Myosin Va is required for normal photoreceptor synaptic activity

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Summary

Myosin Va is an actin-based motor molecule, one of a large family of unconventional myosins. In humans, mutations in *MYO5A* cause Griscelli syndrome type 1 and Elejalde syndrome, diseases characterized by pigmentation defects and the prepubescent onset of severe neurological deficits that ultimately lead to a shortened lifespan. Mutations in the *Myo5a* gene in mouse cause the dilute series of mouse mutants, demonstrating that myosin Va is involved in pigmentation and neural function. Although the reason for the pigmentation abnormalities is well understood, the role of myosin Va in neural function is not. Myosin Va has been found in synaptic terminals in the retina and brain. We report here new physiological evidence for a role of myosin Va in synaptic function. Photoreceptor synapses in neurologically affected myosin Va mutant mice have both anatomical and physiological abnormalities. Thus, myosin Va is required for normal photoreceptor signalling, suggesting that it might function in central nervous system synapses in general, with aberrant synaptic activity potentially underlying the neurological defects observed in dilute lethal mice and patients with Griscelli syndrome type 1 and Elejalde syndrome.

Key Words: Unconventional myosin, Myosin Va, Photoreceptor, Synapse

Introduction

Myosin Va is an unconventional myosin (Kalhammer and Bahler, 2000) that functions as a processive actin-based motor molecule (Mehta et al., 1999). It is known to function in organelle and vesicle transport (Langford, 2002). In humans, mutations in *MYO5A* cause Griscelli syndrome type 1 and Elejalde syndrome, both rare autosomal recessive disorders (Anikster et al., 2002; Bahadoran et al., 2003b; Menasche et al., 2002; Menasche et al., 2003; Pastural et al., 1997). In fact, these two clinically defined diseases probably describe a single disease (Anikster et al., 2002; Bahadoran et al., 2003b; Ivanovich et al., 2001; Menasche et al., 2002). The two syndromes are characterized by hypopigmentation of the skin and hair as well as severe neurological abnormalities. Neurological abnormalities are usually early onset and include seizures, mental retardation and hypotonia. Griscelli syndrome type 1 and Elejalde syndrome patients usually have a shortened life span owing to their neurological abnormalities (Bahadoran et al., 2003a; Duran-McKinster et al., 1999; Elejalde et al., 1979; Pastural et al., 1997; Pastural et al., 2000). The dilute series of mouse mutants is caused by mutations in *Myo5a* and, as in humans, dysfunction of this gene leads to both pigment and neurological abnormalities. Some alleles have a diluted coat color only, whereas others show both diluted coat and progressive ataxia and convulsions leading to early death (Huang et al., 1998a; Huang et al., 1998b; Mercer et al., 1991). The pigmentation defect is believed to be due to abnormal distribution of melanosomes along the dendrites of melanocytes (Provance et al., 1996; Wu et al., 1997).

Although mutations in myosin Va clearly cause severe neurological disease in mammals, its function(s) in the central nervous system (CNS) is not well understood. Myosin Va has been found in abundance in pre- and post-synaptic terminals in the retina and brain, suggesting a role in synaptic activity (Bridgman, 1999; Evans et al., 1998; Prekeris and Terrian, 1997; Schlamp and Williams, 1996; Walikonis et al., 2000). Myosin Va appears to associate with synaptic vesicles, adding support to a role in synaptic transmission (Evans et al., 1998; Prekeris and Terrian, 1997). Also, an abnormal accumulation of presynaptic vesicles are present in cerebellar granular cells of dilute lethal mutants (Bridgman, 1999), but the physiological consequences of these features are not known. In an extensive analysis of hippocampal synaptic function in dilute lethal mice, no functional defects were found in synaptic transmission or plasticity (Schnell and Nicoll, 2001). Lethal dilute mutants also have abnormal Purkinje cell smooth endoplasmic reticulum and no inositol-triphosphate-receptor-laden reticular tubules in the postsynaptic spine of cerebellar neurons (Dekker-Ohno et al., 1993; Petralia et al., 2002; Takagishi et al., 1996). Although no abnormalities were found in either morphology or transmission at this synapse, long-term synaptic depression was absent, suggesting an important role for myosin Va neurophysiology (Miyata et al., 2000).

