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RESEARCH 1

Title Page

The Photopic Negative Response in Autism Spectrum Disorder

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Background: Visual function can be atypical in autism spectrum disorder and structural imaging of the ganglion cell layers has been reported to differ in these individuals. Therefore, we sought to investigate if the photopic negative response of the full field electroretinograms, a measure of ganglion cell function, could help explain the visual perceptual differences in autism spectrum disorder and support the structural changes observed.

Methods: Participants (n=55 autism spectrum disorder, aged 5.4 to 26.7 years) and control (n=87, aged 5.4 to 27.3 years) were recruited for the study. Full field light-adapted electroretinograms using a Troland protocol with ten flash strengths from -0.367 to 1.204 log photopic cd.s.m^{-2} were recorded in each eye. The photopic negative response amplitudes at Tmin and at t=72ms were compared between groups along with the a- and b-wave values.

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Conclusion: The photopic negative response results suggest that there are no significant differences in the summed retinal ganglion cell responses produced by a full field stimulus.

Keywords: Autism Spectrum Disorder; Photopic Negative Response; Electroretinogram; Ganglion cells.

The electroretinogram differs in a variety of neurodevelopmental conditions including schizophrenia and Autism Spectrum Disorder (ASD).[1-5] Evidence is growing that the a- and b-wave amplitudes and timings of the electroretinogram are altered by conditions where depression and anxiety are common features.[6,7] These early parts of the electroretinogram waveform are shaped by the photoreceptors and bipolar cells and suggest the sensory distal retinal processes are atypical. Certainly, individuals with a diagnosis of ASD tend to show sensitivity to sensory visual, somatosensory or auditory stimuli in keeping with the electrophysiological findings.[8-10] The later part of the electroretinogram waveform is called the photopic negative response (PhNR) and has yet to be evaluated in ASD. The PhNR assesses the function of the proximal retina; the ganglion cells, amacrine cells and some glia.[11,12]

Retinal nerve fibre layer thickness change such as thinning is consistently reported in neurodegenerative conditions such as Alzheimer's disease[13] or with mild cognitive delay[14] which supports the model of using the retina as a portal into central nervous system structure and function.[6] The PhNR is reduced in conditions affecting the axons of the retinal ganglion cells such as ischemic optic neuropathy[15] and glaucoma[11,16] that are characterised by retinal ganglion cell loss.

There is some divergence as to whether the structure of the ganglion cell layer and the retinal nerve fibre layer are altered in ASD. Emberti Gialloreti et al.[17] reported the first optical coherence tomography structural profile of the retina in ASD. The authors found a significantly thinner retinal nerve fibre layer in the nasal quadrant between high functioning ASD adult group and controls.[17] In contrast, Garc[í]a-Medina et al.[18], studied the macular cube in young adults with ASD and found the total retinal thickness was increased due to thickening of the inner nuclear and plexiform layers, as well as the peripapillary nerve fibre layers in the inferior and nasal quadrants.[18]

There have been several reports in human[1-3] and mouse models[19-21] of ASD describing differences in the light- and dark-adapted full field electroretinograms in ASD, however, to date no studies have reported the PhNR responses. One study has investigated the pattern electroretinogram, which is a measure of ganglion cell function in the central visual field in ASD adults, but there were no significant differences compared to the control group.[22] The aim of this study was to determine if there were any differences in the PhNR functional measure of retina ganglion cells across the full field in a large cohort of children with a single diagnosis of ASD compared to an age matched cohort. A difference in the timing or amplitude of the PhNR would indicate atypical function in the proximal, inner retinal processes in ASD and may support the psychophysical findings of altered visual function in ASD[8-10,23] and structural changes noted using retinal imaging.[17,18]

METHODS

Participants

A total of 55 ASD and 87 control individuals took part with an age of mean ± standard deviation 13.6y ± 4.7 (range 5.4 to 26.7) and 14.0y ± 4.8 (range 5.4 to 27.3) (p=.17). The gender balance was skewed to a male prevalence in the ASD group with 75% (n=41) male compared to 49 % (n=43) in the control group, ([χ^2], p=.003) which is representative of the typical ASD population where there is a male bias. Children were recruited at two sites from existing databases or local autism groups and via social media. All ASD participants met diagnostic classification for ASD based on the Diagnostic and Statistical Manual-IV text revision or the Diagnostic Statistical Manual-5 on assessment with the Autism Diagnostic Observation Schedule or Autism Diagnostic Observation Schedule-2 and the Developmental, Dimensional and Diagnostic interview.[24] ASD children were assessed by paediatric psychiatrist or clinical psychologists in the social communication disorder clinics at Great Ormond Street Hospital for Children in the United Kingdom or local Child and Adolescent Mental Health clinics in South Australia.

Children were excluded if there was a history of strabismus surgery or other syndromic or metabolic disorders or if there was any history of brain injury or co-morbid diagnosis such as attention deficit hyperactivity disorder or attention deficit disorder. All children were required to be able to follow simple verbal instructions, and with the Full-Scale intelligence quotient of the ASD group was largely in the normal range (mean 99 ± standard deviation 19; range 60 to 136) and ASD severity mean score of 6 ± standard deviation 2 calculated using the method of Gotham et al.[25] The ASD severity scores classify individuals based on diagnostic metrics into three bands with severity scores 1-3 representing non-spectrum autism characteristics, from 4-5 representing an ASD classification and scores of 6-10 representing an autism classification.[25] Parental consent was sought for children under the age of 16 years. The number of ASD participants who had taken a central nervous system acting medication of the day of the study was 9 (16%); one had taken Tegretol for epilepsy (seizure free for the last four years) and the remaining were using medications that targeted dopamine and serotonin levels.

This study was approved by the Flinders University Human Research Ethics Committee and the Human Ethics Committee at University College London. All procedures performed fulfilled the principles of the Declaration of Helsinki (1975), and written parental consent was obtained before participants took part in this study.

Electrophysiology

The recording protocol has been reported previously in more detail[1] and followed the International Society for Clinical Electrophysiology of Vision extended protocol guidelines for the PhNR.[26] All recordings were taken under normal room luminance with the participant seated comfortably. Briefly, white flashes of nine different flash strengths were presented in random sequence on a 30 cd.m^{-2} white background to the right and then left eye at 2 Hz with 60 averages per flash strength. A random nine step Troland protocol was used initially at the following flash strengths: -0.367, -0.119, 0.114, 0.398, 0.602, 0.799, 0.949, 1.114 and 1.204 log photopic cd.s.m^{-2} (See Supplementary Material for conversion table of flash strengths to Td.s). Traces were rejected from the average if they fell above or below the 25th centile. At the end of the sequences for the right and left eyes the International Society for Clinical Electrophysiology of Vision standard flash of log photopic 0.477 cd.s.m^{-2} on a 30 cd.m^{-2} white background at 2Hz was presented with 30 samples averaged from each eye to generate the waveform giving a total of ten flash strengths. Replicates of the recordings were made in each eye as required. The PhNR data, iris colour along with video and images of the electrode below the eye were exported using the extractor ver 2.9.4.1 (LKC Technologies Inc, Gaithersburg, MD, USA) so that differences in pigmentation in the groups[27] and electrode height[28] could be accounted for. If the electrode was positioned more than 4mm below the lower lid the data were not included in the sample.

The iris colour is an automated procedure performed by the RETeval. The calculation is based on the ratio of the 25th centile grey scale values of the pupil to the iris. Typical ranges were 1.10 for pale irises and 1.50 for very dark irises. The vertical height was taken from the photographic image recorded by the RETeval during the recording session. A graticule scaled relative to the electrode dimensions was used to determine the electrode position, (in millimetres) above or below the manufacturer's recommended placement of 2mm below the lower lid. It was not possible to be perfectly accurate to the first decimal place, but the scaling of electrode height provided an additional measure by which the amplitude could be adjusted to compensate for electrode position. All measurements were performed by one author who was unblinded. (See Supplementary Material for further information on these methods).

The mean iris colour and electrode heights were significantly different between the ASD and control groups (one-way analysis of variance) (p<.001) with a mean ± standard deviation of iris colour/electrode height (mm) of 1.20 ± 0.10 / 2.3 ± 0.8 and 1.26 ± 0.12 / 2.4 ± 0.8 respectively.

The amplitude of the PhNR was measured using two methods. The first, measured the amplitude at *t*= 72 ms (p_{72}) post stimulus onset from baseline to the waveform at this time point. The second measured the PhNR amplitude as the most negative point from the baseline in a time window of 55 and 95 ms, using the inbuilt RETeval algorithm and we report this amplitude and the time (Tmin) at which the PhNR occurred within the window as recommended.[29] In addition, the p-ratio which is equal to the PhNR amplitude at *t* = 72 ms divided by the b-wave amplitude as measured from baseline.[27] The w-ratio which is equal to the PhNR amplitude within the 55 to 95 ms time window divided by the b-wave amplitude measured from the baseline.

Only waveforms recorded from the right eye were included in the analysis and all waveforms were excluded if the a-wave was < 1 [μ]V. Where replicates were recorded within the eye the waveform with the largest b-wave amplitude was included in the analysis. There was no significant difference between right or left eye with respect to the b-wave amplitude (*p*=.27).

