Characterising the orientation-specific pattern-onset visual evoked potentials in children with bilateral refractive amblyopia and non-amblyopic controls

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Abstract

Purpose: An orientation-specific visual evoked potential (osVEP) protocol was developed to probe meridional anisotropies in children with refractive amblyopia. The aim was to characterise the osVEP response in children with bilateral refractive amblyopia, evaluate the intra-session repeatability of the main osVEP components (C1, C2 and C3), coefficient of repeatability (CoR) of the response to gratings in different meridians and determine if refractive amblyopes have poorer repeatability as compared with non-amblyopic controls.

Methods: Children aged 4 to 7 years with newly diagnosed and untreated bilateral refractive amblyopia and non-amblyopic controls were recruited. Orientation-specific pattern-onset VEPs were recorded in response to an achromatic sinewave grating stimulus of 4 cycles per degree under monocular and binocular stimulation. The grating lines used for monocular stimulation were parallel with the subjects' most positive and negative astigmatic meridians when considered in sphero-minus cylinder form (Meridians 1 and 2 respectively). In subjects without astigmatism, meridians 1 and 2 were designated horizontal and vertical gratings respectively. Binocular stimuli were presented with grating lines parallel to meridians 45, 90, 135 and 180°. The repeatability of latencies of the main osVEP components (C1, C2 and C3) were investigated using two successive osVEPs recordings for each stimulus meridian and the CoR for each component's latencies were assessed.

Results: Seven amblyopic children (Visual acuity (VA) ranging from 0.08 to 0.40 LogMAR in the less amblyopic eye and 0.26 to 0.52 LogMAR in the more amblyopic eye) and 7 non-amblyopic controls (VA ranging from 0.00 to 0.02

LogMAR in either eye), with a median age of 4.6 and 7.0 years respectively, completed the study. C1 had the highest CoR for most conditions assessed. Ratio of CoRs C1:C2 was >2 for all binocular meridians in controls and the 90 and 180 meridians in the amblyopes; C1:C3 was >2 for the binocularly assessed 45, 90 and 135 meridians in the controls and the 90 and 180 meridians in the amblyopes; C2:C3 were all <2 for all meridians assessed in both groups.

Conclusions: The osVEP waveforms are reliable and useful for future investigations into the meridional anisotropies in children with refractive amblyopia, particularly the C3 component. Component C1 had the poorest repeatability, which consequentially affected C2 amplitude estimation. Only C3 amplitude and latency could be consistently estimated as C2 and C3 latencies were similarly repeatable. Coefficients of repeatability of osVEP latencies did not appear to systematically differ between non-amblyopic and amblyopic children.

Keywords

Amblyopia, Visual Evoked Potentials, Children Visual Development,

Paediatric Ophthalmology and Optometry, Orientation, Meridian, Astigmatism

Introduction

Amblyopia is a neurodevelopmental deficit that is characterised by a loss of spatial vision in the presence of one or more amblyogenic factors, such as media opacities,[1] uncorrected refractive errors[2] and strabismus.[3] Affecting approximately 1 to 4% of the general population,[4-8] the clinical diagnosis of amblyopia requires accurate assessment of visual acuity (VA) and entails a thorough review of the patient's case history and ophthalmic tests (e.g. refraction, ophthalmoscopy and cover test) to identify potential amblyogenic factors and to exclude ocular pathologies.[9] In situations of unexplained loss of vision, electrophysiological testing may be used to assist clinicians in the differential diagnosis.

In clinical practice, electrophysiological techniques are rarely used to monitor amblyopia treatments even though VEPs may be used to assess neural deficits non-invasively and objectively.[9-21] Previous studies have demonstrated reduced peak amplitudes (P100) and delayed peak latencies in amblyopic eyes.[16-27] The attenuation in the P100 amplitude has been attributed to passive signal degradation,[28] undersampling[29] and reduced activity of neurons due to poor quality signals from the amblyopic eye,[30] whereas delayed latencies have been attributed to poor integration within *V1* and the extrastriate cortex,[31-33], perhaps reflecting neuronal connectivity that is slower or less efficient than expected. Most of these discussed findings were observed in unilateral amblyopes who had amblyopia secondary to strabismus, anisometropia or both, but the situation is unclear in children with bilateral refractive amblyopia.

The current clinical guidelines by the International Society for Clinical Electrophysiology of Vision (ISCEV) describes the use of standard checkerboard stimuli in a pattern-reversal mode in monitoring the efficacy of occlusion therapy in amblyopic and fellow eyes, but subjective VA testing, where possible, generally takes priority.[22] While the use of the ISCEV standard pattern-reversal VEP (PRVEP) is known to yield repeatable measurements,[34-36] the standard checkerboard stimuli are not orientation-specific and are therefore not suited to probe refractive meridians individually to determine if any meridional anisotropy may exist in refractive amblyopia. In addition, diagonally oriented grating stimuli may be fundamental for assessing meridional anisotropies that are unrelated to amblyogenic refractive errors.[37-39]

The assessment of meridional anisotropies is believed to be important when evaluating refractive amblyopia because astigmats may develop neural deficits corresponding to the astigmatic meridians,[40] a condition known as meridional amblyopia[41] such that they demonstrate reduced sensitivity along the meridian which experienced the greatest retinal blur.[42] Moreover, non-amblyopic children may exhibit meridional anisotropies that may be developmentally normal for their respective age groups which includes the oblique effect and the horizontal effect, which may be demonstrated psychophysically or electrophysiologically as poorer sensitivity or function in the oblique and horizontal meridians than the other meridians respectively.[37-39] Electrophysiologically, the oblique effect may be manifest as diminished VEP amplitudes and longer latencies for oblique gratings (average of meridians 45° and 135°) as compared to the cardinal meridians (average of horizontal and

vertical), whereas the horizontal effect may be manifest as significantly lower VEP amplitudes and longer latencies when stimulated by horizontal gratings as compared to the rest of the meridians (i.e. vertical and obliques).[39] These meridional anisotropies may be assessed using oriented stimuli; an orientation-specific VEP (osVEP)[9] may be recorded using either pattern-reversal (PRVEP) or pattern-onset (POVEP) stimulation.