In the retina, myosin Va is expressed predominantly in photoreceptor cells. In photoreceptor cells myosin Va is

localized mainly to presynaptic terminals and, to a lesser extent, to inner segments (Schlamp and Williams, 1996). The subcellular localization of myosin Va suggests several roles in photoreceptor function, including in synaptic transmission. We report here new physiological evidence for a role of myosin Va in synaptic function. Electroretinography of dilute lethal mutants revealed prolonged implicit times and disproportionately-reduced b-wave amplitudes, and ultrastructural analysis showed abnormal presynaptic ribbons in the photoreceptor cells. We propose that myosin Va is required for normal photoreceptor synaptic morphology and synaptic physiology.

Materials and Methods

Mice

$Myo5a^d$ and $Myo5a^{d-l}$ mutants were originally obtained on an undefined genetic background from the MRC Mammalian Genetics Unit (Harwell, UK). The two mutations were maintained in a single segregating stock. Maintenance of the stock was facilitated by the visible recessive phenotype of the short ear ($Bmp5^{se}$) mutation that is very closely linked to the $Myo5a$ locus and was carried on the same chromosome as $Myo5a^d$, allowing $Myo5a^d/Myo5a^{d-l}$ mice to be distinguished readily from $Myo5a^d/Myo5a^d$. Homozygous $Myo5a^{d-l}$ mutants were recognized by their progressive ataxia, which begins about postnatal day 12 and is clearly visible by 2 weeks of age. $Myo5a^{d-l}$ mutants die between the third and fourth weeks of life from unknown causes. This phenotype is consistent with all known myosin Va lethal mutations (presumably, it is a null mutation) (Huang et al., 1998a; Huang et al., 1998b). The $Myo7a^{4626SB}$ mutation was maintained on a background comprising approximately 50% CBA/Ca, 50% BS with a small BALBc contribution (Libby and Steel, 2001), and homozygotes were distinguished by their characteristic head-bobbing and circling behavior from about 2 weeks of age. Double mutants were obtained by intercrossing, and the $Myo7a^{4626SB}$ genotype was established by molecular typing as described previously (Holme and Steel, 2002). The control groups included both $Myo5a^d/Myo5a^d$ and $Myo5a^d/Myo5a^{d-l}$ grouped together, because there were no significant differences between these two genotypes for any of the parameters measured. Likewise, the control groups for the cross with $Myo7a^{4626SB}$ included both $+/Myo7a^{4626SB}$ and $+/+$ mice, because no significant differences between these two genotypes were detected. All experiments were carried out in full compliance with UK Home Office regulations and in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Electroretinography

Electroretinography was performed as previously described (Libby et al., 2003; Libby and Steel, 2001). Mice were anaesthetized with urethane and the right pupil was then dilated with atropine and the mice dark adapted for 30 minutes. A series of flashes (photic stimulator PS22; Grass-Telefactor, Quincy, MA) over a 7.8 log unit scale (in 0.6 log unit intervals) were presented to the mice. At the lowest light intensities, 20 flashes were presented at three second intervals and the average response was recorded. As the light intensity increased, the number of flashes was reduced and the interval between stimuli was increased so that the animal would not light adapt. At the highest intensities, only two flashes were averaged and the inter-stimulus time was 1 minute. The unattenuated flash was 466 candela m^{-2}. The a-wave amplitude was measured from the baseline to the lowest point of the initial negative deflection and the b-wave amplitude was measured from the baseline or when the a-wave was present from its trough to the highest maximum positive value. Implicit times were measured from the time of the stimulus to the a- or b-wave maximum.