Statistics

Descriptive statistics were estimated via robust methods (see Mair and Wilcox).[30] The median (*Mdn*) and the median absolute deviation (*MAD*) were used to estimate the data's location and scale parameters, respectively. Other measures of dispersion such as the interquartile range (*IQR*) and 25th and 75th quantiles were also estimated. Approximate 95% confidence intervals around median values (*Mdn*) were computed with the formula $Mdn \pm 1.57[\bullet](IQR/n^{.5})$. Robust measures of skewness (sk_r) were estimated via the medcouple method (see Brys et al.).[31]

Inferential statistics were carried out via linear quantile mixed models. These models enable the effects of covariates on the dependent variable's quantiles while accounting for repeated measurements via random effects.[32] Although, linear quantile mixed models permit selecting one or several quantiles in the dependent variable, in this study the focus was on the .5 quantile; that is the median, as this is a robust estimator of location.[33] Median pairwise comparisons[30]) were then carried out where needed and, in the case of multiple comparisons, *p*-values were corrected via the false discovery rate method (p_{FDR}).

The original set of independent variables considered were (numeric variables are shown in italics): Flash strength (FS), Group (G), Vert: electrode height from lower lid margin (V), *Iris colour* (*I*), Central Nervous System medication taken on the day (M), Control with an ASD sibling (AS), Ethnicity (E), Gender (Ge), and *Age* (*A*). Linear quantile mixed models

including all main effects, and their potential *n*-way interactions would result in non-parsimonious explanatory models. Thus, to increase the parsimony of the models, a background-knowledge variable selection was performed (see Heinze et al.).[34] Different from a traditional statistical variable selection approach, background-knowledge variable selection requires the principal investigators to use their experience and knowledge of the topic to closely examine the variables' qualities such as their quality of measurement or relevance to the field, in order to rank the variables considered as the most relevant predictors.

In this study, background-knowledge variable selection was carried out in three steps: i) each expert (the main authors) ranked all the variables from the most important to the least important by thinking of the potential 'main effect' of each variable on the dependent variables; ii) the first 50% of the variables, (that is the first four variables in the current study) in each ranking list were retained; and, iii) after combining these new lists, the retained variables were sorted from the most common to the least common. The experts were not involved in steps ii) and iii). (See the Supplementary Material for detail of the background-knowledge variable selection methodology).

The variables selected were: FS, V, I, and G. These fixed-effect variables were entered additively, the only interaction considered was that between FS and G, and participants were entered as a random effect. The resulting model was therefore: DV ~ FS + V + I + G + FS[•]G; where DV is the dependent variable. The DVs considered were (all numeric): a-wave time (ms); a-wave amplitude ([μ]V); b-wave time (ms); b-wave amplitude ([μ]V); b-wave : a-wave ratio; PhNR at 72ms ([μ]V) (p_{72}); Tmin of PhNR (ms) (Tmin); PhNR at Tmin ([μ]V); p-ratio; and w-ratio.

p-values ([α] = .05) associated to the variables in each linear quantile mixed models and models' goodness of fit (via Akaike Information Criterion, the lower the Akaike Information Criterion the better the model's fit) are reported.

Data files for the study and R codes for the statistical methods are available at: https://figshare.com/projects/The_photopic_negative_response_in_Autism_Spectrum_Disorder/78798

RESULTS

Descriptive statistics of the dependent variables of interests for each of the groups are presented in Table 1 and the results of the linear quantile mixed models are displayed in Table 2.

_____Insert Tables 1 and 2 Near Here_____

While all other variables are held constant, a main effect of flash strength was observed in almost all dependent variables (except in Tmin of PhNR (ms) and w-ratio). For example, while there were differences between the PhNR at Tmin values of the flash strengths studied, no group differences were observed in this dependent variable (Figure 1). A main effect of 'Vert' was observed in three of the dependent variables; w-ratio, a-wave amplitude ([µ]V), and b-wave amplitude ([µ]V). A main effect of iris was observed in the variables a-wave time (ms) and b-wave time (ms) only. Finally, group differences and a significant interaction between G and FS emerged in the dependent variable b-wave amplitude ([µ]V) only (Figure 2).

The significant FS[•]G interaction was further examined by pairwise comparison of the two groups at each of the ten flash strengths. The corrected *p*-values indicated pairwise differences between ASD and control participants when the flash strength were log photopic 0.602 cd.s.m^{-2} (p_{FDR} =.03) and at log photopic 1.204 cd.s.m^{-2} (p_{FDR} =.03).

_____Insert Figures 1 and 2 Near Here_____

Figure 3 shows the comparison of the electroretinograms produced to flashes of log photopic 0.799 cd.s.m^{-2} from the ASD and control group. The PhNR descends from the b-wave apex typically below the zero [µ]V baseline. The amplitude of the PhNR was measured at 72ms (t72) and as the lowest amplitude between the horizontal arrows within the time window 55-95ms (Tmin). The b-wave amplitude from the individual with ASD (blue graph) is smaller than the control (red) example, but the PhNR amplitudes are similar. (For summary plots of all PhNR and electroretinogram parameters tested see Supplementary Material).

_____Insert Figure 3 Near Here_____

DISCUSSION

This is the first study to investigate the PhNR in children and young adults with ASD. There were no significant differences between case and comparison participants (p>.30). These findings suggest that the differences observed in optical coherence tomography at the macula, such as a thinned retinal nerve fibre layer or thicker inner plexiform layer, do not correspond with any functional deficit that is recorded from the whole retina as the summated signal from the ganglion cell layer. It may be that any structural deficits are localised to the macula region, but there is evidence for normal macular function based on the pattern electroretinogram, which suggests the ganglion cell layer is not affected in ASD.[22]

To date, the main electrophysiological findings in neurodevelopmental disorders have focused on the electroretinogram under dark- and light-adapted conditions.[1-5] As previously reported the electroretinogram b-wave was reduced across the flash strengths (p<.001) with a pair-wise comparison indicating significance at the peak and plateau of the photopic hill at log photopic 0.602 cd.s.m^{-2} and log photopic 1.204 cd.s.m^{-2} consistent with previous findings of reduced b-wave amplitudes at higher flash strengths.[1,2] In a small series of adult subjects with ASD the PhNR responses were also non -significant and the light-adapted b-wave amplitude was reduced at 0.5 log photopic cd.s.m^{-2} consistent with these findings in a younger but larger cohort.[2]

In previous studies, the main electroretinogram findings in adults and children with ASD have been reduced dark adapted and light adapted b-wave amplitudes.[1-3] Similar findings have been made in adults with schizophrenia, with reduced light-adapted a-wave and b-wave amplitudes.[4,5] However, unlike in ASD, a reduced PhNR is also found in schizophrenia suggesting there is a more global dysfunction in retinal signalling from photoreceptors to the ganglion cells[35] under light-adapted conditions in schizophrenia. There is an overlap in the genetic risk factors identified for ASD and schizophrenia and so it may be unsurprising that in these conditions, retinal signalling changes have been identified as a common feature.[36,37]

Recent developments of three murine models for ASD may help our understanding further by being able to explore functional and structural changes alongside specific genotypes. They also assist with identifying the key pathways and proteins implicated in the observed human electroretinogram waveforms. All models exhibit differences in the electroretinograms and structural changes to the retina, although the findings are not always consistent with those reported in the human studies to

date. Unfortunately, none of the models have reported the PhNR response characteristics to compare with the current study.[19-21]

One limitation of this study is that a red flash on a blue background which produces a larger PhNR amplitude was not used for the recordings.[11,26,38,39] However, as there was clearly no significant difference at a group level for any of the PhNR parameters it is unlikely that this factor would have affected the main outcomes. The optical coherence tomography findings of Emberti Gialloreti et al.[17] showed reduced retinal nerve fibre layer thickness only in the nasal quadrant. This limited area may not be sufficient to reduce the full field electroretinogram or demonstrate a functional loss in the PhNR. Similarly, the reduced overall retinal thickness in the macular cube reported by Garc[í]a-Medina et al.[18] might not be reflected in the full field PhNR retinal response. A further limitation of this study was the lack of optical coherence tomography data in these cohorts in order to evaluate the structural and functional findings. One important, finding is that in ASD adults there was no difference in visual acuity or contrast gain when measured with the pattern electroretinogram which support the notion that the macular region functions normally in ASD.[22]

In conclusion, the PhNR, a global measure of retinal ganglion cell function, does not differentiate case from control participants in this study, over the age range 5.4- 26.7 years. This finding implies that ganglion cell activity, summed over the whole retina, is not functionally different in young people with ASD. Previous research reported that the b-wave amplitude is reduced in ASD, indicating independence of the PhNR from the b-wave. A typical PhNR in combination with atypically reduced b-wave amplitude of the electroretinogram in ASD suggests the neurodevelopmental nature of this condition may be related to synaptogenesis between the photoreceptor and bipolar cells primarily.

These PhNR data taken together with pattern electroretinograms findings in ASD[22] and the apparent lack of any noticeable difference in PhNR in illustrative mouse model electroretinograms[19-21] suggests that the summed ganglion cell responses are not substantially affected in ASD. There may be more subtle changes of signal coding within the summed responses as demonstrated by altered sensitivity to contrast at higher spatial frequencies. These may be the result of amacrine cell interactions and detected more sensitively in oscillatory potentials.[40]

Further studies are required to quantify the responses within the electroretinogram such as the oscillatory potentials that may reveal differences that relate to the psychophysical differences

observed in ASD that underlie some of their sensory differences and performance on visual tasks.[9,10] The summed response of the retinal ganglion cell population recorded as the PhNR may conceal the components of retinal processing of contrast, motion and colour for example that may alter visual perception in ASD.