Although the PRVEP is a frequently used protocol for clinical electrophysiology in a wide variety of eye conditions,[34] POVEPs may have advantages as a technique for assessing amblyopic children[9, 43, 44] because they are less prone to fixation errors, eye movements and unintentional defocusing.[34] This is because the POVEP stimuli would tend to be observed to appear and disappear whereas PRVEP targets may appear to move at each reversal. The PRVEP's strengths include high repeatability (consistent inter-individual variations in morphologies and latencies with limited inter-individual variability) and minimal within-subject inter-eye differences.[34-36]

Previous studies have shown that the paediatric POVEP's C1 and C2 peaks (see figure 2 for examples of the C1, C2 and C3 components of POVEPs) tend to be less prominent in the immature visual system compared to adults. For example, *Boon, et al. (2016)*[43] noted that C1 was inconsistently present in children when stimulated by chromatic (magenta-cyan isoluminant) gratings. Typically, a characteristic single broad positive peak[45] may be observed during infancy and becomes more complex with maturation.[45-48] The distinct C1-C2-C3 waveform may take more than 20 years to develop[49] and it is debatable whether this broad peak is equivalent to the C1 or C3 in the adult

VEP. This is because the C2 component is typically unrecordable in infants aged 5 to 10 months and tends to emerge only in about 40% of 20 month-old children.[49] However, C2 is nearly always recordable by 8 years of age.[50]

Since the waveform morphology derived from the POVEP is known to be less repeatable than the PRVEP and more inter-individually variable,[43, 51-53] findings are expected to differ from PRVEPs. The POVEP latencies tend to be more variable in the amblyopic eye compared to the fellow eye[54, 55] given that the amblyopic visual system may have increased internal neural noise.[10, 56-58] However, the quality of having less repeatable inter-individual POVEP responses may be useful in differentiating between amblyopes and non-amblyopes, especially in studies that seek to understand the complexity of the waveform morphology[43]: POVEPs tend to have good intra-individual concordance,[59] allowing inter-ocular comparison to be made. Nevertheless, it must be noted that there is increasing evidence that the VEPs associated with viewing with either eye (amblyopic or fellow) may be abnormal.[60] Hence, both unilateral and bilateral amblyopia should be compared with non-amblyopic control reference data.[9] Overall, POVEP recordings have the potential to differentiate between children with and without amblyopia and provide information that is different from the PRVEP.[44]

A lack of repeatability of the POVEP could potentially be related to neural abnormalities in the development of visual pathways in amblyopic children.[28] The rationale for amblyopes having poorer repeatability is mainly attributed to the increased internal neural noise, which may be related to the changes in spatially and temporally structured neural activities due to prolonged retinal image blur.[61] Through measuring detection thresholds on a noisy

background, it was previously estimated that the equivalent cortical noise could be 1.4 times compared to without amblyopia.[58] A high level of neural noise within the amblyopic visual system would tend to reduce the detectability of the neural signals. This may be related to the loss of synaptic strength of signals from amblyopic eyes, such that the post-synaptic targets may be too weakly activated due to the influence of other synaptic inputs, as described by *Bienenstock, Cooper and Munro's* synaptic modification theory.[62]

As the POVEP components C1 and C2 were previously noted to be inconsistently evident in children[43] and it is not known whether an orientation-specific POVEP (osVEP) methodology will be reliable and repeatable in probing refractive amblyopia, the main purpose of this pilot study was to:

(1) characterise the osVEP's waveform morphology by assessing the main components (C1, C2 and C3),

(2) evaluate the intra-session repeatability of the response to each of the meridians (Monocular: aligned with the principal astigmatic meridians; Binocular: 45, 90, 135 and 180°) so as to ascertain the reliability of the methodology for future studies,

(3) evaluate if children with newly diagnosed and untreated refractive amblyopia have poorer repeatability of osVEP components as compared with non-amblyopic children with normal vision.

Methods

This research was conducted at the visual electrophysiology laboratory in Singapore National Eye Centre (SNEC). The study adhered to the tenets of Helsinki and ethical approval was obtained from the Centralized Institutional Review Board (CIRB) (Registration number: R1083/98/2013) at SingHealth and ratified by the human research ethics committees at the University of New South Wales, Sydney, NSW, Australia (Approval number: 09364). Parents and guardians gave their informed consent and children six years of age and above provided assent.

Subjects: Children aged 4 to 7 years with newly diagnosed and untreated bilateral refractive amblyopia and non-amblyopic controls (VA ≤0.05 LogMAR in each eye, with no history of amblyopia) were recruited from the refraction clinic at a children's hospital in Singapore and by advertisement. Bilateral refractive amblyopia was defined as having best corrected visual acuity (BCVA) of 0.3 LogMAR (6/12) or worse in one eye in the presence of significant bilateral myopia/hyperopia (≥2.00 DS) or astigmatism (≥1.50 DC), or a combination of both spherical and astigmatic ametropias in the absence of strabismus and other ocular conditions. Their suitability for this present study was determined following a series of entrance tests to rule out strabismus, ocular diseases or other abnormalities in both groups: All subjects underwent ocular health examination, LogMAR VA (*HOTV distance LogMAR chart, Good-lite Co, USA*), binocular vision (e.g. cover test and stereopsis, which was measured using the *Near 3-plates Frisby Stereotest, Stereotest Ltd, Fulwood, Sheffield, U.K.*), retinoscopy, autorefraction and manifest subjective refraction assessments using age-appropriate refraction techniques. To reflect real life conditions,

children with refractive errors were corrected using the prescriptions given by their own attending clinicians, who also made the decision if it was necessary or not to conduct cycloplegic refraction. All subjects were able to fluently read the English letter alphabet.

