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Research Article

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Retinal Signal Transmission in Duchenne Muscular Dystrophy: Evidence for Dysfunction in the Photoreceptor/Depolarizing Bipolar Cell Pathway

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Abstract

There have been reports of abnormal retinal neurotransmission determined by electroretinography in boys with Duchenne and Becker muscular dystrophy. Dystrophin may play a role in transmitting signals between photoreceptors and the excitatory synapse of the ON-bipolar cell. These electroretinographic changes appeared to be limited to the rod ON-pathway but we felt there was also similar abnormality in the cone ON-pathway. We used long-duration stimuli to separate ON (depolarizing bipolar cell) and OFF (hyperpolarizing bipolar cell) contributions to the cone-dominated ERG to better understand how the retina functions in boys with Duchenne muscular dystrophy. We recorded the electroretinograms of 11 boys with Duchenne muscular dystrophy and found abnormal signal transmission at the level of the photoreceptor and ON-bipolar cell in both the rod and cone generated responses. The OFF-bipolar cell that responds to the offset of the stimulus continues to function normally. The results support our hypothesis that retinal dystrophin plays a role in receptor function or controlling ion channels at the level of the photoreceptor and depolarizing bipolar cell. (*J. Clin. Invest.* 1994. 93:2425–2430.) **Key words:** sensory neurons • electroretinography • dystrophin • retina • glutamate receptors

Introduction

Photoreceptor cells (rods and cones) continuously release the neurotransmitter L-glutamate in the dark. The synaptic release of L-glutamate is inhibited under light stimulation when the cells hyperpolarize (1). The reduction in neurotransmitter release causes the bipolar cells (second-order neurons) to either depolarize or hyperpolarize dependent upon their unique receptors (2). Depolarizing bipolar cells (DBC)¹ drive ganglion cells that fire at the onset of light whereas hyperpolarizing bipo-

lar cells (HBCs) drive ganglion cells that fire at the offset of light. DBCs and HBCs are also referred to as ON- and OFF-bipolar cells, respectively. Therefore, organization at the level of the retina begins for two parallel pathways: the ON-pathway, which responds to an increase in brightness and the OFF-pathway, which responds to a decrease in brightness (3).

Cones may synapse to either depolarizing ON- or hyperpolarizing OFF-bipolar cells. The cone ON-bipolar cell makes a direct synapse with ON-ganglion cells, and, similarly, the cone OFF-bipolar cell synapses with an OFF-ganglion cell providing direct but distinctly different neural pathways to the visual cortex (4). Conversely, rods synapse to only one bipolar cell type, a depolarizing ON-bipolar cell (5). The output of the rod bipolar cell is not to a ganglion cell, instead, rod signals reach ganglion cells through an intermediate circuit: amacrine cells. The AII amacrine cell receives input from the rod bipolar cell and makes an electrical synapse on the cone ON-bipolar terminal causing it and the ganglion cell it contacts to depolarize (2). Using the inhibitory transmitter glycine, the AII amacrine cell hyperpolarizes the terminals of cone OFF-bipolars and the ganglion cells they contact. Therefore, while cones have a direct bipolar cell pathway to ganglion cells, the rods must use the cone bipolar cell pathway through the AII amacrine cell to send messages to ganglion cells.

There have been recent reports of abnormal retinal transmission in boys with Duchenne muscular dystrophy (DMD), a fatal neuromuscular disease that is the result of mutations in the dystrophin gene (6–8). Dystrophin is absent or nearly absent in DMD and it is possible that dystrophin plays a role in normal retinal function. The studies of retinal function have been done using electroretinography (ERG); a recording of the summed electrical signal produced by the retina when stimulated with a flash of light. The dark-adapted ERGs, recorded under scotopic testing conditions, have shown normal *a*-waves (a response of negative polarity generated by the photoreceptors) but reduced amplitude rod-isolated *b*-waves (a response of positive polarity originating primarily from the ON-bipolar cells) (9). This type of ERG abnormality with profound *b*-wave suppression is commonly associated with night blindness, (10) however, there have not been reports of night blindness or any other visual anomalies in boys with DMD, and dark-adaptometry studies have been normal (7).