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TABLES

Table 1. Descriptive statistics of the measures in this study. The amplitude of the PhNR is represented by the most negative point occurring at Tmin (within the time window 55-95 ms) and the PhNR measured at 72 ms (p_{72}) after stimulus onset.

Dependent Variable	**Group**					
	ASD			*Control*		
	Mdn[‡] ± *MAD*[§] *[lower to upper limit]*	*IQR*[†] *[25th to 75th]*	sk_r[¶]	*Mdn* ± *MAD* *[lower to upper limit]*	*IQR* *[25th to 75th]*	sk_r
a-wave time (ms)	11.59 ± 1.34 [11.46-11.72]	1.87 [10.98-12.86]	.32	11.75 ± 1.35 [11.65-11.85]	1.84 [11.07-12.92]	.30
a-wave amplitude ([μ]V)	-5.70 ± 3.18 [-5.99-5.41]	4.28 [-7.91-3.62]	-.14	-6.75 ± 3.17 [-6.98-6.51]	4.24 [-9.17-4.93]	-.14
b-wave time (ms)	27.85 ± 3.22 [27.49-28.21]	5.27 [24.40-29.68]	-.28	27.60 ± 3.41 [27.33-27.88]	5.01 [24.36-29.38]	-.28
b-wave amplitude ([μ]V)	23.41 ± 11.35 [22.38-24.44]	15.13 [16.53-31.72]	.11	28.02 ± 11.40 [27.19-28.85]	15.20 [19.62-34.82]	-.04
b-wave:a-wave amplitude ratio	4.12 ± 1.47 [3.98-4.26]	1.99 [3.16-5.16]	.09	3.87 ± 1.28 [3.78-3.97]	1.76 [3.16-4.92]	.23
PhNR ([μ]V) at 72 ms (p_{72})	-5.25 ± 4.54 [-5.66-4.83]	6.05 [-8.25-2.17]	.006	-5.51 ± 3.67 [-5.78-5.24]	4.92 [-8.02-3.09]	-.06
Tmin (ms)	75.5 ± 20.58 [73.51-77.59]	29.95 [65.45-95.46]	.20	72.48 ± 17.69 [70.86-74.10]	29.74 [62.45-92.19]	.25
PhNR at Tmin ([μ]V)	-7.27 ± 4.54 [-7.69-6.85]	6.11 [-10.42-4.28]	-.05	-7.36 ± 3.98 [-7.66-7.06]	5.47 [-10.42-4.95]	-.14
p ratio	.28 ± .26	.35	.16	.26 ± .18	.26	.21

	[.26-.30]	[.13-.48]		[.25-.28]	[.16-.42]	
w ratio	1.04 ± .16 [1.03-1.06]	.22 [.95-1.17]	.18	1.02 ± .12 [1.01-1.02]	.16 [.94-1.10]	.13

[†]*IQR = Inter quartile range*

[‡]*Mdn = The median.*

[§]*MAD = The median absolute deviation.*

¶sk_r *=skewness*

Table 2. Results of linear quantile mixed models with selected independent variables applied to each of the dependent variables. Significant estimates and their *p*-values (in brackets) with $p<.05^{*}$, $p<.01^{**}$ and $p<.001^{***}$. *FS=Flash Strength, V=vertical height of electrode, I = iris colour index, G = Group,*

Independent Variables	**Dependent Variables**									
	Photopic Negative Response					*Electroretinogram*				
	PhNR at t72	*Tmin*	*PhNR at Tmin*	*p ratio*	*w ratio*	*a-wave time (ms)*	*a-wave amplitude ([μ]v)*	*b-wave time (ms)*	*b-wave amplitude ([μ]v)*	*b-wave to a-wave amplitude ratio*
FS	$-.55^{***}$ (<.001)	$5\ e^{-5}$ (.99)	$-.49^{***}$ (<.001)	$.01^{**}$ (.01)	-.002 (.63)	$-.26^{***}$ (<.001)	$-.55^{***}$ (<.001)	1.01^{***} (<.001))	1.23^{***} (<.001)	$-.12^{***}$ (<.001)
V	.32 (.13)	1.6 (.10)	.21 (.47)	-.005 (.78)	$.02^{**}$ (.003)	.004 (.93)	$.76^{***}$ (<.001)	.01 (.89)	-2.56^{***} (<.001)	.07 (.52)
I	-.47 (.82)	-5.8 (.43)	-.49 (.83)	.009 (.94)	-.04 (.57)	1.19^{***} (<.001)	-1.25 (.23)	1.57^{***} (<.001)	.91 (.80)	-.34 (.57)
G	-.32 (.51)	-3.7 (.16)	-.06 (.90)	.02 (.75)	-.02 (.36)	.35 (.07)	-.21 (.38)	-.20 (.07)	2.34^{*} (.04)	-.01 (.95)
FS[•]G	-.01 (.81)	.48 (.30)	-.07 (.44)	-.004 (.61)	-.002 (.64)	-.04 (.06)	-.11 (.12)	-.06 (.13)	$.35^{***}$ (<.001)	-.01 (.60)
AIC†	7416	11294	7610	741.6	-720.1	4318	5947	3952	9643	5172

†*AIC= Akaike Information Criterion with eight degrees of freedom.*

FIGURE CAPTIONS

Figure 1. Distribution of the PhNR at Tmin values for each group at different flash strengths in log photopic cd.s.m^{-2}.

Figure 2. Distribution of the b-wave amplitude values for each group at different flash strengths. The flash strengths in log photopic cd.s.m^{-2} at which significant group differences were observed (as indexed by corrected *p*-values) are shown in red font.

Figure 3 Figure 3 Representative trace from the ASD group (blue) and control group (red) showing the reduced b-wave amplitude but normal PhNR when measured from baseline to the trough with a flash strength of log photopic 0.799 cd.s.m^{-2} (6.3 photopic cd.s.m^{-2}). The amplitude of the PhNR at 72ms (p_{72}) was measured at t=72ms (t_{72}) and at the lowest amplitude at Tmin between the horizontal arrows within the time window of 55-95ms.

Title Page

The Photopic Negative Response in Autism Spectrum Disorder

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Clinical Relevance: To ascertain if the Photopic Negative Response of the electroretinogram is different in autism spectrum disorder as a potential clinical marker.

Background: Visual function can be atypical in autism spectrum disorder and structural imaging of the ganglion cell layers has been reported to differ in these individuals. Therefore, we sought to investigate if the photopic negative response of the full field electroretinograms, a measure of ganglion cell function, could help explain the visual perceptual differences in autism spectrum disorder and support the structural changes observed.

Methods: Participants (n=55 autism spectrum disorder, aged 5.4 to 26.7 years) and control (n=87, aged 5.4 to 27.3 years) were recruited for the study. Full field light-adapted electroretinograms using a Troland protocol with ten flash strengths from -0.367 to 1.204 log photopic cd.s.m^{-2} were recorded in each eye. The photopic negative response amplitudes at Tmin and at t=72ms were compared between groups along with the a- and b-wave values.

Results: There were no significant interactions between groups for the Photopic Negative Response measures of amplitude or time (p>.30). There was a group interaction between groups and flash strengths for the b-wave amplitude as previously reported (p<.001).

Conclusion: The photopic negative response results suggest that there are no significant differences in the summed retinal ganglion cell responses produced by a full field stimulus.

Keywords: Autism Spectrum Disorder; Photopic Negative Response; Electroretinogram; Ganglion cells.

The electroretinogram differs in a variety of neurodevelopmental conditions including schizophrenia and Autism Spectrum Disorder (ASD).[1-5] Evidence is growing that the a- and b-wave amplitudes and timings of the electroretinogram are altered by conditions where depression and anxiety are common features.[6,7] These early parts of the electroretinogram waveform are shaped by the photoreceptors and bipolar cells and suggest the sensory distal retinal processes are atypical. Certainly, individuals with a diagnosis of ASD tend to show sensitivity to sensory visual, somatosensory or auditory stimuli in keeping with the electrophysiological findings.[8-10] The later part of the electroretinogram waveform is called the photopic negative response (PhNR) and has yet to be evaluated in ASD. The PhNR assesses the function of the proximal retina; the ganglion cells, amacrine cells and some glia.[11,12]

Retinal nerve fibre layer thickness change such as thinning is consistently reported in neurodegenerative conditions such as Alzheimer's disease[13] or with mild cognitive delay[14] which supports the model of using the retina as a portal into central nervous system structure and function.[6] The PhNR is reduced in conditions affecting the axons of the retinal ganglion cells such as ischemic optic neuropathy[15] and glaucoma[11,16] that are characterised by retinal ganglion cell loss.

