigmoid for sucrose-control in *black* (a), and FAE in *red* (b). The parameter of the sigmoid that corresponds to the maximum amplitude of the fit function, demonstrated by the height of the *arrows* in (a) and (b), was significantly affected by age. In (c) we plotted the maximum amplitude parameter (i.e., the height of the *arrow*) as a function of monkey age (*error bars* represent SEM). *Square data points* are averages for each age group, plotted as a function of the mean age of the group, and *circles* are data from each individual monkey, plotted as a function of the monkey's actual age.

14.6  $\mu\text{V}$  higher in older monkeys ( $\bar{x}_{7y} = -102.1 \pm 5.6 \mu\text{V}$ ) than younger monkeys ( $\bar{x}_{3y} = -87.4 \pm 4.6 \mu\text{V}$ ). There was also a significant interaction of flash \* alcohol consumption ( $F_{5,195} = 2.54$ ,  $P = 0.030$ , partial  $\eta^2 = 0.061$ ) that we followed-up with post hoc tests. Alcohol-exposed monkeys had larger responses than control monkeys at the following flash intensities: significantly larger at 0.0 log cd.s.m<sup>-2</sup>: mean difference = 15.4  $\mu\text{V}$ ,  $P = 0.037$ ; and 1.4 log cd.s.m<sup>-2</sup>: mean difference = 25.7  $\mu\text{V}$ ,  $P = 0.05$ , respectively; marginally significant at 0.6 log cd.s.m<sup>-2</sup>: mean difference = 20.4  $\mu\text{V}$ ,  $P = 0.081$ , see Figure 2. An increase of 15 to 25  $\mu\text{V}$  is about the difference that is observed between the flashes. That is, when presented with a flash intensity of 0.0 or 0.6 log cd.s.m<sup>-2</sup>, FAE monkeys and older monkeys are over responding (compared with sucrose control or younger monkeys) as though the stimulus was 0.4 or 1.4 log cd.s.m<sup>-2</sup>, respectively.

In sum, the effect of alcohol exposure on the a-wave amplitude under dark-adapted conditions is quite similar to the effect of maturation—both causing an increased amplitude compared with data collected on the youngest control monkeys. As a result, as can be seen in Figure 2, the data from the youngest control monkeys has a much smaller a-wave than the other samples. Therefore, the effect of alcohol on the amplitude of the a-wave is an increase in amplitude, which resembles an early maturation.

### A-Wave Amplitude: Photopic Condition

The amplitude of the a-wave was plotted as a function of the flash luminance and fit with a 3-parameter sigmoid (Fig. 3). For the parameter that corresponds to the maximum amplitude, there was a significant interaction effect between age and alcohol intake ( $F_{1,70} = 5.170$ ,  $P = 0.008$ , partial  $\eta^2 = 0.13$ ). This interaction was present because in the control group there is a significant difference between the youngest and the two older age groups ( $P < 0.001$  in both cases), but no difference between the two older age groups (Fig. 3a). In contrast, there were no significant differences across the age groups for the FAE population (Fig. 3b). While the control group shows an increase in the maximum amplitude of the a-wave with increased age, the FAE monkeys resemble the mature controls even at the first testing; FAE do not show any reliable change in the amplitude of the a-wave with age (Fig. 3b).