The cone-mediated ERG recorded under photopic testing conditions has been reported as normal in DMD subjects (7, 11) or slightly reduced in amplitude, (8) however, we have observed the absence or attenuation of a small wavelet on the ascending *b*-wave of this response in our DMD subjects. Two small wavelets (oscillatory potentials O1, O2) are routinely seen on the ascending *b*-wave of the photopic ERG in our normal subjects but the second oscillatory potential, O2, was consistently absent in the DMD photopic ERG. Other evidence that pointed to a dysfunction at the level of the cone and its DBC was an unusual sawtooth-shaped waveform in the DMD

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1. *Abbreviations used in this paper:* CSNB, congenital stationary night blindness; DBC, depolarizing bipolar cell; DMD, Duchenne muscular dystrophy; ERG, electroretinography; HBC, hyperpolarizing bipolar cell.

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30 Hz flicker response. These clinical ERGs are recorded to short-duration stimuli of .1 ms and such brief flashes of light do not separate cone DBC and HBC contributions; the clinical photopic ERG is a mixture of both ON- and OFF-responses. Long-duration stimuli are needed to isolate cone DBC and HBC activity in the retina.

Long-duration photopic ERGs were used by other investigators to study subjects with retinal ON-pathway disorders such as CSNB (12–14) and paraneoplastic night blindness (15). The authors found absent or severely reduced rod and cone DBC responses that suggested these disorders were specific to ON-pathways. The clinical ERG showed reduced amplitude or absent rod-dominated *b*-waves with an abnormal cone response; both O1 and O2 oscillatory potentials were missing. While the ERGs in both CSNB and paraneoplastic night blindness are similar to what we have described in our DMD subjects, there were significant exceptions that, in our opinion, distinguished the DMD ERG as a clinical entity distinct from CSNB and other negative ERGs: the photopic O1 oscillatory potential was intact, the photopic O2 oscillatory potential was attenuated or absent and sawtooth-shaped waveform in the 30 Hz flicker response. We questioned whether the abnormal ERG in DMD was limited to just the rod pathway as suggested by the absence of rod-dominated *b*-waves. The purpose of this study was to use long-duration stimuli to isolate DBC and HBC contributions to the photopic ERG in order to determine the source of the apparent ERG abnormalities. Here we show functional evidence that would support the hypothesis that retinal dystrophin may localize to the postsynaptic membrane of the DBCs.

Methods

Subjects. The subjects were recruited from The Children's Mercy Hospital Departments of Ophthalmology and Genetics after the tenets of the Declaration of Helsinki and Internal Review Board approval. Subjects were 11 male children between the ages of 2 yr, 4 mo, and 21 yr (mean; 9 yr) who met the standard criteria for DMD: muscle weakness, elevated CPK, a positive family history when present, or a consistent muscle biopsy. All subjects had Southern blot analysis of genomic DNA and 75% had demonstrable deletions in the DMD gene. The remaining 25% had non-detectable deletions, and the diagnosis of DMD was based on the clinical presentation and muscle biopsies. A complete eye examination was performed before the ERG.

ERG. All subjects underwent a clinical ERG to short-duration stimuli of varying wavelengths before the long-duration photopic ERG. The clinical ERGs were recorded following published guidelines (16). Methods of the clinical ERGs are described in a previous paper (6).

After the clinical ERG, long-duration photopic ERGs were recorded. After 10 min of light adaptation to a rod-desensitizing background of 20 ft-L, a 2.2-log cd-s/m² stimulus (Dolan-Jenner Industries, Inc., Woburn, MA) was delivered through a 3-mm fiberoptic cable (Dolan-Jenner Industries, Inc. Woburn, MA) which was presented on a 30-ft-L Ganzfeld background (Nicolet Biomedical Instrument Corp., Madison, WI). Flash and background luminance were calibrated with a photometer (model 350, United Detector Technology, Hawthorne, CA). A Uniblitz shutter (Vincent Associates, Rochester, NY) was used to control the duration of the stimulus. ERGs were recorded to stimulus durations of 100, 80, 60, 40, 20, 10, and .1 ms to visualize the OFF-response merging with the ON-response. The ERG was recorded with a monopolar "jet" contact lens electrode (Universo SA, La Chaux-De-Fonds, Switzerland), referenced to the patients ipsilateral mastoid with a forehead ground. Recordings were performed with a Nicolet CA-1000 signal averaging system (Nicolet Biomedical Instru-