There is some divergence as to whether the structure of the ganglion cell layer and the retinal nerve fibre layer are altered in ASD. Emberti Gialloreti et al.[17] reported the first optical coherence tomography structural profile of the retina in ASD. The authors found a significantly thinner retinal nerve fibre layer in the nasal quadrant between high functioning ASD adult group and controls.[17] In contrast, Garc[í]a-Medina et al.[18], studied the macular cube in young adults with ASD and found the total retinal thickness was increased due to thickening of the inner nuclear and plexiform layers, as well as the peripapillary nerve fibre layers in the inferior and nasal quadrants.[18]

There have been several reports in human[1-3] and mouse models[19-21] of ASD describing differences in the light- and dark-adapted full field electroretinograms in ASD, however, to date no studies have reported the PhNR responses. One study has investigated the pattern electroretinogram, which is a measure of ganglion cell function in the central visual field in ASD adults, but there were no significant differences compared to the control group.[22] The aim of this study was to determine if there were any differences in the PhNR functional measure of retina ganglion cells across the full field in a large cohort of children with a single diagnosis of ASD compared to an age matched cohort. A difference in the timing or amplitude of the PhNR would indicate atypical function in the proximal, inner retinal processes in ASD and may support the psychophysical findings of altered visual function in ASD[8-10,23] and structural changes noted using retinal imaging.[17,18]

METHODS

Participants

A total of 55 ASD and 87 control individuals took part with an age of mean ± standard deviation 13.6y ± 4.7 (range 5.4 to 26.7) and 14.0y ± 4.8 (range 5.4 to 27.3) (p=.17). The gender balance was skewed to a male prevalence in the ASD group with 75% (n=41) male compared to 49 % (n=43) in the control group, ([χ^2], p=.003) which is representative of the typical ASD population where there is a male bias. Children were recruited at two sites from existing databases or local autism groups and via social media. All ASD participants met diagnostic classification for ASD based on the Diagnostic and Statistical Manual-IV text revision or the Diagnostic Statistical Manual-5 on assessment with the Autism Diagnostic Observation Schedule or Autism Diagnostic Observation Schedule-2 and the Developmental, Dimensional and Diagnostic interview.[24] ASD children were assessed by paediatric psychiatrist or clinical psychologists in the social communication disorder clinics at Great Ormond Street Hospital for Children in the United Kingdom or local Child and Adolescent Mental Health clinics in South Australia.

Children were excluded if there was a history of strabismus surgery or other syndromic or metabolic disorders or if there was any history of brain injury or co-morbid diagnosis such as attention deficit hyperactivity disorder or attention deficit disorder. All children were required to be able to follow simple verbal instructions, and with the Full-Scale intelligence quotient of the ASD group was largely in the normal range (mean 99 ± standard deviation 19; range 60 to 136) and ASD severity mean score of 6 ± standard deviation 2 calculated using the method of Gotham et al.[25] The ASD severity scores classify individuals based on diagnostic metrics into three bands with severity scores 1-3 representing non-spectrum autism characteristics, from 4-5 representing an ASD classification and scores of 6-10 representing an autism classification.[25] Parental consent was sought for children under the age of 16 years. The number of ASD participants who had taken a central nervous system acting medication of the day of the study was 9 (16%); one had taken Tegretol for epilepsy (seizure free for the last four years) and the remaining were using medications that targeted dopamine and serotonin levels.

This study was approved by the Flinders University Human Research Ethics Committee and the Human Ethics Committee at University College London. All procedures performed fulfilled the principles of the Declaration of Helsinki (1975), and written parental consent was obtained before participants took part in this study.

Electrophysiology

The recording protocol has been reported previously in more detail[1] and followed the International Society for Clinical Electrophysiology of Vision extended protocol guidelines for the PhNR.[26] All recordings were taken under normal room luminance with the participant seated comfortably. Briefly, white flashes of nine different flash strengths were presented in random sequence on a 30 cd.m^{-2} white background to the right and then left eye at 2 Hz with 60 averages per flash strength. A random nine step Troland protocol was used initially at the following flash strengths: -0.367, -0.119, 0.114, 0.398, 0.602, 0.799, 0.949, 1.114 and 1.204 log photopic cd.s.m^{-2} (See Supplementary Material for conversion table of flash strengths to Td.s). Traces were rejected from the average if they fell above or below the 25th centile. At the end of the sequences for the right and left eyes the International Society for Clinical Electrophysiology of Vision standard flash of log photopic 0.477 cd.s.m^{-2} on a 30 cd.m^{-2} white background at 2Hz was presented with 30 samples averaged from each eye to generate the waveform giving a total of ten flash strengths. Replicates of the recordings were made in each eye as required. The PhNR data, iris colour along with video and images of the electrode below the eye were exported using the extractor ver 2.9.4.1 (LKC Technologies Inc, Gaithersburg, MD, USA) so that differences in pigmentation in the groups[27] and electrode height[28] could be accounted for. If the electrode was positioned more than 4mm below the lower lid the data were not included in the sample.

The iris colour is an automated procedure performed by the RETeval. The calculation is based on the ratio of the 25th centile grey scale values of the pupil to the iris. Typical ranges were 1.10 for pale irises and 1.50 for very dark irises. The vertical height was taken from the photographic image recorded by the RETeval during the recording session. A graticule scaled relative to the electrode dimensions was used to determine the electrode position, (in millimetres) above or below the manufacturer's recommended placement of 2mm below the lower lid. It was not possible to be perfectly accurate to the first decimal place, but the scaling of electrode height provided an additional measure by which the amplitude could be adjusted to compensate for electrode position. All measurements were performed by one author who was unblinded. (See Supplementary Material for further information on these methods).

The mean iris colour and electrode heights were significantly different between the ASD and control groups (one-way analysis of variance) (p<.001) with a mean ± standard deviation of iris colour/electrode height (mm) of 1.20 ± 0.10 / 2.3 ± 0.8 and 1.26 ± 0.12 / 2.4 ± 0.8 respectively.

The amplitude of the PhNR was measured using two methods. The first, measured the amplitude at *t*= 72 ms (p_{72}) post stimulus onset from baseline to the waveform at this time point. The second measured the PhNR amplitude as the most negative point from the baseline in a time window of 55 and 95 ms, using the inbuilt RETeval algorithm and we report this amplitude and the time (Tmin) at which the PhNR occurred within the window as recommended.[29] In addition, the p-ratio which is equal to the PhNR amplitude at *t* = 72 ms divided by the b-wave amplitude as measured from baseline.[27] The w-ratio which is equal to the PhNR amplitude within the 55 to 95 ms time window divided by the b-wave amplitude measured from the baseline.

Only waveforms recorded from the right eye were included in the analysis and all waveforms were excluded if the a-wave was < 1 [μ]V. Where replicates were recorded within the eye the waveform with the largest b-wave amplitude was included in the analysis. There was no significant difference between right or left eye with respect to the b-wave amplitude (*p*=.27).

Statistics

Descriptive statistics were estimated via robust methods (see Mair and Wilcox).[30] The median (*Mdn*) and the median absolute deviation (*MAD*) were used to estimate the data's location and scale parameters, respectively. Other measures of dispersion such as the interquartile range (*IQR*) and 25th and 75th quantiles were also estimated. Approximate 95% confidence intervals around median values (*Mdn*) were computed with the formula $Mdn \pm 1.57[\bullet](IQR/n^{.5})$. Robust measures of skewness (sk_r) were estimated via the medcouple method (see Brys et al.).[31]

Inferential statistics were carried out via linear quantile mixed models. These models enable the effects of covariates on the dependent variable's quantiles while accounting for repeated measurements via random effects.[32] Although, linear quantile mixed models permit selecting one or several quantiles in the dependent variable, in this study the focus was on the .5 quantile; that is the median, as this is a robust estimator of location.[33] Median pairwise comparisons[30]) were then carried out where needed and, in the case of multiple comparisons, *p*-values were corrected via the false discovery rate method (p_{FDR}).

The original set of independent variables considered were (numeric variables are shown in italics): Flash strength (FS), Group (G), Vert: electrode height from lower lid margin (V), *Iris colour* (*I*), Central Nervous System medication taken on the day (M), Control with an ASD sibling (AS), Ethnicity (E), Gender (Ge), and *Age* (*A*). Linear quantile mixed models

including all main effects, and their potential *n*-way interactions would result in non-parsimonious explanatory models. Thus, to increase the parsimony of the models, a background-knowledge variable selection was performed (see Heinze et al.).[34] Different from a traditional statistical variable selection approach, background-knowledge variable selection requires the principal investigators to use their experience and knowledge of the topic to closely examine the variables' qualities such as their quality of measurement or relevance to the field, in order to rank the variables considered as the most relevant predictors.

In this study, background-knowledge variable selection was carried out in three steps: i) each expert (the main authors) ranked all the variables from the most important to the least important by thinking of the potential 'main effect' of each variable on the dependent variables; ii) the first 50% of the variables, (that is the first four variables in the current study) in each ranking list were retained; and, iii) after combining these new lists, the retained variables were sorted from the most common to the least common. The experts were not involved in steps ii) and iii). (See the Supplementary Material for detail of the background-knowledge variable selection methodology).