Orientation-specific VEP (osVEP) protocol: Single channel transient pattern-onset VEPs were recorded monocularly and binocularly, in response to a 12° field-size achromatic sinewave grating stimulus of 4 cycles per degree (cpd) displayed within a square window, with the subjects wearing their full prescribed spectacles prescription, if any, during the recording. The spatial frequency of 4 cpd was chosen because (1) it is near the peak of the normal contrast sensitivity function and (2) the stimulus should be resolvable by a large proportion of amblyopes as it corresponds to Snellen VA of 6/45. Further, previous experiments have demonstrated that meridional anisotropies may be evident at approximately this spatial frequency.[42] The gratings were aligned to match the two principal astigmatic meridians (Meridians 1 and 2) such that the grating lines used for monocular stimulation were parallel with the subjects' most positive and negative astigmatic meridians of their refractive error: For the current sample, meridians 1 and 2 respectively which were made up of horizontal and vertical lines respectively as most of the subjects had with-the-rule astigmatism, as is expected in children.[63] Figure 1 provides a representation of a stimulus with the luminance contrast modulated at 45°, hence oriented at 45°, but with the grating lines aligned along the 135° meridian. In this paper, the naming convention used for the meridian refers to the alignment of the grating lines, which is 135°. Binocular stimuli were presented with grating lines parallel to meridians 45, 90, 135 and 180°. Two averages of

30 sweeps of one second duration were recorded in succession for each stimulus condition. The stimulus onset duration was 100 ms and offset 400 ms, which was presented at a temporal frequency of 2 Hz, which is the same as previous experiments by *Boon, et al.* (2016)[28] but differs from the 2016 ISCEV POVEP standard. Michelson contrast was 54% to reduce monitor-related luminance artefacts[64] and the gratings were presented against a background of the same space-averaged luminance of approximately 54 cd/m^2. Room lighting was turned off during testing in order to minimize reflections off the screen.

During electrophysiological testing, the subjects were instructed to look at a central fixation target (black dot with a 2 mm diameter) at 1-metre viewing distance without any additional lenses to control their accommodation. Viewing distance was maintained by ensuring that the subjects leaned back against their seat's backrest. Fixation was monitored visually by the examiner. Subjects were reminded to observe the target on the screen. When fixation losses were observed or fidgeting occurred, the recordings were paused and repeated. For those who were excessively fidgeting or non-attentive resulting in sweeps contaminated by artefacts, individual sweeps were evaluated and those contaminated were manually removed post recording. Impedance was maintained below 8 kΩ throughout each recording.

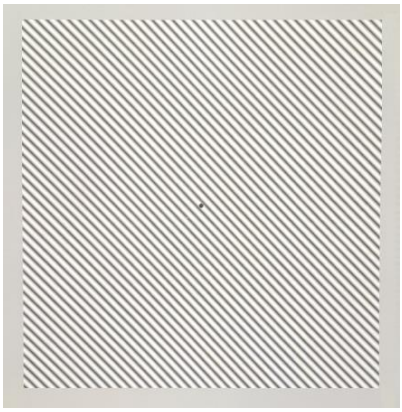

Figure 1: Representation of the achromatic grating stimulus of 4 cycles per degree at 54% Michelson contrast within a 12° field-size and surrounded by an isoluminant border. Fixation target was a 2 mm dot in the middle of the stimulus. In this example, the grating stimuli's luminance contrast was modulated at 45° but with the grating lines were aligned along the 135° meridian. Hence, this is labelled 135° based on the alignment of the grating lines.

Equipment: The electrode montage was based on the International 10 – 20 configuration,[65] but the placement differs from the standards[59, 66] as it was intended to maximize comparability with other parallel studies conducted in the *University of New South Wales, Sydney, Australia,* and for which analysis with the novel mathematical methods have been found to work.[48, 53] Three gold-cup surface electrodes (9 mm) were used during VEP recording, where the active electrode was applied at O_z (occipital midline), reference electrode at C_z and ground electrode at F_z, using EEG conductance paste and micropore tape. The Espion System (*Diagnosys, Cambridge, UK*) was used for electrophysiological recording at a sampling rate of 5 kHz with a band-pass filter of 0.312 – 100 Hz and a recording window of 1 second per sweep. The stimuli were generated using the ViSaGe Mk II (*Cambridge Research Systems, UK*), a 14-bit system, and were presented on a calibrated gamma-corrected high-performance cathode ray tube (CRT) monitor (*Sony CPD-G500 21-inch*

Trinitron monitor; Maximum Resolution 2048 x 1536 @ 75Hz; Horizontal and Vertical Scan Range 30 – 121 kHz and 48 – 160 Hz respectively). The mains frequency in Singapore is 50 Hz.

Analysis: The repeatability of the main osVEP components (C1-C2-C3) and the stimuli orientations were investigated using two successive osVEP recordings for each stimulus meridian. The first of the two responses from each separate recording was analysed. To be expedient, the responses from the same window were not compared as they are more likely to produce similar timings. If the osVEP component from the first response was ambiguous (e.g. the peaks were not obvious), the second response from the same time window was used during the analysis. If both responses in the same window were ambiguous, the recording that had the most obvious peak of the two was chosen for the analysis. The C3 component was defined as the largest peak that was present in both waveforms in the 1 second time window. By working backwards, the C2 component may be defined as the preceding trough and the C1 component as the preceding positive peak. Each osVEP component's amplitude was computed from the peak of the preceding component, except for C1 which was computed from the end of the pre-stimulus recording period (which had a duration of 12 ms) which was then regarded as the baseline (0 μV). Each component's peak latency was calculated as the time taken between stimulus onset and the peak. In situations where C1 was difficult to identify, C2 amplitude was measured from the baseline. Repeatability was assessed using the coefficient of repeatability (CoR) as computed for each meridian and each osVEP component (C1, C2 and C3) in two groups – amblyopes and non-amblyopic controls. The CoR provides an indication of the magnitude of the

95% limits of agreement (LOA) between the successive osVEP recordings. Derived from a *Bland-Altman* analysis, the LOAs are calculated as the mean of differences between the two successive measures of latencies of each osVEP components (i.e. bias) ± *k* x standard deviation,[67] where *k* is the calculated *coverage factor (k value)*, which was drawn from the statistical *critical t-distribution table*, for degree of freedom equivalent to *n – 1*, where *n* is the number of differences measured. Note the *k* is 1.96 for large sample sizes.