ment Corp., Madison, WI). Ten repetitions were averaged using a 1–1,500 Hz (–3 dB points) bandpass in a 200 ms window. Results were stored on a floppy disk for later analysis. The *a*-wave amplitude was measured from the baseline to the trough of the *a*-wave. ON-response amplitude was measured from the trough of the *a*-wave to the peak of the *b*-wave. The *b/a* amplitude ratio was obtained by dividing *b*-wave amplitude by *a*-wave amplitude.

Results

The eye examinations in all eleven subjects were normal with no abnormalities of the cornea, anterior chamber, iris, lens, vitreous, optic disk, retinal vessels, macula, or peripheral retina. Eye alignment was normal with no incidence of strabismus. There were no significant refractive errors. The subjects had no visual complaints. Their parents did not observe behavior associated with night blindness.

The clinical short-duration (.1 ms) ERG to both scotopic and photopic conditions is illustrated in Fig. 1. Representative normal and CSNB ERGs are shown for comparison to the DMD ERG. The normal subject shows a positive *b*-wave rise to very dim blue stimuli. This response is rod dominated, and, only when the intensity increases, do cone contributions become apparent. The negative polarity *a*-wave generated from both rod and cone photoreceptors is best seen at higher intensity white light. The *a*-wave is followed by the positive polarity *b*-wave that rises above the baseline yielding *b*-wave amplitudes that are approximately twice the amplitude of the *a*-wave. The scotopic oscillatory potential is filtered to remove slower frequencies giving a better view of the faster frequency components. After light-adaptation, cone dominated photopic responses are recorded in the presence of steady background light. To a short-duration (.1 ms) flash, a shallow *a*-wave of negative polarity is followed by a positive polarity *b*-wave of approximately four times the amplitude of the *a*-wave. Two small oscillatory potentials (O1, O2) are seen on the ascending limb of the *b*-wave. The response to a 30 Hz flickering light is sinusoidal in waveform.

The representative CSNB ERG is typical for this disorder with no *b*-wave to scotopic stimuli. The response to a bright white flash is often termed "negative" because of the lack of positive-polarity *b*-wave rise. Scotopic oscillatory potentials are attenuated and of particular interest, the photopic oscillatory potentials, O1 and O2, are missing. The response to 30 Hz flicker is sawtooth in waveform.

The DMD ERG shares scotopic similarities to CSNB such as attenuated *b*-waves, a negative response to bright white stimuli and attenuated oscillatory potentials but the photopic response is unique because the O1 oscillatory potential is preserved and the O2 oscillatory potential is attenuated to absent. The 30 Hz flicker response is also sawtooth in its waveform but less so than CSNB.

Photopic ON- and OFF-responses to long-duration stimuli are illustrated in Fig. 2. In normal subjects, the negative-polarity *a*-wave is followed by the positive-polarity *b*-wave (ON-response) that rises above the stimulus baseline. This response occurs at the same place in time to all stimulus durations for the obvious reason that the onset of light occurs at the same time. The response to light offset (OFF-response) is seen shifting to the left as stimulus duration decreases. At shorter intensities, ON- and OFF-responses merge; both make contributions to the morphology of the short-duration photopic ERG and