The variables selected were: FS, V, I, and G. These fixed-effect variables were entered additively, the only interaction considered was that between FS and G, and participants were entered as a random effect. The resulting model was therefore: DV ~ FS + V + I + G + FS[•]G; where DV is the dependent variable. The DVs considered were (all numeric): a-wave time (ms); a-wave amplitude ([μ]V); b-wave time (ms); b-wave amplitude ([μ]V); b-wave : a-wave ratio; PhNR at 72ms ([μ]V) (p_{72}); Tmin of PhNR (ms) (Tmin); PhNR at Tmin ([μ]V); p-ratio; and w-ratio.

p-values ([α] = .05) associated to the variables in each linear quantile mixed models and models' goodness of fit (via Akaike Information Criterion, the lower the Akaike Information Criterion the better the model's fit) are reported.

Data files for the study and R codes for the statistical methods are available at: https://figshare.com/projects/The_photopic_negative_response_in_Autism_Spectrum_Disorder/78798

RESULTS

Descriptive statistics of the dependent variables of interests for each of the groups are presented in Table 1 and the results of the linear quantile mixed models are displayed in Table 2.

_____Insert Tables 1 and 2 Near Here_____

While all other variables are held constant, a main effect of flash strength was observed in almost all dependent variables (except in Tmin of PhNR (ms) and w-ratio). For example, while there were differences between the PhNR at Tmin values of the flash strengths studied, no group differences were observed in this dependent variable (Figure 1). A main effect of 'Vert' was observed in three of the dependent variables; w-ratio, a-wave amplitude ([µ]V), and b-wave amplitude ([µ]V). A main effect of iris was observed in the variables a-wave time (ms) and b-wave time (ms) only. Finally, group differences and a significant interaction between G and FS emerged in the dependent variable b-wave amplitude ([µ]V) only (Figure 2).

The significant FS[•]G interaction was further examined by pairwise comparison of the two groups at each of the ten flash strengths. The corrected *p*-values indicated pairwise differences between ASD and control participants when the flash strength were log photopic 0.602 cd.s.m^{-2} (p_{FDR} =.03) and at log photopic 1.204 cd.s.m^{-2} (p_{FDR} =.03).

_____Insert Figures 1 and 2 Near Here_____

Figure 3 shows the comparison of the electroretinograms produced to flashes of log photopic 0.799 cd.s.m^{-2} from the ASD and control group. The PhNR descends from the b-wave apex typically below the zero [µ]V baseline. The amplitude of the PhNR was measured at 72ms (t72) and as the lowest amplitude between the horizontal arrows within the time window 55-95ms (Tmin). The b-wave amplitude from the individual with ASD (blue graph) is smaller than the control (red) example, but the PhNR amplitudes are similar. (For summary plots of all PhNR and electroretinogram parameters tested see Supplementary Material).

_____Insert Figure 3 Near Here_____

DISCUSSION

This is the first study to investigate the PhNR in children and young adults with ASD. ~~The PhNR was not able to detect any~~There were no significant differences between case and comparison participants (p>.30). These findings suggest that ~~there are no functional deficits that relate to~~the differences observed in optical coherence tomography at the macula, such as a thinned retinal nerve fibre layer or thicker inner plexiform layer, do not correspond with any functional deficit that is recorded from the whole retina as the summated signal from the ganglion cell layer. It may be that any structural deficits are localised to the macula region, but there is evidence for normal macular function based on the ~~profile of the~~ pattern electroretinogram ~~responses to contrast and visual acuity~~, which suggests the ganglion cell layer is not affected in ASD.[22]

To date, the main electrophysiological findings in neurodevelopmental disorders have focused on the electroretinogram under dark- and light-adapted conditions.[1-5] As previously reported the electroretinogram b-wave was reduced across the flash strengths (p<.001) with a pair-wise comparison indicating significance at the peak and plateau of the photopic hill at log photopic 0.602 cd.s.m^{-2} and log photopic 1.204 cd.s.m^{-2} consistent with previous findings of reduced b-wave amplitudes at higher flash strengths.[1,2] In a small series of adult subjects with ASD the PhNR responses were also non -significant and the light-adapted b-wave amplitude was reduced at 0.5 log photopic cd.s.m^{-2} consistent with these findings in a younger but larger cohort.[2]

In previous studies, the main electroretinogram findings in adults and children with ASD have been reduced dark adapted and light adapted b-wave amplitudes.[1-3] Similar findings have been made in adults with schizophrenia, with reduced light-adapted a-wave and b-wave amplitudes.[4,5] However, unlike in ASD, a reduced PhNR is also found in schizophrenia suggesting there is a more global dysfunction in retinal signalling from photoreceptors to the ganglion cells[35] under light-adapted conditions in schizophrenia. There is an overlap in the genetic risk factors identified for ASD and schizophrenia and so it may be unsurprising that in these conditions, retinal signalling changes have been identified as a common feature.[36,37]

Recent developments of three murine models for ASD may help our understanding further by being able to explore functional and structural changes alongside specific genotypes. They also assist with identifying the key pathways and proteins implicated in the observed human electroretinogram waveforms. All models exhibit differences in the electroretinograms and structural changes to the retina, although the findings are not always consistent with those reported in the human studies to

date. Unfortunately, none of the models have reported the PhNR response characteristics to compare with the current study.[19-21]

One limitation of this study is that a red flash on a blue background which produces a larger PhNR amplitude was not used for the recordings.[11,26,38,39] However, as there was clearly no significant difference at a group level for any of the PhNR parameters it is unlikely that this factor would have affected the main outcomes. The optical coherence tomography findings of Emberti Gialloreti et al.[17] showed reduced retinal nerve fibre layer thickness only in the nasal quadrant. This limited area may not be sufficient to reduce the full field electroretinogram or demonstrate a functional loss in the PhNR. Similarly, the reduced overall retinal thickness in the macular cube reported by Garc[í]a-Medina et al.[18] might not be reflected in the full field PhNR retinal response. ~~A further limitation of this study was the lack of optical coherence tomography data in these cohorts in order to evaluate the functional findings reported here.~~ One important, finding is that in ASD adults there was no difference in visual acuity or contrast gain when measured with the pattern electroretinogram which support the notion that the macular region functions normally in ASD.[22]

In conclusion, the PhNR, a global measure of retinal ganglion cell function, does not differentiate case from control participants in this study, over the age range 5.4- 26.7 years. This finding implies that ganglion cell activity, summed over the whole retina, is not functionally different in young people with ASD. Previous research reported that the b-wave amplitude is reduced in ASD, indicating independence of the PhNR from the b-wave. A typical PhNR in combination with atypically reduced b-wave amplitude of the electroretinogram in ASD suggests the neurodevelopmental nature of this condition may be related to synaptogenesis between the photoreceptor and bipolar cells primarily.

These PhNR data taken together with pattern electroretinograms findings in ASD[22] and the apparent lack of any noticeable difference in PhNR in illustrative mouse model electroretinograms[19-21] suggests that the summed ganglion cell responses are not substantially affected in ASD. There may be more subtle changes of signal coding within the summed responses as demonstrated by altered sensitivity to contrast at higher spatial frequencies. These may be the result of amacrine cell interactions and detected more sensitively in oscillatory potentials.[40]

Further studies are required to quantify the responses within the electroretinogram such as the oscillatory potentials that may reveal differences that relate to the psychophysical differences

observed in ASD that underlie some of their sensory differences and performance on visual tasks.[9,10] The summed response of the retinal ganglion cell population recorded as the PhNR may conceal the components of retinal processing of contrast, motion and colour for example that may alter visual perception in ASD. ~~The PhNR was unable to reveal a significant group difference between ASD and control participants and additional studies using the multifocal electroretinogram with optical coherence tomography maybe able to construct a more accurate picture of any anatomical changes at the macula with localised electrophysiological measurements.~~

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TABLES

Table 1. Descriptive statistics of the measures in this study. The amplitude of the PhNR is represented by the most negative point occurring at Tmin (within the time window 55-95 ms) and the PhNR measured at 72 ms (p_{72}) after stimulus onset.

Dependent Variable	**Group**					
	ASD			*Control*		
	Mdn[‡] ± *MAD*[§] *[lower to upper limit]*	*IQR*[†] *[25th to 75th]*	sk_r[¶]	*Mdn* ± *MAD* *[lower to upper limit]*	*IQR* *[25th to 75th]*	sk_r
a-wave time (ms)	11.59 ± 1.34 [11.46-11.72]	1.87 [10.98-12.86]	.32	11.75 ± 1.35 [11.65-11.85]	1.84 [11.07-12.92]	.30
a-wave amplitude ([μ]V)	-5.70 ± 3.18 [-5.99-5.41]	4.28 [-7.91-3.62]	-.14	-6.75 ± 3.17 [-6.98-6.51]	4.24 [-9.17-4.93]	-.14
b-wave time (ms)	27.85 ± 3.22 [27.49-28.21]	5.27 [24.40-29.68]	-.28	27.60 ± 3.41 [27.33-27.88]	5.01 [24.36-29.38]	-.28
b-wave amplitude ([μ]V)	23.41 ± 11.35 [22.38-24.44]	15.13 [16.53-31.72]	.11	28.02 ± 11.40 [27.19-28.85]	15.20 [19.62-34.82]	-.04
b-wave:a-wave amplitude ratio	4.12 ± 1.47 [3.98-4.26]	1.99 [3.16-5.16]	.09	3.87 ± 1.28 [3.78-3.97]	1.76 [3.16-4.92]	.23
PhNR ([μ]V) at 72 ms (p_{72})	-5.25 ± 4.54 [-5.66-4.83]	6.05 [-8.25-2.17]	.006	-5.51 ± 3.67 [-5.78-5.24]	4.92 [-8.02-3.09]	-.06
Tmin (ms)	75.5 ± 20.58 [73.51-77.59]	29.95 [65.45-95.46]	.20	72.48 ± 17.69 [70.86-74.10]	29.74 [62.45-92.19]	.25
PhNR at Tmin ([μ]V)	-7.27 ± 4.54 [-7.69-6.85]	6.11 [-10.42-4.28]	-.05	-7.36 ± 3.98 [-7.66-7.06]	5.47 [-10.42-4.95]	-.14
p ratio	.28 ± .26	.35	.16	.26 ± .18	.26	.21