Results

Seven bilateral amblyopic and seven non-amblyopic control children completed the pilot study. The median (range) ages for controls and amblyopes were 7.0 (4.7 to 7.7) and 4.6 (4.3 to 6.6) years respectively. In the amblyopes, LogMAR VA was 0.21 ± 0.13 (mean ± SD) for the right eye (OD) and 0.30 ± 0.15 for the left eye (OS), whereas the non-amblyopic controls had equal VA in each eye (0.00 ± 0.00). The refractive profiles, ages and BCVAs of the subjects are presented on Table 1.

All the amblyopes in this present study were bilateral refractive amblyopes with moderately high magnitudes of astigmatism ranging from -1.25 to -5.00 dioptres cylinder (DC). The amblyopes' median dioptric spherical (DS) and cylindrical refractive errors were OD plano / -2.25 OS +0.50 / -3.50 with cylinder axes ranging from 5 to 165 °; controls' median refractive errors OD +0.63 / -0.13 OS -1.25 / -0.25 with cylinder axes ranging from 5 to 165 °.

The group averaged osVEP waveforms were plotted for the non-amblyopic controls and refractive amblyopes, both monocularly (Figure 2) and binocularly

(Figure 3), in Meridians 1 and 2 for the monocular results and Meridians 45, 90, 135 and 180° for the binocular results. Waveform morphologies of the non-amblyopic controls and refractive amblyopes appeared similar in overall shape. Amplitudes and latencies of the main osVEP components (C1-C2-C3) for each tested meridian are presented for the monocular (Table 2) and binocular results (Table 3).

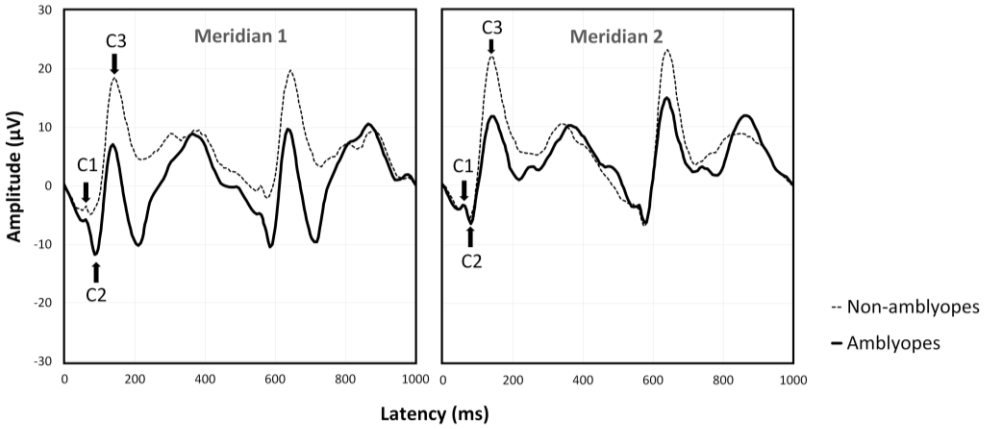

Figure 2: Group averaged waveforms of the orientation-specific pattern-onset visual evoked potential (osVEP) recordings in response to monocular stimulation using gratings orientated at Meridians 1 and 2 in children with (a) bilateral refractive amblyopia [n = 7] (solid lines) and (b) non-amblyopic controls [n = 7] (dotted lines). The main osVEP components (C1, C2 and C3) are labelled to indicate their respective amplitudes and latencies of the group-averaged waveform, which may differ from the actual average which was determined statistically from the individual waveforms.

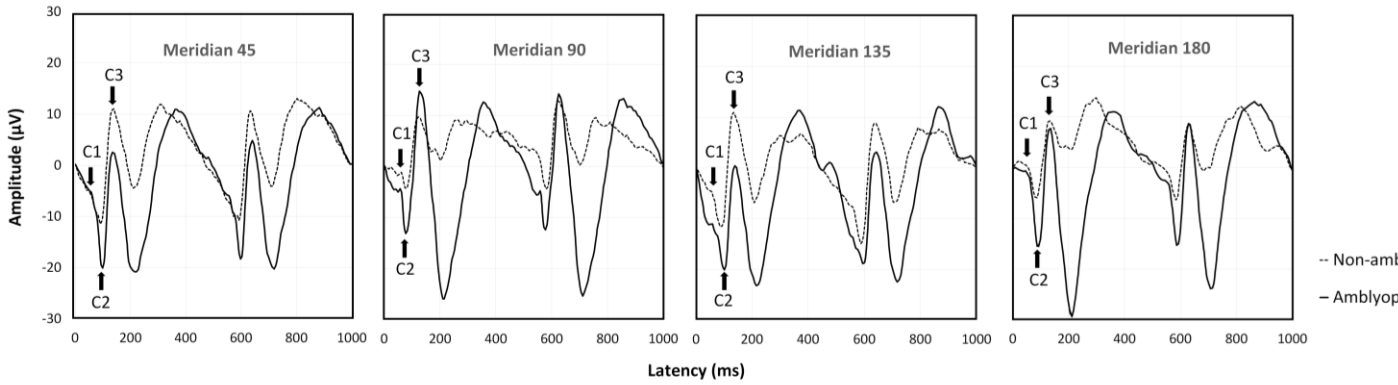

Figure 3: Group averaged waveforms of the orientation-specific pattern-onset visual evoked potential (osVEP) recordings in response to binocular stimulation using gratings oriented at 45, 90, 135 and 180° in children with (a) bilateral refractive amblyopia [n = 7] (solid lines) and (b) non-amblyopic controls [n = 7] (dotted lines). The main osVEP components (C1, C2 and C3) are labelled to indicate their respective amplitudes and latencies of the group-averaged waveform, which may differ from the actual average which was determined statistically from the individual waveforms.

Table 1: Refractive profile, age and best corrected visual acuity of subjects in order of recruitment.