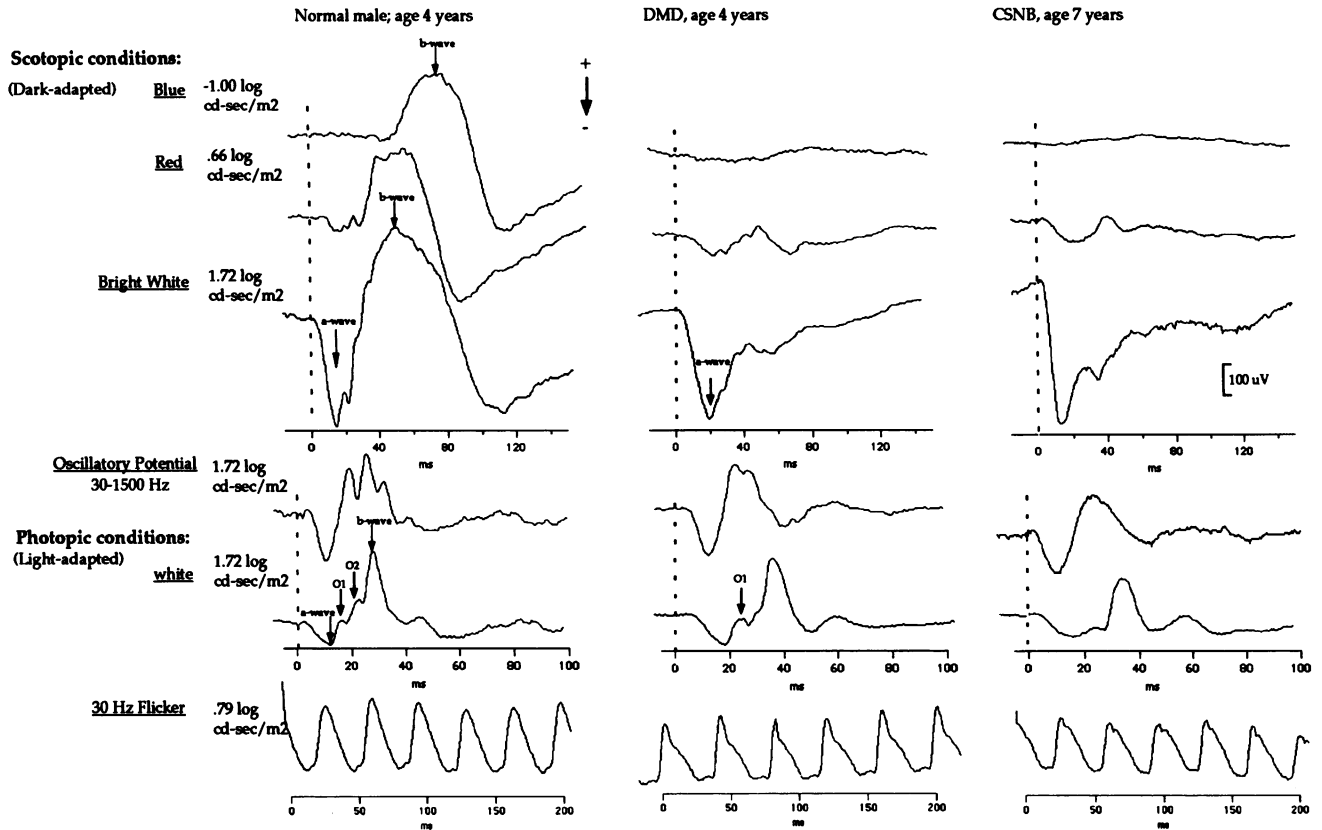


Figure 1. Clinical scotopic and photopic ERGs from representative normal, DMD and CSNB subjects. (DMD) Note the absence of a rod-mediated *b*-wave to blue, red, and white stimuli under scotopic testing conditions. Only cone oscillations can be seen in the response to the red stimulus. Response to a bright white flash shows only the photoreceptor generated *a*-wave. Under photopic testing conditions, the second oscillatory potential (O2) is missing. The response to a 30 Hz flickering light shows a steeper leading edge followed by a less abrupt descending slope. (CSNB) Scotopic responses are similar to DMD with no rod contribution and a negative ERG to a bright white stimulus. Under photopic testing conditions, both O1 and O2 oscillatory potentials are missing. This has been identified as loss of the cone ON-response.