	[.26-.30]	[.13-.48]		[.25-.28]	[.16-.42]	
w ratio	1.04 ± .16 [1.03-1.06]	.22 [.95-1.17]	.18	1.02 ± .12 [1.01-1.02]	.16 [.94-1.10]	.13

[†]*IQR = Inter quartile range*

[‡]*Mdn = The median.*

[§]*MAD = The median absolute deviation.*

[¶]sk_r *=skewness*

Table 2. Results of linear quantile mixed models with selected independent variables applied to each of the dependent variables. Significant estimates and their *p*-values (in brackets) with $p<.05^{*}$, $p<.01^{**}$ and $p<.001^{***}$. *FS=Flash Strength, V=vertical height of electrode, I = iris colour index, G = Group,*

Independent Variables	**Dependent Variables**									
	Photopic Negative Response					*Electroretinogram*				
	PhNR at t72	*Tmin*	*PhNR at Tmin*	*p ratio*	*w ratio*	*a-wave time (ms)*	*a-wave amplitude ([μ]v)*	*b-wave time (ms)*	*b-wave amplitude ([μ]v)*	*b-wave to a-wave amplitude ratio*
FS	$-.55^{***}$ (<.001)	$5\ e^{-5}$ (.99)	$-.49^{***}$ (<.001)	$.01^{**}$ (.01)	-.002 (.63)	$-.26^{***}$ (<.001)	$-.55^{***}$ (<.001)	1.01^{***} (<.001))	1.23^{***} (<.001)	$-.12^{***}$ (<.001)
V	.32 (.13)	1.6 (.10)	.21 (.47)	-.005 (.78)	$.02^{**}$ (.003)	.004 (.93)	$.76^{***}$ (<.001)	.01 (.89)	-2.56^{***} (<.001)	.07 (.52)
I	-.47 (.82)	-5.8 (.43)	-.49 (.83)	.009 (.94)	-.04 (.57)	1.19^{***} (<.001)	-1.25 (.23)	1.57^{***} (<.001)	.91 (.80)	-.34 (.57)
G	-.32 (.51)	-3.7 (.16)	-.06 (.90)	.02 (.75)	-.02 (.36)	.35 (.07)	-.21 (.38)	-.20 (.07)	2.34^{*} (.04)	-.01 (.95)
FS[•] G	-.01 (.81)	.48 (.30)	-.07 (.44)	-.004 (.61)	-.002 (.64)	-.04 (.06)	-.11 (.12)	-.06 (.13)	$.35^{***}$ (<.001)	-.01 (.60)
AIC†	7416	11294	7610	741.6	-720.1	4318	5947	3952	9643	5172

†*AIC= Akaike Information Criterion with eight degrees of freedom.*

FIGURE CAPTIONS

Figure 1. Distribution of the PhNR at Tmin values for each group at different flash strengths in log photopic cd.s.m^{-2}.

Figure 2. Distribution of the b-wave amplitude values for each group at different flash strengths. The flash strengths in log photopic cd.s.m^{-2} at which significant group differences were observed (as indexed by corrected *p*-values) are shown in red font.

Figure 3 Figure 3 Representative trace from the ASD group (blue) and control group (red) showing the reduced b-wave amplitude but normal PhNR when measured from baseline to the trough with a flash strength of log photopic 0.799 cd.s.m^{-2} (6.3 photopic cd.s.m^{-2}). The amplitude of the PhNR at 72ms (p_{72}) was measured at t=72ms (t_{72}) and at the lowest amplitude at Tmin between the horizontal arrows within the time window of 55-95ms.

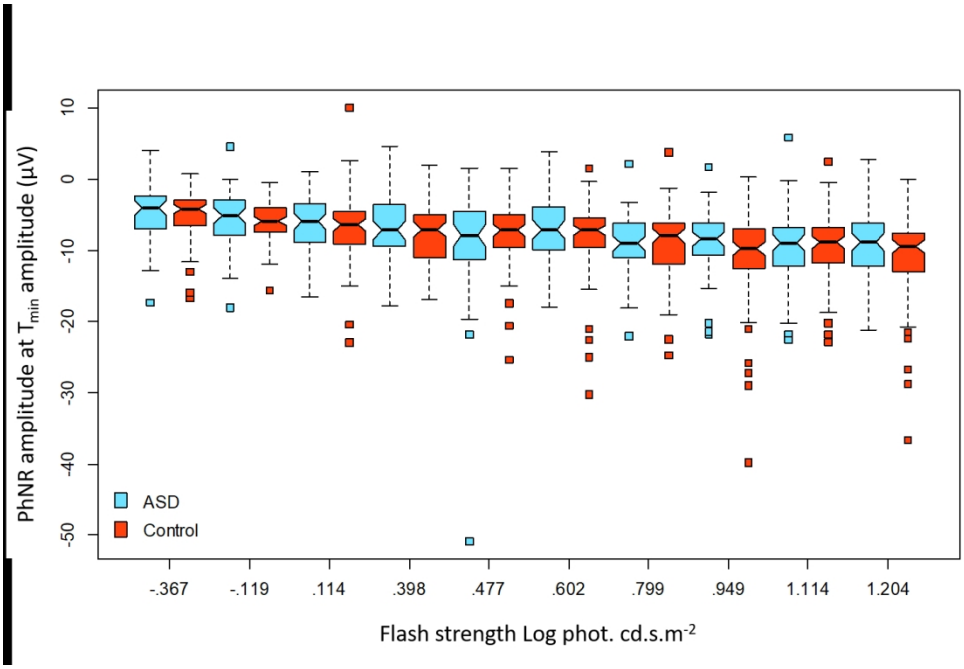

Figure 1. Distribution of the PhNR at Tmin values for each group at different flash strengths in log photopic cd.s.m^{-2}.

263x174mm (150 x 150 DPI)

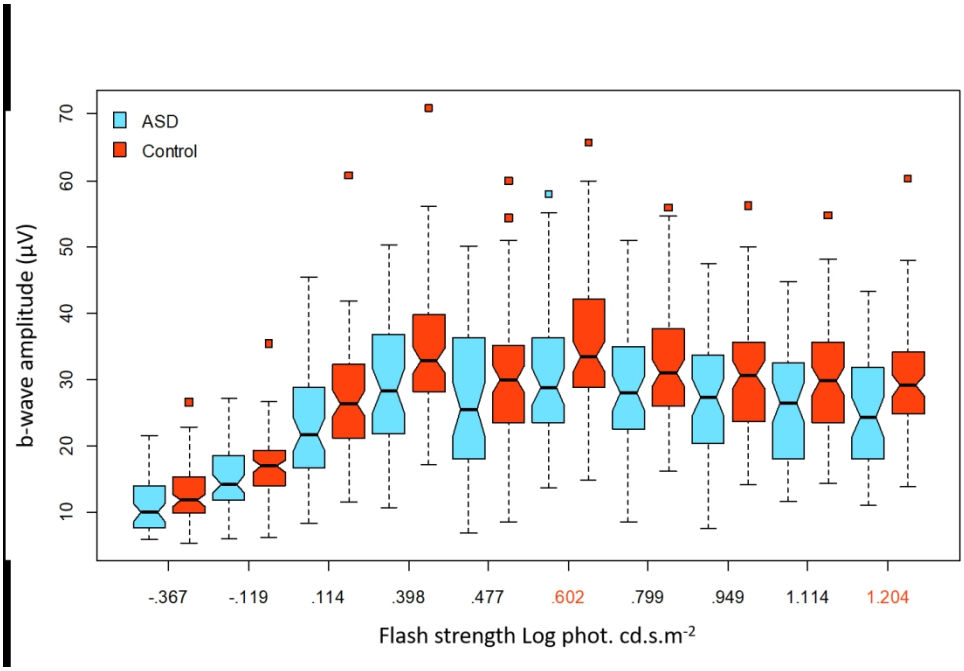

Figure 2. Distribution of the b-wave amplitude values for each group at different flash strengths. The flash strengths in log photopic cd.s.m^{-2} at which significant group differences were observed (as indexed by corrected p-values) are shown in red font.