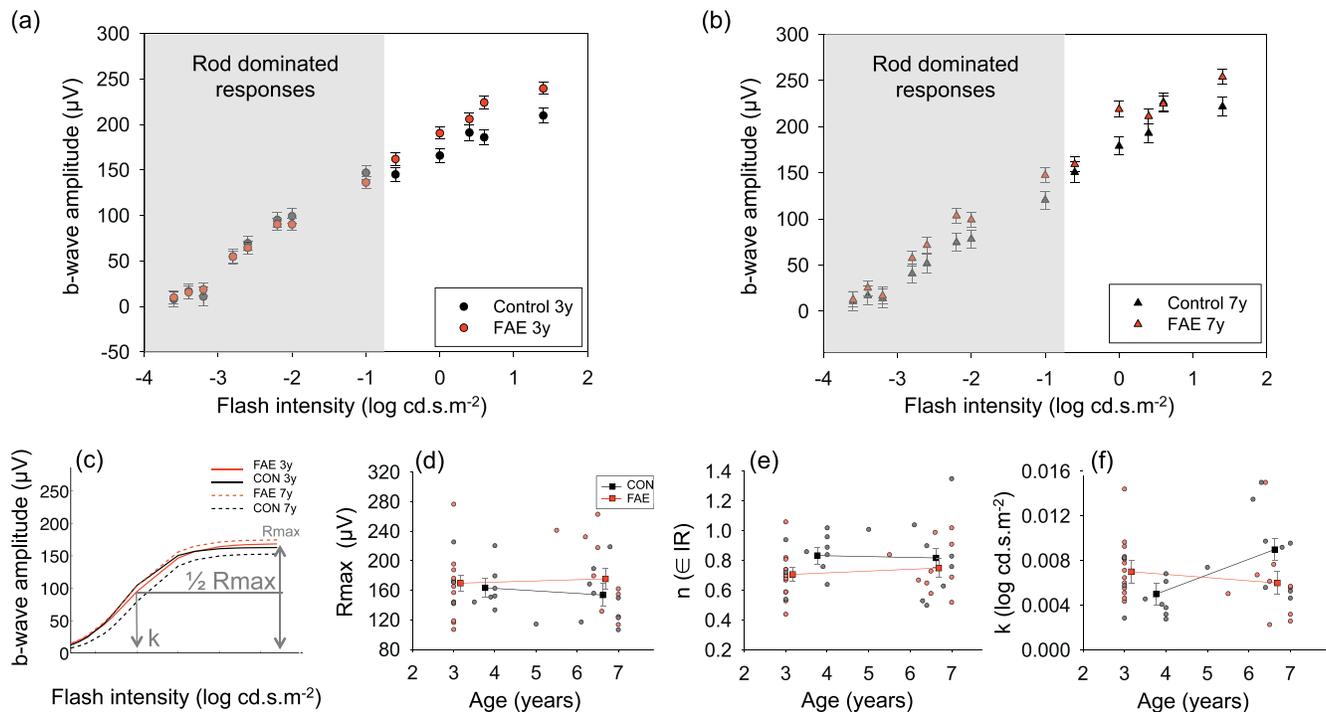
This pattern is also seen with the other parameters of the fit function: the inflection point, and the slope. While there were no significant effects on the slope, for the inflection point there was a significant interaction effect between age and

alcohol intake ( $F_{2,70} = 9.17$ ,  $P < 0.001$ , partial  $\eta^2 = 0.21$ ). Post hoc test demonstrated that for the FAE group, the young monkeys were significantly ( $P = 0.038$ ) or borderline significantly different ( $P = 0.058$ ) from the two older age groups, but the monkeys in the two older age groups were not different from each other. In contrast, in the control group, it was the middle-age group that was different from the younger and older age groups ( $P = 0.001$ ,  $P = 0.017$ , respectively), while the youngest and oldest monkeys were not different from each other (see means in Table 2). Here again, the data from the youngest FAE monkeys resemble data from the next age group of the control monkeys.