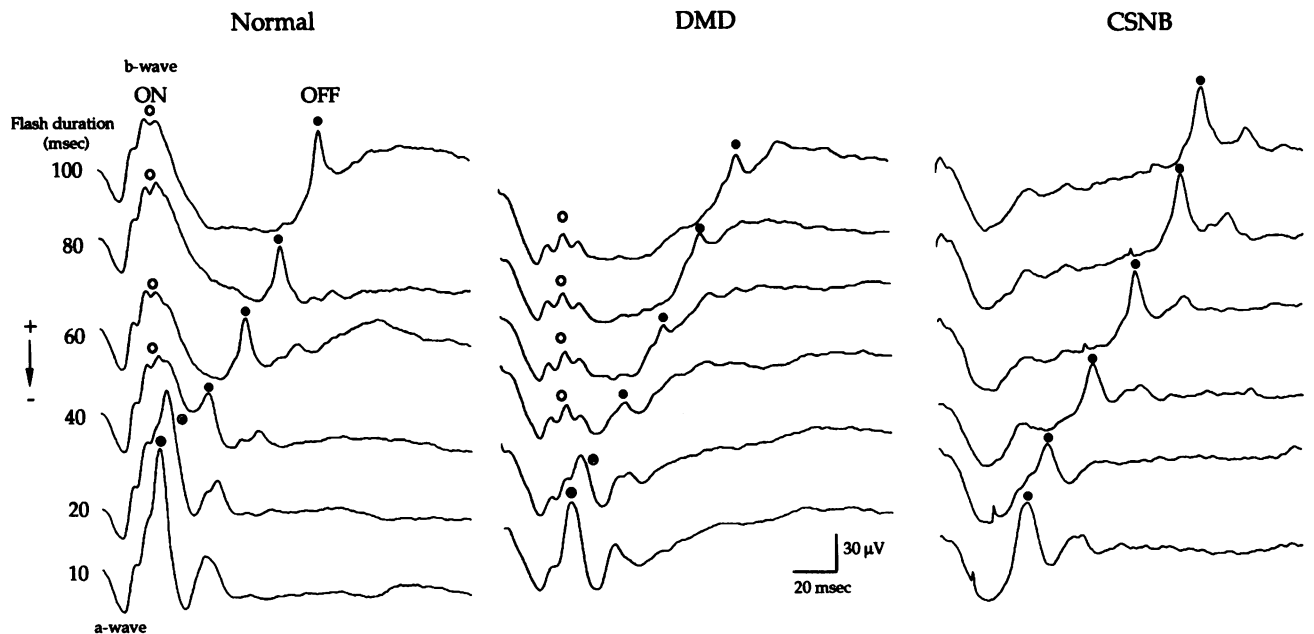


Figure 2. Long-duration cone-dominated photopic ERGs from representative normal, DMD and CSNB subjects. Open circles signify ON-responses, Solid circles signify OFF-responses, and the open circle with a dot is a mixture of both ON- and OFF-responses. The CSNB is shown for comparison to demonstrate an ON-pathway selective disorder; there is no response to stimulus onset. A small, decreased amplitude response to stimulus onset remains for DMD subjects but increased negativity of the *a*-wave and decreased amplitude ON-responses leads to abnormal ERG parameters to all stimulus durations.

Table I. Mean and P-values for Long-duration (100–10 msec) and Short-duration (.1 msec) Photopic ERG

Flash duration	a-wave amplitude			b-wave amplitude			b/a amplitude ratio		
	Normal	DMD	P-value*	Normal	DMD	P-value	Normal	DMD	P-value
<i>msec</i>									
100	44.6	75.0	.0024	86.4	43.1	.0002	2.01	.66	<.0001
80	45.6	62.7	.0077	94.8	39.9	<.0001	2.13	.63	<.0001
60	49.8	64.2	.0043	91.1	42.8	<.0001	1.87	.65	<.0001
40	45.8	64.0	.0006	90.2	42.7	<.0001	2.00	.67	<.0001
20	48.9	69.9	.0055	128.9	92.9	.0281	2.75	1.36	.0014
10	50.9	72.1	.0004	160.2	128.9	.0713	3.23	1.75	<.0001
.1	50.2	72.9	.0028	230.2	159.6	.0111	4.77	2.25	<.0001

* LOS = .01

oscillatory potentials are seen on the ascending limb of the *b*-wave.

The CSNB photopic ERG to long-duration stimuli is shown for comparison as a disorder affecting ON-pathways. The *a*-wave is of increased negative-polarity, the ON-response is highly attenuated to absent while the OFF-response is preserved. At short durations, the supposed *b*-wave is really the OFF-response and no oscillatory potentials can be seen on the ascending limb.