	Age (years)	**Refractive error and best corrected visual acuity** (logMAR)			
Amblyopic subjects		Right Eye		Left Eye	
1	4.4	+2.25 -3.00 x 170	0.40	+2.25 -4.00 x 170	0.52
2	4.6	plano -3.50 x 180	0.16	plano -4.00 x 175	0.26
3	4.4	plano -1.50 x 5	0.14	+0.50 -3.00 x 165	0.34
4	4.3	plano -1.50 x 5	0.12	+0.50 -2.75 x 5	0.32
5	5.2	plano -5.00 x 5	0.40	plano -3.25 x 5	0.38
6	5.5	+0.25 -1.25 x 165	0.30	plano -2.00 x 180	0.32
7	6.6	+1.00 -2.00 x 5	0.30	+1.50 -2.75 x 180	0.32
Non-amblyopic subjects					
1	6.6	-3.50 D.S.	0.00	-3.75 -0.50 x 160	0.00
2	7.6	-1.00 -0.25 x 20	0.00	-2.25 D.S.	0.00
3	5.0	plano	0.00	plano	0.00
4	7.0	-1.75 D.S.	0.00	-1.75 D.S.	0.00
5	7.0	plano -2.25 x 5	0.00	plano -1.75 x 150	0.00
6	4.6	-0.25 -2.25 x 15	0.00	-0.75 -1.00 x 160	0.00
7	7.4	+0.50 -1.25 x 180	0.00	+1.00 -1.50 x 180	0.00

Table 2: Monocular orientation-specific visual evoked potentials in children with bilateral refractive amblyopia and non-amblyopic controls.

Amplitudes (μV)		**Non-amblyopes** (n = 7)			**Amblyopes** (n = 7)		
		Median	Min.	Max.	Median	Min.	Max.
C1	Meridian 1	1.56	-6.08	12.55	-3.02	-21.73	6.85
	Meridian 2	2.44	-10.53	23.51	-0.63	-6.27	7.22
C2	Meridian 1	-5.89	-51.38	-2.14	-8.31	-21.88	-1.98
	Meridian 2	-7.76	-76.57	-1.68	-6.10	-18.50	-1.06
C3	Meridian 1	23.73	10.91	12.99	21.72	11.88	57.19
	Meridian 2	27.53	13.00	74.00	21.71	5.40	62.66
Latencies (ms)							
C1	Meridian 1	69.75	36.20	83.00	55.35	8.20	108.90
	Meridian 2	61.70	22.10	68.40	61.30	1.85	136.70
C2	Meridian 1	92.75	69.80	135.50	91.60	48.70	133.90
	Meridian 2	91.05	68.00	132.60	88.05	46.70	173.20
C3	Meridian 1	155.75	130.20	229.00	139.40	106.50	195.10
	Meridian 2	151.75	123.50	217.80	149.55	122.40	219.70

Meridians 1 & 2 corresponds to the most positive & negative astigmatic meridians respectively.
Meridians 1 & 2 are horizontal & vertical gratings respectively in non-astigmatic subjects.

Table 3: Binocular orientation-specific visual evoked potentials in children with bilateral refractive amblyopia and non-amblyopic controls.

Amplitudes (μV)		**Non-amblyopic subjects** (n = 7)			**Amblyopic subjects** (n = 7)		
	Meridians (°)	Median	Min	Max	Median	Min	Max
C1	45	-4.05	-21.27	3.70	-5.33	-10.27	13.44
	90	3.38	-13.40	16.66	-3.93	-9.86	5.64
	135	-4.12	-18.17	5.62	-5.43	-25.36	5.64
	180	1.98	-1.96	13.61	-0.48	-6.90	5.36
C2	45	-6.98	-46.80	-4.26	-8.40	-58.15	-5.46
	90	-9.06	-69.25	-4.07	-6.60	-34.11	4.58
	135	-8.20	-52.91	-3.31	-4.63	-53.68	-1.74
	180	-11.80	-50.72	-2.56	-15.13	-30.45	-5.06
C3	45	42.98	25.15	91.95	22.88	3.69	61.67
	90	36.45	20.68	120.60	17.85	3.46	105.86
	135	38.72	14.31	78.72	27.32	3.18	62.02
	180	34.68	2.86	91.08	22.24	8.01	69.95
Latencies (ms)							
C1	45	57.10	30.90	84.30	59.00	38.50	94.20
	90	60.10	43.10	70.30	59.30	47.30	67.20
	135	58.50	38.40	78.90	84.00	46.30	96.80
	180	57.30	26.80	74.40	58.60	24.90	70.10
C2	45	96.20	69.00	128.70	101.00	65.60	117.30
	90	81.80	70.20	128.70	84.90	72.70	111.20
	135	92.80	70.90	127.50	106.10	82.70	121.20
	180	88.10	70.10	130.10	95.60	79.20	102.70
C3	45	141.70	117.60	228.30	135.90	88.80	164.00

90	129.40	112.50	218.30	129.30	102.30	147.60
135	138.00	121.20	229.20	143.00	108.70	160.80
180	132.00	92.00	227.00	131.60	124.80	144.50

The Bland-Altman plots of the latencies for the main osVEP components (C1, C2 and C3) are presented on Figures 4 and 5. Out of all the components, C1 has the highest CoR for most of the meridians tested (Figure 6). The CoR of C1 is two times greater than C2's for all binocularly assessed meridians in non-amblyopic controls and for the binocularly assessed 90 and 180 meridians in amblyopes. The CoR of C1 is two times greater than C3's for the binocularly assessed 45, 90 and 135 meridians in controls and 90 and 180 meridians in amblyopes. In contrast, the CoR of C2 was not greater than 2 x C3's for any of the meridians assessed, binocularly or monocularly. Hence, binocular C1 components have the poorest repeatability in most circumstances.