Histology

All eyes were harvested at least 3 hours after light onset and before lights out. The eyes were fixed in 2% paraformaldehyde + 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.6. Eyecups were processed for embedding in Epon 812 resin. Semithin sections (0.7 µm) were obtained along the entire dorsoventral axis, passing through the optic nerve head, and stained with 0.25% toluidine blue in 0.1% sodium borate. The number of photoreceptor cells was determined by counting the number of nuclei in an 85 µm length of retina at 0.5 mm intervals from the optic nerve head. Ultrathin sections were mounted on copper grids and stained with uranyl acetate and lead citrate before observation in a Philips (model 208) electron microscope.

Results

Electroretinography can be used to measure several parameters of retinal physiology, including photoreceptor stimulation and synaptic activity (Peachey and Ball, 2003). The a-wave of the electroretinogram (ERG) is a result of photoreceptor activation and the b-wave is the result of interneuron activity (Peachey and Ball, 2003). ERGs were recorded from mice at postnatal day 19-25, when dilute lethal mice are clearly ataxic. ERG waveforms showed clear reductions in amplitudes of both a- and b-waves in dilute lethal homozygotes ($Myo5a^{d-l}/Myo5a^{d-l}$), which have diluted coat color and neurological defects compared with $Myo5a^d/Myo5a^d$ and $Myo5a^d/Myo5a^{d-l}$ mice, which have pigmentation defects alone (the two control genotypes were physiologically indistinguishable; Fig. 1A). Measurements of a-wave amplitudes showed significant reductions of around 40% across a range of stimulus intensities, whereas b-wave amplitudes were reduced by around 50% (Fig. 1B). Light microscopy of retinas from these mice revealed no obvious structural defects in the retina (Fig. 1C). Photoreceptor outer segments did not appear to be any shorter in the dilute lethal mutants, and photoreceptor cell numbers were actually a little larger (by approximately 10%) in the dilute lethal group compared with the non-lethal group (data not shown).

Inspection of the ERG waveform shape suggested that b-waves were affected to a greater degree than a-waves in dilute lethal mutants (Fig. 1). We measured implicit times (which represent synaptic activity) for both a- and b-waves, and found significantly longer times for both waves in dilute lethal mutants, suggesting a deficiency in synaptic activity (Fig. 2A,B). Furthermore, b:a wave amplitude ratios were smaller in dilute lethal mutants than in the control group. This observation suggests that transmission of activity from photoreceptors to bipolar and other inner retinal cells is less efficient than it should be (Fig. 2C).