In contrast to CSNB, the DMD ERG retains some ON-response. The *a*-wave is of increased negative-polarity and a small positive response with oscillations can be seen following the *a*-wave. This ON-response is attenuated and does not rise above the baseline until the OFF-response contributes to the amplitude.

The ERGs were compared to age and gender-matched normal control subjects. Comparisons between groups were made using Student's *t* test for unpaired measures (two-tailed). The means and P-values for ERG parameters to all stimulus durations are presented in Table I and graphically represented in Figs. 3–5. Subjects with DMD show significantly increased *a*-wave amplitudes (Fig. 3) and low *b/a* ratios (Fig. 4) to all flash

durations. *b*-Wave amplitude is significantly decreased to all flash durations except 20, 10, and .1 ms (Fig. 5). DMD photopic *a*-waves, like the scotopic *a*-wave, exhibits a strong negative trend resulting in increased *a*-wave amplitude. In addition, DMD subjects exhibit decreased amplitude of the photopic ON-response (*b*-wave) which represents the activity of the cone DBC. *b*-Wave amplitude varies significantly from normal except at 20, 10, and .1 msec. At these shorter durations, the biphasic OFF-response merges with the ON-response increasing the amplitude of the *b*-wave; the shorter the duration, the steeper the *b*-wave. At .1 ms (clinical photopic ERG) the ascending slope is very steep, resulting in what appears to be a normal *b*-wave. Traditionally, the *b*-wave is measured from the trough of the *a*-wave to the peak of the *b*-wave. If only *b*-wave values are considered, as in most clinical situations, the short-duration clinical photopic ERG would be considered normal. However, the ON-response is decreased in amplitude to all stimulus durations. This, coupled with increased negativity of the *a*-wave and summation of the OFF-response, adds to the

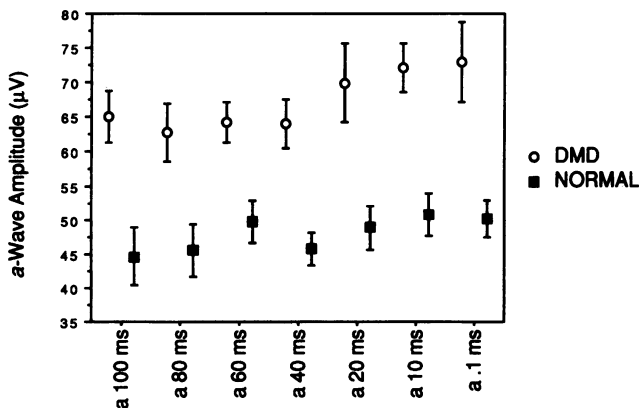


Figure 3. *a*-Wave amplitude of DMD subjects and normal controls to long- and short-duration stimuli. DMD *a*-wave amplitudes to long-duration stimuli are significantly increased in amplitude when compared to normal. The DMD *a*-wave continues to greater negativity because of the lack of a positive going ON-response. Error bars represent ± 1 SEM.

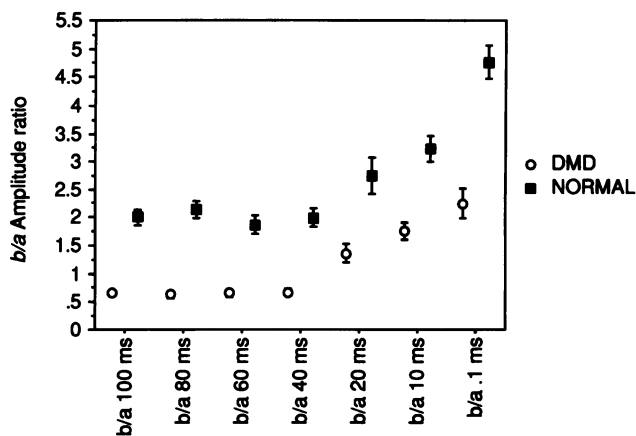


Figure 4. *b/a* Amplitude ratios of DMD subjects and normal controls to long- and short-duration stimuli. In normal subjects the *b/a* amplitude ratio is > 1.0 to all stimulus durations. In contrast, DMD subjects show *b/a* amplitude ratios that are < 1.0 except at 20, 10, and .1 ms when the amplitude of the OFF-response increases the amplitude of the *b*-wave which in turn gives higher *b*-wave values. Despite the addition of the OFF-response, the *b/a* amplitude ratios are significantly lower than normal controls to all stimulus durations. Error bars represent ± 1 SEM.