### B-Wave Amplitude: Scotopic Condition (Dark Adapted)

The amplitude of the b-wave was plotted as a function of the flash luminance (Figs. 4a, 4b) and fit with Naka-Rushton functions (Fig. 4c). We found no significant difference in the peak amplitude at any ages (means across ages given here:  $R_{\text{maxFAE}} = 172.6 \pm 9.1$ ,  $R_{\text{maxControl}} = 158.5 \pm 10.0$ ,  $F_{1,39} = 1.08$ ,  $P = 0.304$ , partial  $\eta^2 = 0.027$ , Fig. 4d). There was also no significant difference in the slopes of the Naka-Rushton function at any ages (means across ages given here:  $n_{\text{FAE}} = 0.729 \pm 0.04$ ,  $n_{\text{Control}} = 0.826 \pm 0.04$ ,  $F_{1,39} = 2.71$ ,  $P = 0.108$ , partial  $\eta^2 = 0.065$ , Fig. 4e). There was, however, a significant interaction between age and alcohol exposure on  $k$ , the flash illumination yielding the half-amplitude,  $F_{1,39} = 7.80$ ,  $P = 0.008$ , partial  $\eta^2 = 0.16$  (Fig. 4f). Older FAE monkeys reached the half-amplitude at basically the same flash intensity as young FAE monkeys (FAE:  $k_{3y} = 0.007 \pm 0.001$ ,  $k_{7y} = 0.006 \pm 0.001$ ,  $P = 0.330$ ), while older control monkeys needed a brighter flash to reach the half-amplitude compared with the young control monkeys (control:  $k_{3y} = 0.005 \pm 0.001$ ,  $k_{7y} = 0.009 \pm 0.001$ ,  $P = 0.006$ ).

Because the Naka-Rushton only fits the first limb of the data,<sup>45</sup> we ran a repeated measures (RM) ANOVA at the six brightest scotopic flashes: flash intensity (6 levels) \* alcohol intake (2 levels) \* age (2 levels) \* eye (2 levels). There were no significant interactions with eye. The RM ANOVA revealed a significant three way interaction effect: flash (6 levels) \* age (2 levels) \* alcohol consumption (2 levels) ( $F_{5,195} = 2.45$ ,  $P = 0.035$ , partial  $\eta^2 = 0.059$ ). This interaction demonstrates that aging (which is associated with an increase in amplitudes) has a smaller effect on the FAE monkeys (who already had high



**FIGURE 4.** Scotopic b-wave amplitude and Naka-Rushton fits. *Red data points and lines* demonstrate FAE data, while *black data points and lines* represent data from sucrose-controls. In (a), the mean amplitudes for the youngest age group (3–5 years) are plotted as a function of flash intensity. In (b), the mean amplitudes for the older age group (6–7 years) are plotted as a function of flash intensity. (a, b) The shaded region indicates responses that are dominated by the rod system, while the nonshaded area indicates mixed rod and cone responses. In (c), mean Naka-Rushton curves, fit to the first limb of the data, are plotted for each alcohol and age group: 3–5 years = solid lines; 6–7 years = dashed line. In (d, f), the average parameters for the Naka-Rushton equation are individually plotted for each alcohol group, across the two age groups tested. (d) The maximum amplitude of the Naka-Rushton function was not significantly altered by age or alcohol intake. (e) The exponent parameter (where  $n$  is a real number) was not significantly affected by age or alcohol exposure. (f) The intensity that yielded the half-maximum intensity (known as  $k$ ) was significantly affected by an interaction of age and alcohol exposure.

amplitudes in the youngest age group tested) compared with the sucrose-control monkeys.