Photoreceptors have an unusual presynaptic specialization: a ribbon of electron-dense material surrounded by a cluster of synaptic vesicles. These ribbons are found mainly in cells that need to pass on information very rapidly, such as photoreceptors and inner hair cells in the cochlea (Morgans, 2000). In photoreceptors, these ribbons are normally located at the presynaptic complex, immediately opposite the synaptic contact with bipolar and horizontal cell terminals (Fig. 3A). In dilute lethal mutants, the localization of the ribbons did not appear to be altered, but their morphology was abnormal. In approximately half of all rod spherules, one end of the ribbon or the entire ribbon was enlarged to give the ribbon a club-like
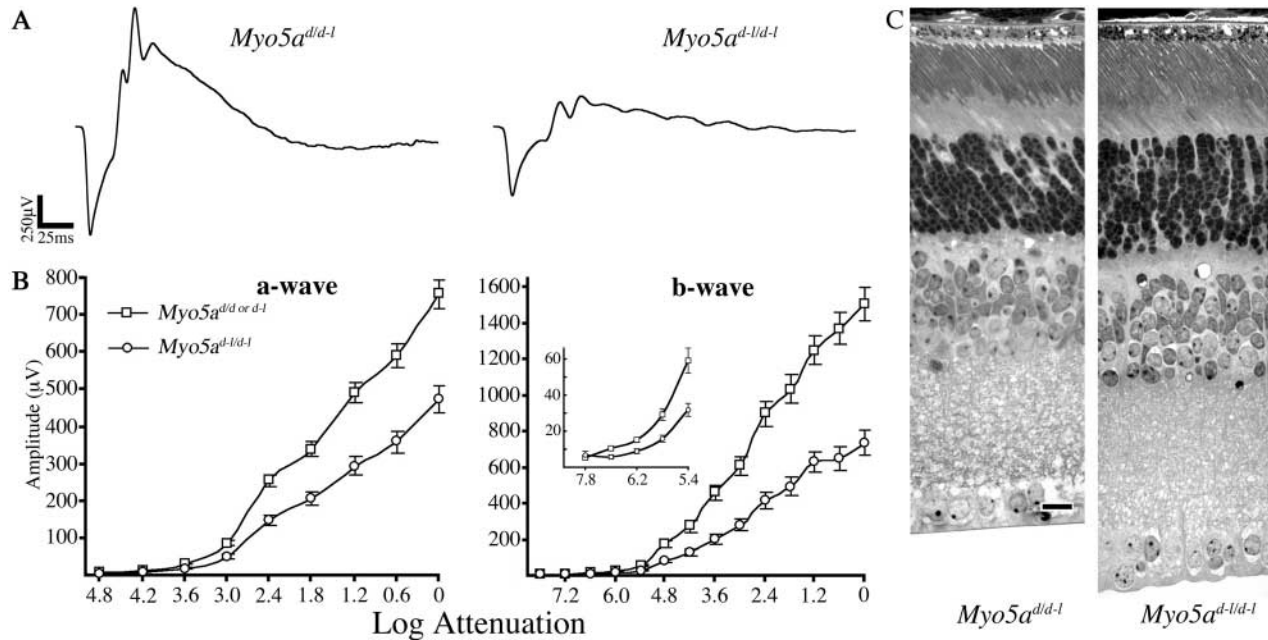
Fig. 1. Mutants in dilute lethal have severely disrupted electroretinographic responses. (A) Waveforms of electroretinograms from a dilute mouse ($Myo5a^d/Myo5a^d$) on the left and a dilute lethal mutant ($Myo5a^{d-l}/Myo5a^{d-l}$) on the right, at the maximum light intensity. The a-wave is the first negative (downward) deflection and the b-wave is the later positive deflection, with oscillatory potentials caused by retinal interneuron activity superimposed. The dilute lethal waveforms show reduced amplitudes of response and an anomalous shape caused by disproportionate reduction of the b-wave. Scale bar for both waveforms shown on left. (B) Response amplitudes ± standard error plotted as a function of light intensity: (left) a-waves; (right) b-waves. There is a significant difference between dilute and dilute lethal amplitudes for a-waves from 4.2 log units of attenuation (4.2-3.6, $P<0.01$; 3.3-0, $P<0.001$) and from 7.2 log units of attenuation for the b-waves (7.2-4.8, $P<0.005$; 4.2-0, $P<0.001$; see inset). At maximum flash intensity, a-wave amplitudes are reduced by 38% and b-wave amplitudes by 51% in $Myo5a^{d-l}/Myo5a^{d-l}$ ($n=12$) compared with $Myo5a^d/Myo5a^{d\ or\ d-l}$ ($n=12$; includes six $Myo5a^d/Myo5a^d$ and six $Myo5a^d/Myo5^{d-l}$; there was no significant difference between these two genotypes for any electroretinographic parameter analysed, $P>0.25$ for all). All mice were between postnatal days 19 and 25 (both groups averaged 21 days of age), ages when the $Myo5a^{d-l}/Myo5a^{d-l}$ are clearly afflicted with the neurological phenotype. (C) Light microscopy of retinas from a $Myo5a^d/Myo5a^d$ dilute control (left) and a $Myo5a^{d-l}/Myo5a^{d-l}$ dilute lethal mutant (right), at 20 days of age. No obvious differences were seen in outer segment length or any other feature of gross structure. Scale bar, 10 µm.