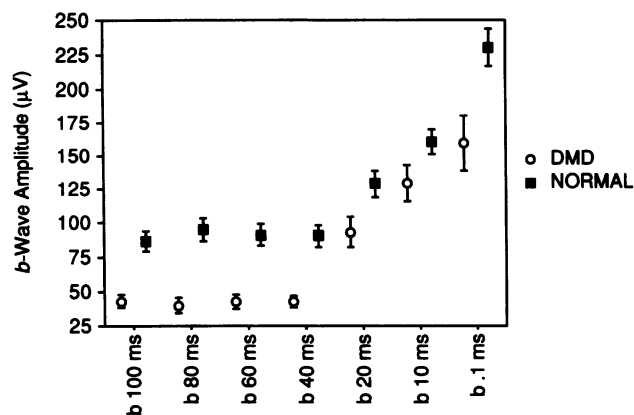


Figure 5. *b*-Wave amplitude of DMD subjects and normal controls to long- and short-duration stimuli. *b*-Wave amplitude is traditionally measured from the trough of the negative *a*-wave to the peak of the positive *b*-wave. *b*-Wave amplitude in DMD differs significantly from normal at the longer durations, but, as the OFF-response is added into the *b*-wave at shorter durations, the difference between groups is no longer significant. This gives the false impression that the clinical photopic ERG in DMD is normal, however, *a*-wave amplitude and *b/a* amplitude ratios must be considered to fully analyze the photopic ERG. Error bars represent ± 1 SEM.

value of the *b*-wave giving the false impression that *b*-wave amplitude is normal. This abnormality can only be detected in the clinical ERG by examining the *b/a* amplitude ratios. In normal subjects, the *b/a* amplitude ratio is > 1.0 to all stimulus durations. In contrast to this, the *b/a* amplitude ratio in DMD subjects is < 1.0 to all stimulus durations except 20, 10, and .1 ms, but, even with the contribution of the OFF-response, the *b/a* ratio is significantly different at these shorter durations because of increased negativity of the *a*-wave and attenuation of the ON-response.

Discussion

The ERG in DMD is characterized by a negative response to a bright flash under scotopic testing conditions suggesting dysfunction at the level of the rod DBC. The clinical short-duration photopic ERG appears normal when only *b*-wave amplitude and implicit time are considered, however, the missing O2 oscillatory potential, increased negativity of the *a*-wave and decreased *b/a* amplitude ratio points to cone DBC dysfunction as well. While the cone DBC response is highly attenuated, some function remains. This unique ERG could be the result of abnormal regulation of ion channels secondary to changes in function or structure of the DBC-specific receptor. Our ERG findings in ON-pathway disorders have been consistent with the results of selectively blocking DBC and HBC contributions in the primate retina. These recent studies described below have added much to our understanding of the photopic ERG.

When ON-pathways (DBCs) are blocked with 2-amino-4-phosphonobutyric acid (APB) the *a*-wave becomes more negative, the *b*-wave is suppressed, and the OFF-response is enhanced (17). This phenomenon is similar to the DMD and CSNB ERGs illustrated in Fig. 2. Subsequent blocking of HBCs, the OFF-pathway, with cis-2,3-piperidine dicarboxylic acid (PDA) reversed this effect and the *a*-wave becomes less negative, therefore, HBCs may make a contribution to the nega-

tivity of the *a*-wave. When PDA and kynurenic acid were used alone to block the OFF-pathway, an early large amplitude light-evoked positive response was recorded that corresponded with the normal *b*-wave but was twice as large. It appears that the DBC ON-response contributes to the rising positive slope of the *b*-wave but the early persistent negativity of the HBC OFF-response truncates this large-amplitude positive wave. In a continuation of this work, Bush and Sieving (18) investigated the 30 Hz flicker in primates to determine whether the response might contain contributions from the inner retina. When ON-responses were blocked, they describe a major phase delay of the flicker that gives the waveform a sawtooth appearance, consistent with the 30 Hz flicker responses in DMD we report in this paper.