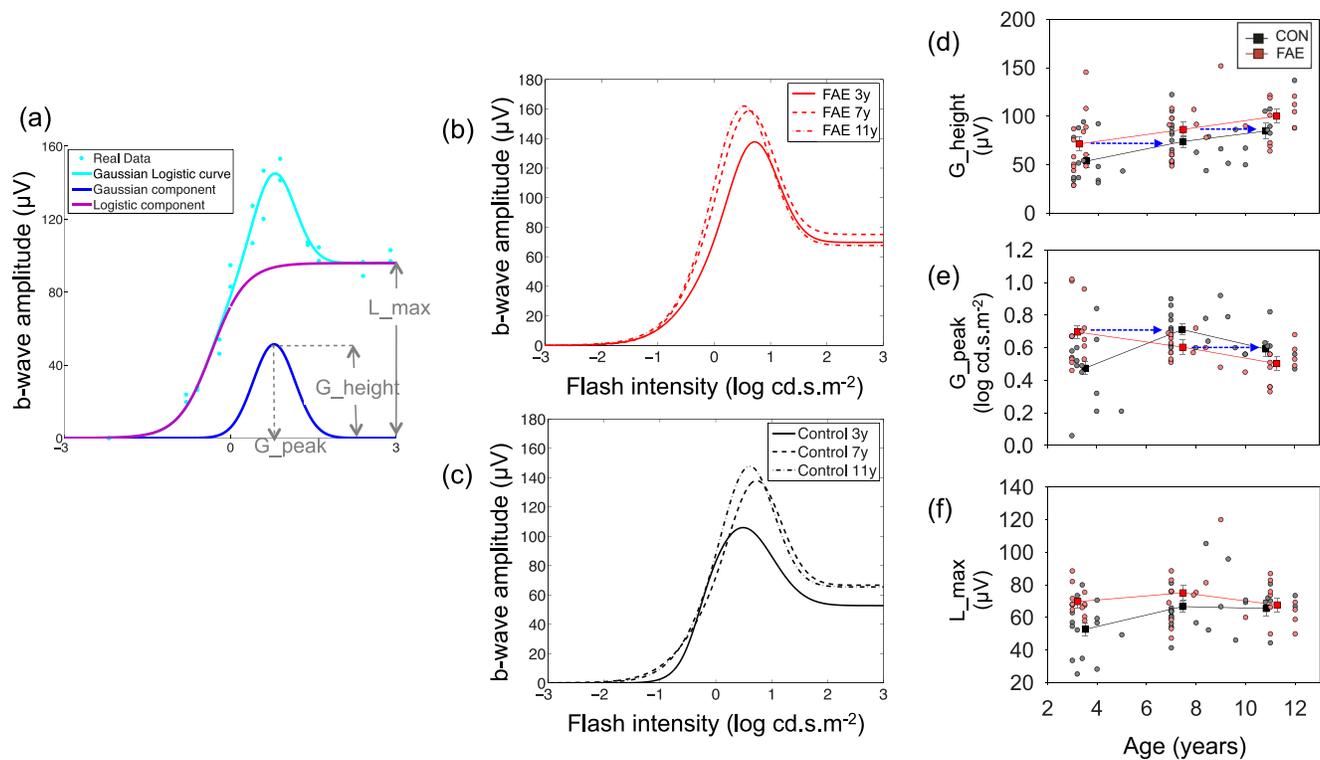
In summary, the pattern in the controls and FAE varied differently for responses normally dominated by rod responses versus the mixed rod-cone responses. At flashes generally arising from mixed rod responses (brighter flashes), FAE had higher amplitudes than controls, and older monkeys had higher amplitudes than younger monkeys (i.e., the same pattern as was reported for the cone-dominated responses in the photopic conditions). Responses that were rod-dominated, however, showed an important difference. The 7-year-old FAE monkeys had higher amplitudes than their age-matched controls, while the 3-year-old FAE monkeys had lower amplitudes than their age-matched controls. Together these results demonstrate the importance of age when assessing the effects of alcohol exposure on the rod system, while the effect of maturation in the cone responses (an increase with age) seems to be consistent across the ages tested.

### B-Wave Amplitude: Photopic Condition

The amplitude of the b-wave was plotted as a function of the flash luminance and fit with a Gaussian-logistic function (Figs. 5a–c). Changes in the Gaussian portion (related to the OFF-pathway) demonstrate effects of both age and alcohol. While there was no interaction effect between the two factors ( $F < 1$ ), both factors significantly affected the height of the Gaussian (Fig. 5d). The maximum Gaussian amplitude was significantly larger in the FAE group than in the age-matched controls ( $G_{\text{height}_{\text{FAE}}} = 84.8 \pm 4.3$ ,  $G_{\text{height}_{\text{Control}}} = 72.5 \pm 4.0$ ,  $F_{1,71} = 4.33$ ,  $P = 0.041$ , partial  $\eta^2 = 0.06$ ). The maximum Gaussian