or completely circular profile (Fig. 3B,E; >95% of the ribbons in control retinas had a normal ribbon shape). Many of the rod spherules with normal-looking ribbons were still abnormal. In these spherules, clusters of presumed synaptic vesicles were ectopically located, within the spherule but not opposite the synapse (Fig. 3C,D,F). We also saw abnormal ribbons and ectopic clusters of vesicles in cone photoreceptors (data not shown). The ribbon synapses appeared normal in the non-lethal dilute genotypes. Moreover, the ribbon synapses of bipolar cells, in the inner plexiform layer, appeared normal in dilute lethal and non-lethal mutants (Fig. 3G).

Myosin Va is one of several unconventional myosin molecules expressed in the retina. We have demonstrated previously that ERG amplitudes are reduced by around 20% in $Myo7a^{4626SB}$ mutants (Libby and Steel, 2001), and that myosin VIIa is expressed in photoreceptor cells (Liu et al., 1997; Wolfrum et al., 1998). We asked if myosin VIIa and myosin Va showed any signs of interactions by intercrossing the two mutant stocks and deriving mice that were homozygous for both dilute lethal and the $Myo7a^{4626SB}$ mutation, which is believed to be a null allele (Hasson et al., 1997; Liu et al., 1999). These double mutants were viable and could be distinguished by their dilute coat color and their