We believe the anomaly seen in the DMD photopic ERG and 30 Hz flicker can be explained by this "push-pull" mechanism between the ON- and OFF-pathways. The DBC ON-response has a fast velocity of positive polarity while the HBC OFF-response shows early negative polarity followed by a positive wave. The DMD photopic ERG shows increased negative *a*-wave amplitude and attenuation of the positive O2 oscillatory potential. If there is dysfunction of the cone DBC it would stand to reason that the DBC contribution would be dominated by the more negative HBC component leading to increased negativity and suppression of the leading edge of the *b*-wave, which possibly explains the missing O2 oscillatory potential.

Dystrophin is a large (427 kD) protein with an amino acid sequence comparable to the spectrin family of membrane-associated cytoskeletal proteins (19). It exists in many isoforms, due to promoter selection and RNA splicing (20). Dystrophin localizes to the sarcolemma of skeletal, cardiac, and smooth muscle, and is thought to mechanically stabilize the plasma membrane, similar to erythrocyte spectrin (21). Another hypothesis is that dystrophin regulates the normal closure or voltage dependence of Ca^{+2} permeable membrane channels (22). Dystrophin has also been identified at cerebral cortical synapses but its role has yet to be elucidated (23). Recently, multiple isoforms of dystrophin were identified in the normal human retina (8).

It is possible that dystrophin isoforms play different roles in different tissues. Dystrophin binds to several glycoproteins in the sarcolemma membrane that surround muscle myofibrils. These associated proteins interact with dystrophin and may not exist in the same form in different tissues; indeed, it may prove that there will be dystrophin-glycoprotein complex models specific for separate tissues (24). We have not seen a correlation between the severity of the ERG abnormality and the severity of the muscle disease which suggest that the muscle and retinal products have different promoters and function (K. M. Fitzgerald, G. W. Cibis, A. H. Gettel, S. A. Giambrone, D. J. Harris, and R. A. White, manuscript in preparation).

If retinal dystrophin expression is required for normal function, our results give support to the idea that the dystrophin-glycoprotein complex may play a role in signal transmission or formation of an ion channel. This is a reasonable hypothesis as a functionally related cytoskeletal protein, ankyrin, is associated with brain Na^{+} channels (25). Monoclonal antibodies to an epitope of L-type (long-lasting) Ca^{+2} channels in hippocampal pyramidal neurons found the subcellular localization to be identical to the location of dystrophin (26). Alterations in mature skeletal myocyte Ca^{+2} channel physiology and increased

levels of intracellular Ca^{+2} concentrations have been found in boys with DMD (27). Mouse retinal bipolar cells have been found to have T-type (transient) Ca^{+2} channels (28). Ca^{+2} currents were found to be generated at both the perikaryon and at the axon terminal indicating that this current may participate in synaptic transmission. It is not known whether there are increased Ca^{+2} concentrations in the retina of DMD boys. Histology and immunohistochemical studies of DMD and normal human retina are needed to improve our understanding of retinal dystrophin.

We believe the abnormal ERG in DMD represents an ON-pathway defect and is not a form of CSNB. Unlike CSNB, the cone DBC ON-pathway retains some function. We suggest that the cone DBC response, while abnormal, maintains enough function to serve as a pathway for rods to transmit signals to ON-ganglion cells via AII amacrine cells, sparing DMD subjects from night blindness. We are currently investigating this hypothesis. The fact that this abnormal retinal function occurs in DMD, a well-studied population with no history of retinal disease and normal dark-adaptometry, suggests that dystrophin plays a role in normal retinal function. By using long-duration flashes to separate DBC and HBC activity we give functional evidence to support the hypothesis that retinal dystrophin may localize to depolarizing bipolar cells. This was suggested by Tamura et al. (29) and our results support that hypothesis. The results of this study give functional evidence to that role and favor localization of retinal dystrophin to the postsynaptic membrane of the DBC.

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