263x174mm (150 x 150 DPI)

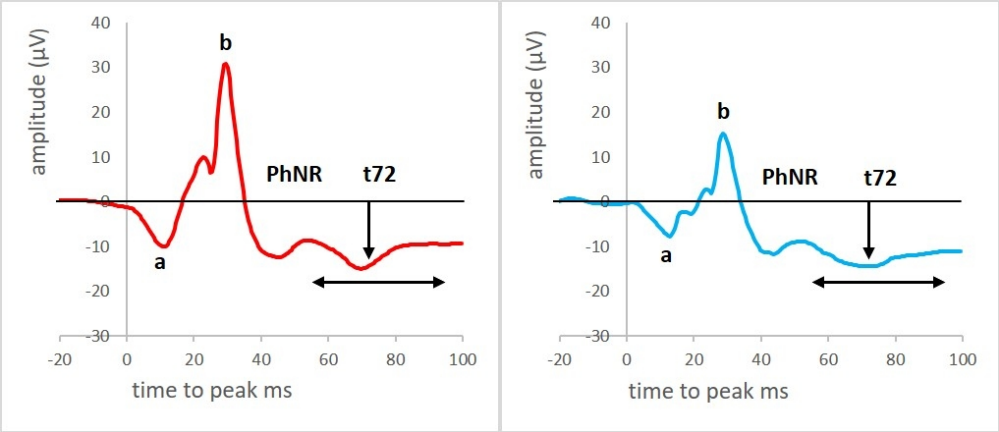

Figure 3 Representative trace from the ASD group (blue) and control group (red) showing the reduced b-wave amplitude but normal PhNR when measured from baseline to the trough with a flash strength of log photopic 0.799 cd.s.m^{-2} (6.3 photopic cd.s.m^{-2}). The amplitude of the PhNR at 72ms (p_{72}) was measured at t=72ms (t72) and at the lowest amplitude at Tmin between the horizontal arrows within the time window of 55-95ms.

172x74mm (150 x 150 DPI)

Supplementary Material

The Photopic Negative Response in Autism Spectrum Disorder

Contents

Background-knowledge variable selection (BKVS) process

- *Principal investigators (PIs) experts in the field*: Paul Constable (P.C.), Irene Lee (I.L.), and Dorothy Thompson (D.T.)
- *Variables considered*: Flash strength (FS), Group (G), Vert (V), Iris colour (I), CNS med take (M), ASD sibling (AS), Ethnicity (E), Gender (Ge), and Age (A).
- *PIs' rationale*:
    - P.C.:

FS: Owing to the 1 x log range on flash strengths the interaction with the ERG waveform parameter amplitudes will be most significantly affected by FS.

G: A group effect on the ERG b-wave and a-wave amplitudes has previously been shown [1,2] and as such a group effect is expected in these parameters.

V: Electrode height is critical to the measured amplitude of the ERG waveform as previously shown [3].

I: Iris colour or pigmentation of the choroid reduced the amplitude of the ERG waveform under dark and more significantly under light-adapted conditions and so iris colour would have an effect of the measured amplitudes [4].

M: A drug targeting the CNS has the potential to alter the underlying neurochemistry of the retina.

AS: Given the heterogeneity of the genetics associated with ASD it is unlikely that a strong genetic trait will exist that modifies the ERG waveform within a family [5].

E: Similar to the association of a sibling of an individual with ASD the varied genetic background amongst ethnic groups means that it is unlikely that there would be a single strong genetic factor based on ethnic background.

Ge: In one study a higher 30 Hz flicker amplitude has been shown in females. However, in this study the 30 Hz was not investigated and so it is unknown if gender affects the single flash ERG waveform [6].

Age: No real impact as all participants are below 27 years of age and so there are unlikely to be reductions in the amplitudes owing to differences in optical transmission.

- I.L.:

FS: If all other factors are unchanged, light strength will produce the widest range of ERG amplitudes.

V: Hobby et al [3] and our practical experience have shown the important effect of the electrode position on ERG results.

G: Constable et al 2016 [1] and 2020 [2] show the significant differences between ASD and control individuals. Other observations of a larger effect on another group (unpublished) also support the importance of this variable.

I: A darker iris absorbs light, resulting in a smaller amplitude of the ERG waveform when compared to lighter irises, so iris colour is a significant variable affecting the ERG results [4].

E: Ethnicity is linked to iris colour, however, as Caucasians can have light or dark iris colouration, ethnicity has a lesser effect on ERG amplitudes.

M: Medication can have effects on ERG amplitudes.

Ge: Females have higher ERG amplitudes than males in a study [6].

AS: In this study, ERG amplitudes of ASD siblings are similar to that of typical-developed controls.

Age: No significant changes of ERG amplitudes have been observed in this study.

- D.T.:

As to the variables and their impact on the b-wave variable:

FS: Flash strength has strong, but predictable, effects on the amplitude and time to peak of the ERG as evidenced by many published LA photopic hill and the DA ERG luminance response series.

G: We expect a group effect on LA b-wave amplitude as previously reported.

V: Based on the findings of Hobby et al (2018) [3] with lower amplitudes recorded when the electrode position is more than 2mm below the lower lid as recommended by the manufacturers. Care in the positioning of skin ERGs is long recognised e.g. Kriss A (1994) [7].

I and E: Darker irises give smaller b-waves [4] and as pigmentation is associated with ethnicity then these two factors are important.

AS: Siblings show a high level of association of amplitude, e.g. monozygotic and dizygotic twin pairs with ISCEV ffERGs twins the highest heritability was found for the photopic single-flash a-wave and b-wave amplitudes,~ 85% hereditability, including PhNR, when normalised on b-wave and i-wave [8].

Ge: Kato et al (2017) [6] found larger amplitudes for the 30Hz ERG in females, but others have not replicated this in their populations [9].

M: medications and drugs may alter the ERG, for example inhalation anaesthesia can reduce the b-wave by 50%, but ERGs in this study were carried out in alert children and very few took any medications.

Age: Minimal impact in our study age groups – babies and infants have with immature mall ERGs, but the LA ERGs amplitudes are adult like by 5yrs, peak times adult like by 6months of age. When subjects are aged 55yrs+ amplitude begins to reduce and is approximately 25% to 40% smaller in over 75yr olds – between these ages little variation is reported. [10-14].

- *Results*

PIs were asked to sort the variables from the most to the least important by thinking of the potential 'main effect' of each variable on the dependent variables (i.e. PIs did not have to consider the importance of potential interactions). The only interaction the PIs expressed interest on was that between FS and G. This interaction enables to know at which FS there are any significant differences between Gs. The results of the PIs ranking are shown below.

Rank	**PIs**		
	P.C.	*I.L.*	*D.T.*
1	FS	FS	FS
2	G	V	V
3	V	G	E
4	I	I	I
5	M	E	AS
6	AS	M	Ge
7	E	Ge	M
8	Ge	AS	A

9	A	A	G

Note: rank: 1=most important; 9=least important. The first 4 variables (i.e. the first ~50% of the variables) were sorted by their modes; i.e. FS (3), V (3), I (3), G (2), E (1). See R code for automating this BKVS.

After extra discussions among the PIs, it was decided to remove E from the final model. The model examined was thus DV ~ FS + V + I + G + FS•G.

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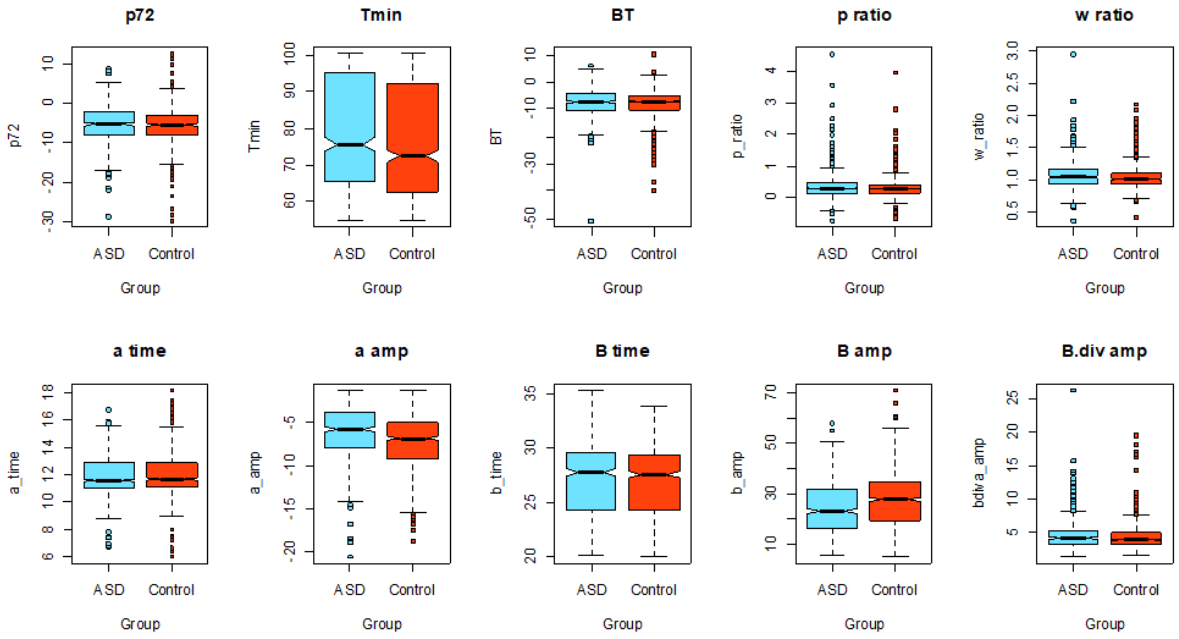
Overall results

No significant group x flash strength differences were observed for the a- wave time to peak (p=.06), amplitude (p=.12, the b-wave time to peak (p=.13) or the b:a wave amplitude ratio (p=.60) or the PhNR parameters p>.30. The interaction for b-wave amplitude was significant p<.001). Summary plots are shown below.