amplitude was also significantly larger as the monkeys in both groups matured ( $G_{\text{height}_{3y}} = 62.5 \pm 4.9$ ,  $G_{\text{height}_{7y}} = 80.2 \pm 4.9$ ,  $G_{\text{height}_{11y}} = 93.3 \pm 5.6$ ,  $F_{2,71} = 8.86$ ,  $P < 0.001$ , partial  $\eta^2 = 0.20$ ). Following this up with post hoc test, the maximum amplitude of the b-wave at the youngest age (3-years old) was significantly lower than that of the 7- ( $P = 0.038$ ) and the 11-year-old monkeys ( $P < 0.001$ ). There was no significant difference between the 7- and the 11-year-old monkeys ( $P = 0.242$ ). These mean Gaussian height parameters are plotted in Figure 5d, and the pattern is clear: as the monkeys mature, the maximum amplitude (at the peak) increases, and at each time point the FAE have a higher amplitude than the matched controls. In sum, the FAE data look most similar to the control data at the subsequent (older) time point suggesting a premature aging effect.

We also analyzed the flash intensity that generates the peak in the b-wave amplitudes ( $G_{\text{peak}}$ , see means plotted in Fig. 5e). Once again, the FAE data look like the data collected from the control monkeys that are a few years older. The statistics demonstrate reliable differences; there was a significant interaction between age and alcohol on the flash luminance at which the peak occurs ( $F_{2,71} = 10.17$ ,  $P < 0.001$ , partial  $\eta^2 = 0.22$ ). Because of the interaction, post hoc test for alcohol at each age group were justified. There was a significant difference only for the youngest age group (3-years old):  $G_{\text{peak}_{\text{FAE}}} = 0.70 \pm 0.04$ ,  $G_{\text{peak}_{\text{Control}}} = 0.47 \pm 0.04$ ,  $P < 0.001$ . Post hoc comparisons within the two alcohol groups revealed the following differences: the youngest FAE peaked at a brighter flash intensity than the oldest FAE group tested ( $P = 0.006$ ). On the other hand, amongst the controls, the middle



**FIGURE 5.** Photopic b-wave amplitude as a function of flash intensity. Gaussian logistic curves were individually fit to each animal's b-wave amplitudes. In (a) the data from one subject (cyan dots) are fit with the Gaussian logistic function (cyan curve), which is the sum of the Gaussian component (blue curve) and the logistic component (magenta). In (b, c), the mean parameters of the function were used to recreate the Gaussian logistic function for each age group (3 years = solid lines; 7 years = dashed line; 11 years = dot dashed line), and each alcohol group. Control data are plotted in black (c, d-f), and FAE data are plotted in red (b, d-f). In (d-f), the parameters are plotted for each monkey (circles: light pink = FAE; gray = control), with the mean parameters (squares with error bars representing SEM) plotted for each alcohol group, across the three ages tested. (d) The height of the Gaussian was significantly higher in older monkeys, as well as FAE monkeys. (d-f) Across all parameters, FAE data look most similar to the control data at the subsequent (older) time point (a horizontal shift, see blue arrow) suggesting a premature aging effect.

age group (7 years) peaked at a significantly higher flash intensity than the youngest control group ( $P < 0.001$ ). While the pattern in Figure 5e looks different, the conclusions for the flash at which the peak occurs are the same: as with the height parameter detailed above, the FAE data are similar to control data except shifted to the right, suggesting that the effect of FAE mimics the effect of aging.