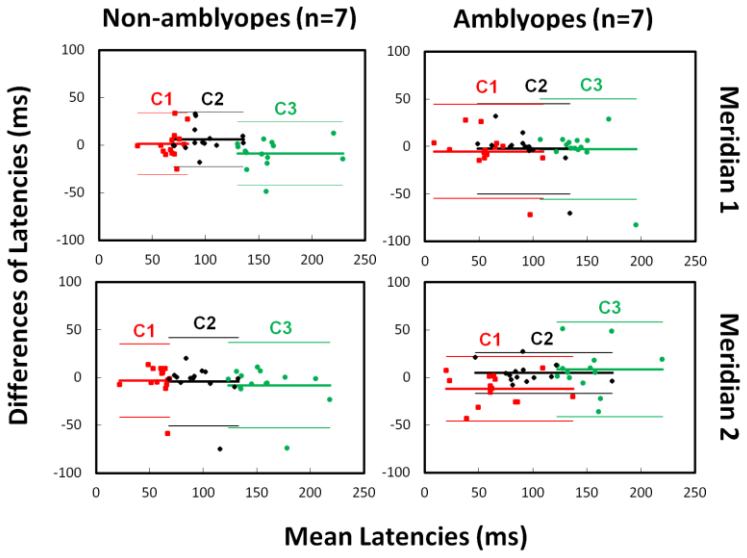

Figure 4: Bland-Altman plots of C1, C2 and C3 latencies in children with bilateral refractive amblyopia and non-amblyopic controls from two successive recordings of the orientation-specific visual evoked potentials (osVEPs) in response to monocular stimulation of gratings in Meridians 1 and 2. Data were plotted from each eye of each subject. The thick lines represent the mean latencies differences between the two successive recordings (i.e. bias) and the thin lines represent the 95% upper and lower limits of agreements between the two successive osVEP recordings.

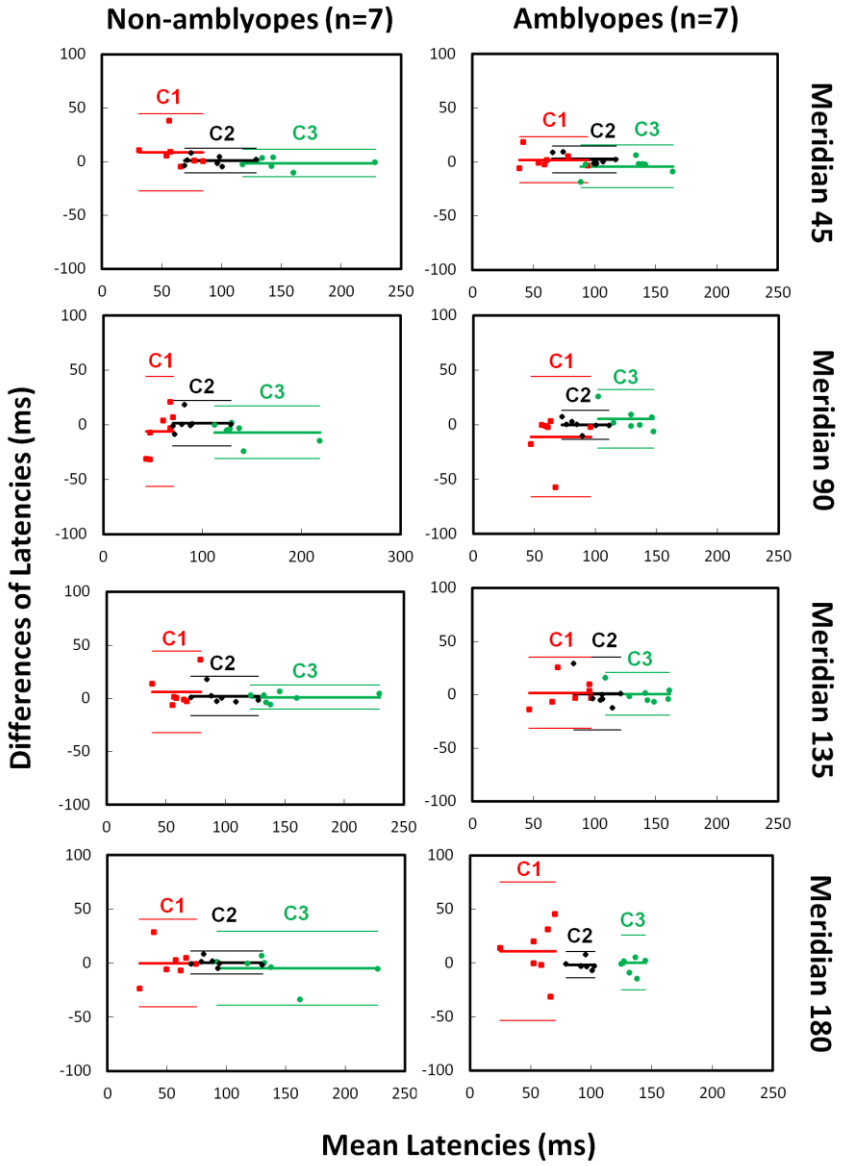

Figure 5: Bland-Altman plots of C1, C2 and C3 latencies in children with bilateral refractive amblyopia and non-amblyopic controls from two successive recordings of the orientation-specific visual evoked potentials (osVEPs) in response to binocular stimulation of gratings oriented at 45, 90, 135 and 180°. The thick lines represent the mean latencies differences between the two successive recordings (i.e. bias) and the thin lines represent the 95% upper and lower limits of agreements between the two successive osVEP recordings.