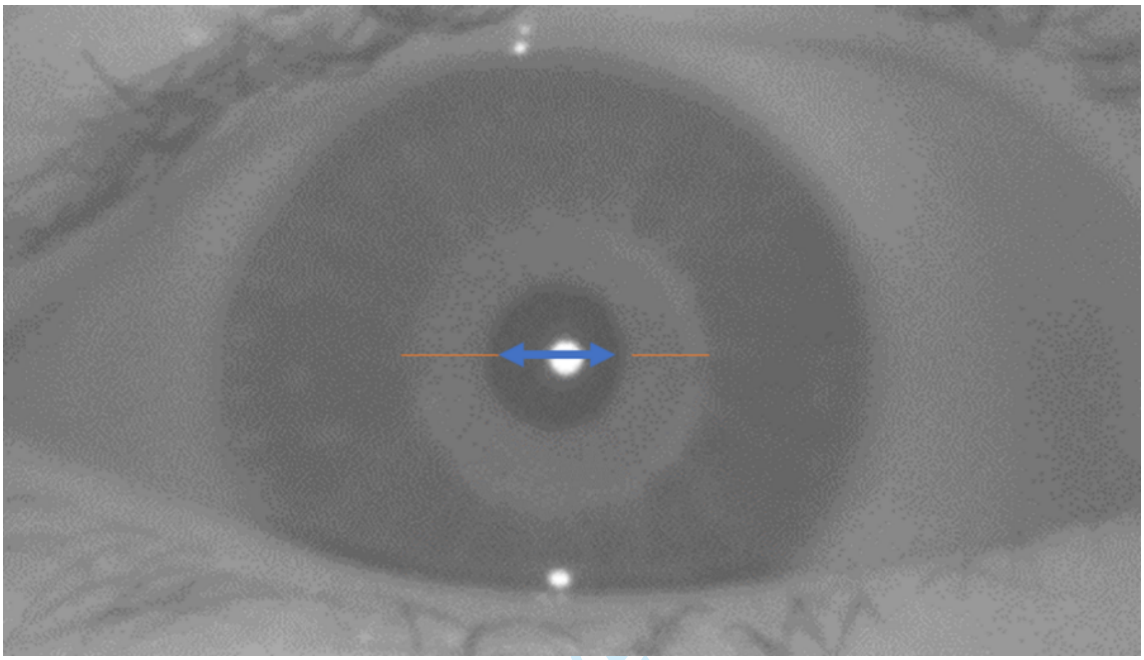


Group differences for all ten flash strengths for the PhNR (upper row) and ERG parameters (lower row).

Iris Colour

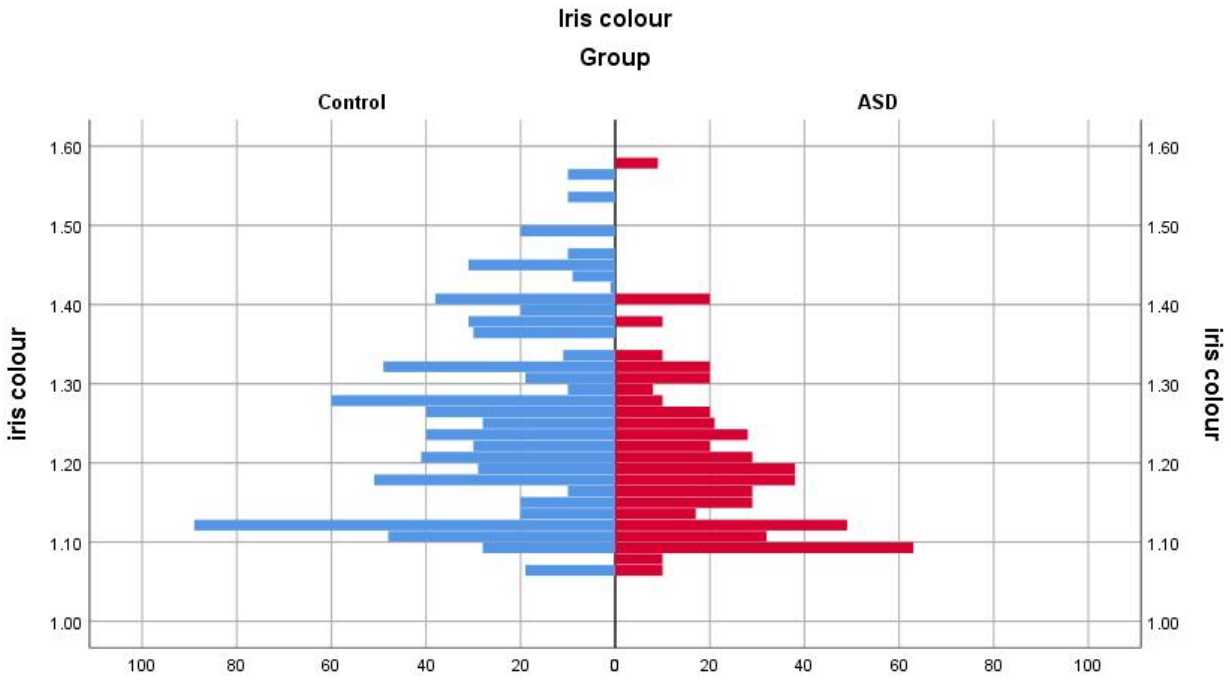
The iris colour ratio or index is an automated procedure performed by the RETeval to estimate the pigmentation of the iris so that iris colour can be accounted for in the statistical analyses (3). The calculation is based on the image acquired by the RETeval during the recording and is the ratio of the 25th centile grey scale values in 1mm segments at the 3 and 9 o'clock position relative to the 25th centile grey scale value of the pupil. The figure below illustrates the image and line segments used for the calculation. The iris colour index has no units and typically varies from 1.10 for pale irises to 1.50 for dark irises.

The ratio is extracted from the video taken of the eye during a Troland protocol recording by the RETeval. The ratio is an estimate of the colour of the iris, where darker irises appear brighter in the infrared and therefore have a larger iris/pupil colour ratio. Because of the automatic gain control of the exposure in the RETeval, the absolute values for the iris and pupil colour are not meaningful.



The iris colour is the 25th percentile of the grey values found in the two 1 mm long horizontal line segments starting at the left and the right edges of the pupil cantered vertically. The pupil colour is the 25th percentile of the grey values across the horizontal diameter of the pupil, excluding saturated values. Orange bars represent the 2 x 1mm line segments and blue arrow the diameter of the pupil. Image is in the infrared and extracted from the video recorded during each recording.

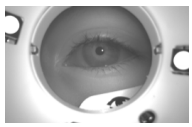
The distribution of iris colours is shown below for the participants in this study. The ASD group has slightly lighter irises. 1.20 ± 0.10 compared to 1.26 ± 0.12 for the control group.



Distribution of iris colour for the ASD and Control Group.

Electrode Position

The electrode position with respect to the lower lid margin was determined using the photographic images of the eye taken by the REТeval at the time of recording, A graticule, that was created by scaling the millimetre grid to the actual size of the electrode was overlaid across the photograph to determine the vertical height of the upper edge of the electrode to the lower lid margin. If the height was more than 4mm the recordings for that eye were discarded. A nominal scale of 1-4 was used to adjust the amplitudes recorded with the height of the electrode with a reference level of 2 set to zero as 2mm is the recommended height for the electrode placement. A series of images and the scaled graticule are shown below.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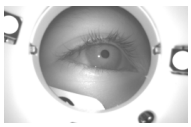
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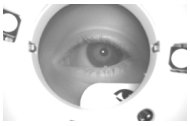
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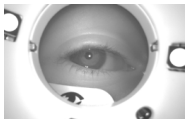
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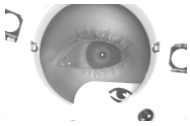
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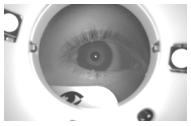
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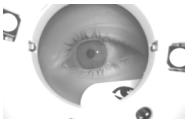
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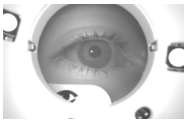
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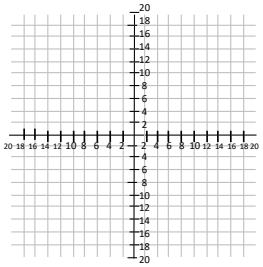

Electrode height was assessed using the graticule scaled to the electrode's dimensions.

Conversion Table for Flash Strength

The below table converts the Td.s flash strengths used by the RETeval into log photopic cd.s.m^{-2} and photopic cd.s.m^{-2}.

Flash Strength		
Td.s	**log photopic cd.s.m^{-2}**	**photopic cd.s.m^{-2}**
12.15	-0.367	0.43
21.48	-0.119	0.76
35.60	0.114	1.30
70.65	0.398	2.50
85.00	0.477	3.00
113.04	0.602	4.00
178.00	0.799	6.30
251.00	0.949	8.90
356.00	1.114	13.00
446.00	1.204	16.00

Conversion table for flash strengths used in this study using the Troland based RETeval protocol.