In addition, the peak amplitude of the logistic portion (representing the ON pathway) was also significantly affected by alcohol and age, with no interaction between the two. The significant main effect of alcohol demonstrates that in the three age groups the logistic peak was larger in the FAE group than in the controls ( $L_{max_{FAE}} = 70.7 \pm 2.5$ ,  $L_{max_{Control}} = 61.9 \pm 2.3$ ,  $F_{1,71} = 6.65$ ,  $P = 0.012$ , partial  $\eta^2 = 0.09$ , Fig. 5f). There was also a marginally significant main effect of Age ( $F_{2,71} = 2.97$ ,  $P = 0.058$ , partial  $\eta^2 = 0.08$ ). These results demonstrate that the ON-pathway was also affected by the alcohol exposure, and again the effect of alcohol exposure (increase photopic amplitude) is similar to the effect of aging suggesting that the youngest FAE monkeys may have prematurely aged.

## DISCUSSION

We found that ERG amplitudes were higher in older monkeys, and in FAE monkeys compared with sucrose controls; this applied in the photopic condition to the parameters related to both the OFF and ON pathways, and in the scotopic condition this applied to the mixed rod-cone responses (see Table 3). In

contrast, in the scotopic condition with dim flashes that only elicit rod responses, we found that young FAE monkeys had lower amplitudes than age-matched controls (as previously reported), while older FAE monkeys had higher amplitudes than age-matched controls. Thus, the effect of alcohol exposure was different depending on the ages of the monkeys. In general, the data from the youngest FAE monkeys appeared to resemble the data from the control monkeys in the next age group. These results suggest that FAE ages the visual system more quickly than under control conditions.

The two previous studies that have investigated retinal function in human FASD have reported data from a much smaller sample (e.g., 4 children in Chan et al.<sup>9</sup>; 11 children in Hug et al.<sup>11</sup>). While Chan et al.<sup>9</sup> reported no abnormalities in the ERGs, Hug et al.<sup>11</sup> found that 10 of 11 participants had abnormally low scotopic rod-derived ERGs, but no corresponding decrease in the b-wave at brighter (cone-activating) scotopic flashes. Here, we have also demonstrated that the rod-derived ERGs are sometimes lower in FAE (depending on age), while mixed rod-cone responses to brighter flashes are associated with an increase in the ERGs of the FAE monkeys. The inconsistency between the previous results is likely related to the small samples in the human-based studies, and their inability to draw from an exposure-controlled sample (exposure may have been at any point during gestation). While the humans FASD are likely to have been exposed during the critical period for alcohol teratogenicity (i.e., first trimester, before people know that they are pregnant<sup>46</sup>), the monkey

TABLE 3. Significance Table of *P* Values

		Significant Effect(s)	<i>P</i> Value	Description
a-wave				
Scotopic	Amplitude	n.s.		
	Inflection	n.s.		
	Slope	n.s.		
	Individual points	Age	0.049	Older > younger
Photopic	Amplitude	Flash * Alc	0.030	FAE > control (at bright flashes)
		Age * Alc	0.008	FAE does not show aging Young FAE resemble the older control monkeys
	Inflection	Age * Alc	<0.001	Young FAE resemble the older control monkeys
	Slope	n.s.		
b-wave				
Scotopic	Rmax	n.s.		
	k	Age * Alc	0.008	FAE does not show aging. Controls: older > younger
	Slope	n.s.		
	Individual points	Flash * Age * Alc	0.035	FAE > control Older > younger (at brighter flashes)
Photopic	Gaussian Height (OFF-pathway)	Age	<0.001	Older > younger
		Alc	0.041	FAE > controls
	Gaussian peak (OFF-pathway)	Age * Alc	<0.001	Young FAE resemble the older control monkeys
	Logistic (ON-pathway)	Age	0.058	Older > younger
		Alc	0.012	FAE > controls

n.s. indicates that there were no significant main effects or interaction effects. Alc refers to the exposure to alcohol 2 levels: FAE or sucrose control.

FASD sample studied here was only exposed in the third trimester. The exposure limited to the third trimester may have caused smaller effect sizes in the current study (~15–80  $\mu$ V, depending on the condition) compared with approximately 200  $\mu$ V in Hug et al.<sup>11</sup> The strength of the current data lies in the sample size (providing statistical power able to identify small effect sizes), and the experimental design, which allowed us to record exactly how much, and when, alcohol was consumed while these monkeys were in utero.