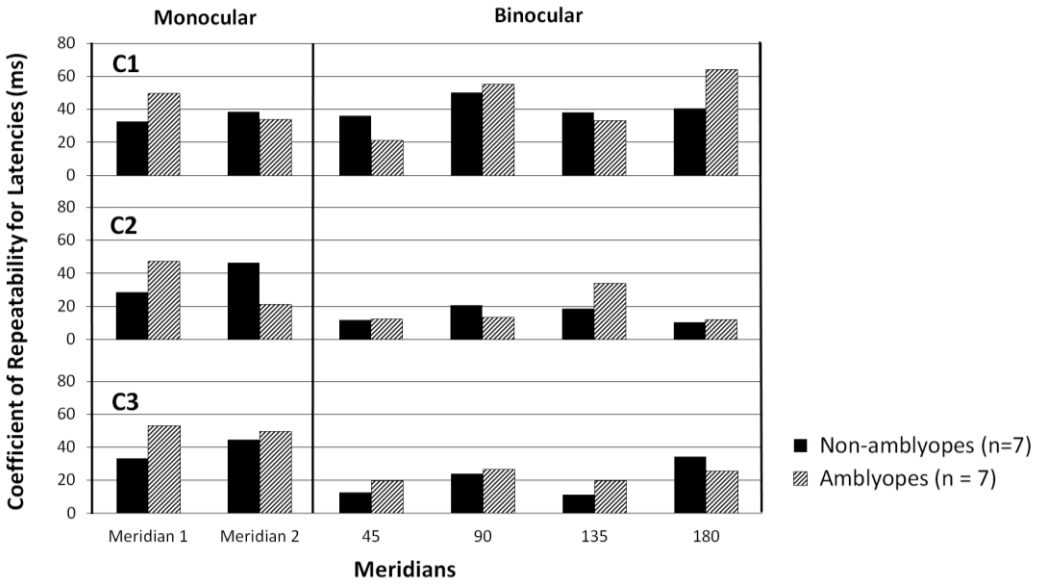

Figure 6: Coefficient of repeatabilities of C1, C2 and C3 latencies in children with bilateral refractive amblyopia and non-amblyopic controls from two successive recordings of the orientation-specific visual evoked potentials in response to monocular and binocular stimulations using gratings of different meridians (°).

To confirm that an offset response did not interfere with the onset response, a validation experiment was conducted by shifting the stimulus offset timing, from 200 ms to 300 ms and to 400 ms, in an adult osVEP to check for any shift in the responses (Figure 7).

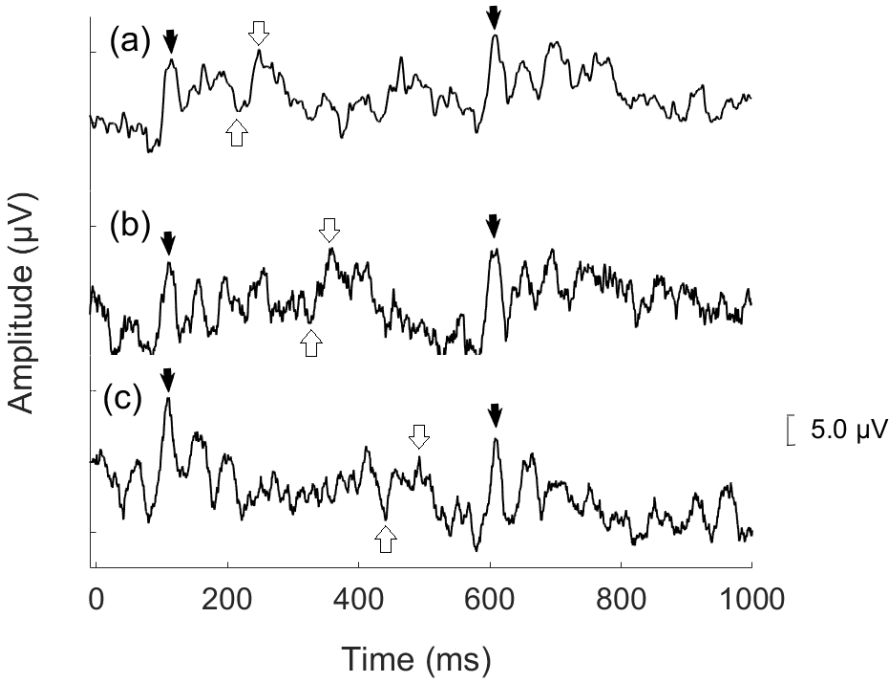

Figure 7: Validation experiment demonstrating that the offset timing of the orientation-specific pattern onset-offset visual evoked potentials (osVEPs) would not interfere with the C3 component. The osVEP waveforms were measured binocularly from an adult, aged 46 years (OD -6.00 -1.00 x 180 VA 6/6; OS -6.50 -0.50 x 180 VA 6/6). The osVEP was assessed in response to horizontal grating stimuli using the following duty cycle timings: (a) 100 – 400 ms, (b) 200 – 300 ms and (c) 300 – 200 ms. The peaks of interest are the two C3 peak latencies, as indicated by the black arrows. The peak and troughs of the offset responses are indicated by the white arrows. It was observed that the C3 peaks did not shift with the changes in offset timing that was made to the stimulus, suggesting that the osVEP C3 component is part of the onset response and not the offset response.

Discussion

This study demonstrated that osVEP waveforms, as stimulated by 4 cpd gratings with a 2 Hz temporal frequency, are repeatable in not only non-amblyopic children, but also refractive amblyopes. The osVEP waveforms were repeatable in all the meridians that were tested, suggesting that the osVEP is reliable to support future investigation of meridional anisotropies in children with refractive amblyopia.

The C1 component was found to be the least repeatable component (Figure 6), in agreement with previous POVEP studies that suggested that the C1 component of the immature visual system tends to be less prominent or absent.[43, 49, 50] Although C1 is the first peak, there were instances where this component was a negative value relative to the baseline in this cohort of amblyopic and non-amblyopic children (Figure 2). In contrast, adults are generally expected to have a positive value for C1.[59, 65, 66] Hence, the paediatric osVEP waveforms can differ substantially[39, 43, 49] from the standard ISCEV waveforms.[59, 65, 66]

It was observed that the repeatability of the C3 component was not very different in CoR from either C1 or C2. A consistently repeatable C1 is required in order to reliably estimate C2 amplitude. Unfortunately, the C2 amplitude proved to be difficult to assess due to the poor repeatability of the C1 and taking an amplitude measurement from baseline may not be ideal. For this reason, the C2 component amplitude may be less consistent than C3 for analysis, although C2 latency could be considered. In the amblyopic group, it was observed that there was greater number of positive C2

components compared to the non-amblyopic control group. As C2 is more positive, it may tend to produce lower C3 amplitudes and this trend was observed in the amblyopic subjects. The high repeatability of C2 component latency was beneficial for the analysis of C3 amplitude as it is a necessary component to determine the C3 amplitude.