There are a handful of FAE studies in rodents, where prenatal alcohol was rigorously measured, and administered at high levels early in gestation, which demonstrate cranial, ocular, cardiovascular, and neural malformations as a result of FAE.<sup>12,17,47</sup> In terms of retinal functioning, rodent models (nocturnal animals) have demonstrated that FAE causes a depression in ERG amplitudes, especially in the scotopic conditions.<sup>13,19</sup> The stimuli used in these aforementioned studies correspond to the scotopic rod-derived ERGs in the current study (shaded region in Figs. 4a, 4b), which also showed a decrease in the youngest age group tested. We extended this result using a primate model (with a more evolved retina), by demonstrating cone-derived ERGs (bright scotopic flashes and photopic conditions) were increased in FAE compared with controls. Given the difference between the cone- and rod-derived ERG results, it appears even more important to use primate models of FAE in order to generalize results from animal models to human populations. That said, the decrease reported in the animal studies was also much larger than we found, which is likely related to when and how much alcohol was administered (see factors affecting neurogenesis).<sup>18</sup> Our data are novel because we demonstrate that age and flash intensity (rod or cone activating) are both critical factors in assessing the effects of FAE on ERGs; changes in scotopic ERGs need to be looked at over a range of flash intensities, and within the context of age.

Several papers have reported that there is an important increase in the amplitude of ERG waves during childhood (both photopic and scotopic), which levels out and remains

stable well into adulthood.<sup>30,31,33</sup> The peak occurs around 3- to 15-years old in humans.<sup>30,31</sup> Then, as humans age, the amplitudes of the ERGs begin to decrease at roughly 40-years old,<sup>30</sup> though older individuals can have more intense cone responses than younger individuals.<sup>32</sup> We demonstrate that the effects of FAE on ERGs are not always an increase or a decrease compared with controls. Rather, the effects of FAE appear to systematically follow the effects of aging.

Premature aging of the central nervous system as a result of alcohol is a hypothesis that has been well supported in the literature.<sup>48,49</sup> Premature aging is generally associated with chronic (adult) alcoholism,<sup>50</sup> although neurological dysfunctions found in alcoholics can be quite similar to those seen in individuals with prenatal alcohol exposure.<sup>51,52</sup> The most prominent similarity is paucity of neurons in the frontal cortex, reported in prenatal ethanol exposed monkeys,<sup>26</sup> and as a result of alcoholism,<sup>53</sup> and normal healthy aging.<sup>54</sup> While the causes might be different (not necessarily due to atrophy in the case of FAE) the changes to the brain are strikingly similar in prenatal alcohol exposure, alcoholism, and aging. Here, for the first time, we present data that suggests premature aging in the visual system of monkeys exposed prenatally to alcohol. This premature aging effect might be due to morphologic and/or biochemical differences in the retina (and cortex) of fetal ethanol-exposed monkeys, such as changes in GABAergic and glutamatergic systems<sup>55</sup> or the endocannabinoid system.<sup>56–59</sup>

In conclusion, this study has found that FAE alters ERG responses in a large sample of nonhuman primates. FAE seems to be related to an increase in the ERGs associated with cone function, and this increase is similar to the changes seen over the course of retinal maturation. ERGs associated with rod function show a decrease with age, and alcohol exposure. A common factor might be mediating the effects of alcohol and age on retinal function, (such as energy metabolism, or mitochondrial dysfunction) and this common factor could then cause the ERG responses of the youngest FAE monkeys to appear more similar to older controls than age-matched controls. For example, energy metabolism in general, or

progressive mitochondrial dysfunction, could mediate the effects reported here. However, this hypothesis remains to be verified experimentally. ERG results across the lifespan may be used as an important marker in identifying and contributing to a diagnosis of human FAS.

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