The results suggest that the most suitable component for amplitude estimation is C3, which agrees with the findings by *Boon, et al. (2016)* [43] and *Thompson, et al*. (2017).[49] It is worthy to note that the stimulus onset timing in this present study (100 ms) differs from the 2016 ISCEV POVEP standard (200 ms) however there was no evidence that an offset response interfered with visualization of the C3 component (Figure 7). When the stimulus offset timing was shifted from 100 ms to 200 ms and to 300 ms in an adult osVEP to check for any shift in the responses, it was clear that the C3 component was unaffected. For these reasons, further work seeking a consistently measurable and repeatable component using osVEP should utilise the C3 component. The C2 component's latency is also repeatable.

Refractive amblyopes and non-amblyopic controls in this present study did not show any systematic differences in terms of their CoR (Figure 6). However, it must be noted that the number of participants assessed was low and a larger sample may reveal different trends. On the other hand, it was evident that the amblyopes' C3 amplitudes were diminished bilaterally which may be the key diagnostic factor in cases of bilateral refractive amblyopia. Interestingly, amblyopes in this cohort had shorter monocular C3 latencies to meridian 1 stimulation (mostly horizontal) than non-amblyopes (Table 2), although binocular appeared similar, which is unlikely

to be related to age as VEP latency tends to decrease with increasing age over the tested age range[68] and may instead reflect different monocular and binocular processing of visual signals from normal.

The finding from this present study differs from the observations by *Boon, et al. (2016)*[43] who found that amblyopes tend to have less repeatable components compared to non-amblyopic children, although CoR was not specifically assessed in that study. The lack of difference in their CoR may be related to subject characteristics; as in the study by *Boon, et al.*, the children were older in range than this present study and most had already started amblyopia treatment, which includes spectacle wear and occlusion of the better seeing eye.[69] The commencement of treatment would encourage active change in the state of the visual system, which might be reflected in the changed timing of electrophysiological processes which may impact on repeatability. In contrast, children in this present study had not received spectacle correction and/or occlusion therapy prior to the osVEP recording so their visual systems were likely to be in a more stable state. Another possible explanation may be related to the different types of stimuli used in these studies, as *Boon, et al. (2016)*[43] used chromatic (magenta-cyan isoluminant) gratings, rather than the achromatic luminance gratings used in the present study. The use of CoR assumes that the differences between each of the two measurements are approximately normally distributed,[70]. This was not found to be a limitation in this present study as the regression lines from the Bland Altman plots did not have any systematic significant proportional differences (Figures 4 and 5).

Poorer repeatability might be expected in amblyopes as there is evidence that they are experiencing elevated levels of internal neural noise which may be related to spatial aliasing.[71] As the stimulation of this present study was conducted at suprathreshold levels (4 cpd), to maximized the signal amplitude, this would allow higher signal-to-noise ratios, even in the presence of neural noise. It is also extremely unlikely that this cohort of bilateral refractive amblyopes would produce the same magnitude of abnormal lateral interactions[72] and spatial distortions[73-76] compared to unilateral amblyopes (e.g. strabismus and/or anisometropic) that were excluded from this present study. Such lateral interactions could arise when activities in neighbouring neurons are not tuned to the same stimulus,[77] which could cause the detectors flanking the stimulated neuron to respond more strongly. Furthermore, bilateral refractive amblyopes would be less likely to produce topographical mismatches of cortical receptive fields between the two eyes,[73, 78-81] that tend to be observed in strabismic and unilateral amblyopes. This present study suggests that for this sample of children newly diagnosed with refractive amblyopia, the neural abnormality in amblyopia is not related to variability in the timing of visual signal processing for suprathreshold stimuli.

The main advantage of the osVEP is the ability to assess meridional anisotropies in amblyopic and non-amblyopic children. In refractive amblyopia, the assessment of meridional anisotropies monocularly determines if neural deficits correspond to the individual's astigmatic refractive errors.[40] In addition, C3 amplitudes produced from binocular stimulation with 4 cpd sinusoidal gratings at the cardinal and oblique

meridians facilitates the assessment of other types of meridional anisotropy such as the horizontal effect or oblique effect.

The horizontal effect has previously been observed in infants in response to gratings[82] and adults when viewing natural scenes,[83] where horizontal stimuli produce poorer responses than stimuli aligned with other meridians. Our previous work on a larger group of subjects, based on analysis of the C3 component only, suggests that the horizontal effect may be a normal finding in children aged 3 to 9 years.[39, 44] In contrast, C3 amplitudes of refractive amblyopes tend to be depressed across all the meridians tested, with varying types of meridional anisotropies in each case.[44] These trends are similarly observed in this present study, suggesting that the horizontal effect may be an indicator of normality in children.

Conclusion

The osVEP waveform is reliable and useful for future investigations into the meridional anisotropies in children with refractive amblyopia, particularly the C3 component. Component C1 had the poorest repeatability, which consequentially affected C2 amplitude estimation. Only C3 amplitude and latency could be consistently estimated as C2 and C3 latencies were similarly repeatable. The repeatability of osVEP components in children with newly diagnosed and untreated amblyopia and children with normal vision and no history of amblyopia were not found to differ.

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Compliance with Ethical Standards

Funding: No funding was received for this research.

Conflict of Interest: T.P. Yap declares that he has no conflict of interest. C.D. Luu declares that he has no conflict of interest. C.M. Suttle declares that she has no conflict of interest. A. Chia declares that she has no conflict of interest. M.Y. Boon declares that she has no conflict of interest.

Ethical approval: All procedures performed in studies involving human subjects were in accordance with the ethical standards of the Centralized Institutional Review Board (CIRB) at SingHealth and human research ethics committees at the University of New South Wales, Sydney, NSW, Australia and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors.

Informed consent: Informed consent was obtained from all individual subjects included in the study.

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