behavior, which included head bobbing and circling as well as progressive ataxia and paralysis. ERG a- and b-wave responses from the double mutants were reduced to around 40% and 50%, respectively, of the level of responses of the control group (Fig. 4). The same reduction in a- and b-wave response amplitude was measured in dilute lethal homozygotes that were either homozygous mutant, heterozygous or wild type at the $Myo7a$ locus, suggesting that there was no additive effect. Mice that were homozygous for $Myo7a^{4626SB}$ but carried at least one wild-type allele at the $Myo5a$ locus showed a 20% reduction in both a- and b-waves (Fig. 4), similar to the reduction in $Myo7a$ mutants that we have reported previously (Libby and Steel, 2001). Finally, the control group included a mixture of mice heterozygous or wild type at both the $Myo5a$ and $Myo7a$ loci, and were thus wild type for coat color (agouti). The relationship between dilute lethal ERG amplitudes and those of this non-dilute control group was the same as for the comparison of dilute lethal and dilute non-lethal ($Myo5a^d$) ERGs shown in Fig. 1. This observation confirms that the ERG abnormalities we see in dilute lethal mutants are associated with the neurological effects of the mutation rather than the pigmentation effects.
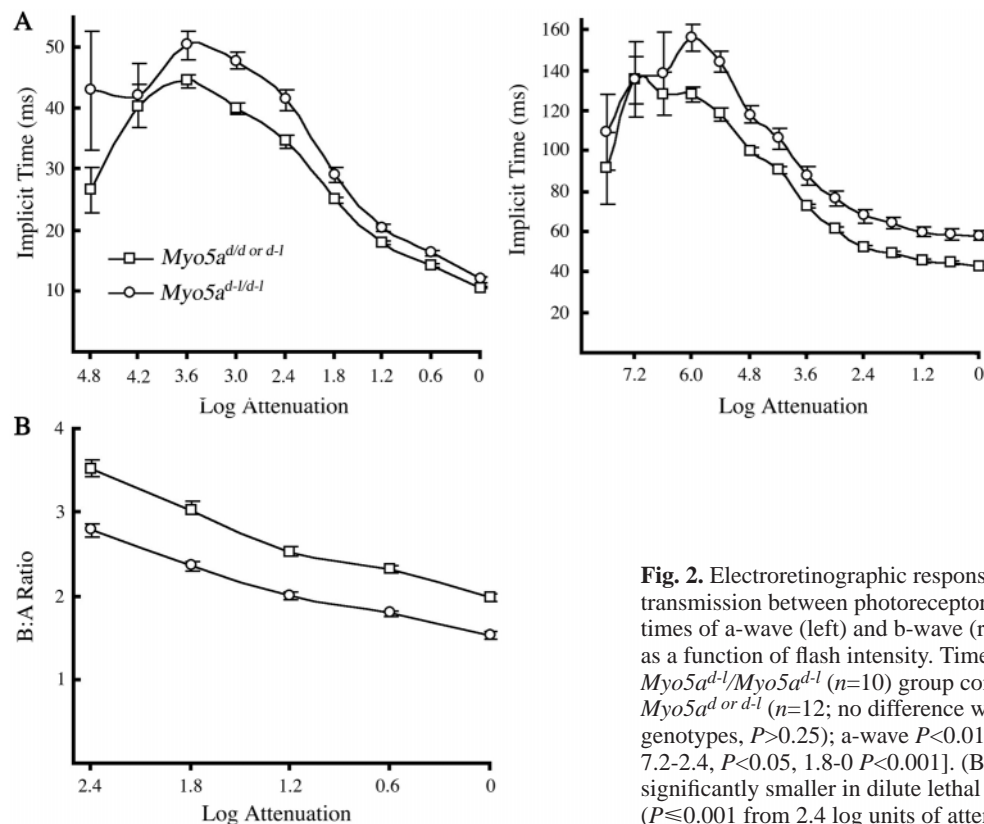
Fig. 2. Electroretinographic responses suggest abnormal synaptic transmission between photoreceptors and retinal interneurons. (A) Implicit times of a-wave (left) and b-wave (right) peaks, with standard errors, plotted as a function of flash intensity. Times are significantly longer in the $Myo5a^{d-l}/Myo5a^{d-l}$ (n=10) group compared with the controls [$Myo5a^{d}/Myo5a^{d\ or\ d-l}$ (n=12; no difference was observed between the two control genotypes, P>0.25); a-wave P<0.01 from 3 log units of attenuation; b-wave, 7.2-2.4, P<0.05, 1.8-0 P<0.001]. (B) Ratios of b- to a-wave amplitudes are significantly smaller in dilute lethal mutants than the control dilute group (P≤0.001 from 2.4 log units of attenuation).

Discussion

We report here clear evidence of a role for myosin Va in synaptic function in the retina. In the dilute lethal mutant, we observed a large reduction in ERG a-wave amplitude, a larger decrease in b-wave amplitude and increased implicit times for both waves. We also observed defective synaptic ultrastructure in the photoreceptor cells, which probably underlies the aberrant electrophysiology.

Interestingly, the ribbon synapses of the bipolar cells were normal, consistent with the retinal localization of myosin Va, which is concentrated in the photoreceptor but not second-order neuron synapses (Schlamp and Williams, 1996). Myosin Va has also been detected at the photoreceptor inner segment as well as the synapse (Schlamp and Williams, 1996), so that a role in inner segment function, in addition to its role in synaptic activity, might explain our observation of a reduced ERG a-wave in mutant mice. Alternatively, there might be some feedback from post-synaptic activity upon photoreceptor function.

Mice with the dilute lethal mutation show deteriorating condition and are undersized. It is possible that the physiological and anatomical abnormalities observed are a secondary result of this progressive illness. However, a rat model of desmosterolosis, a lethal disease involving cholesterol biosynthesis, has several similarities to dilute lethal mutants, including small size and death by 4 weeks of age (only 18% survival rate after postnatal day 28). There were no electroretinographic abnormalities found in these animals (Fliesler et al., 2000). Therefore, it is unlikely the phenotypes described here are the result of the general health of the dilute mutants, but rather are the result of a direct role of myosin Va

(consistent with its expression pattern) in photoreceptor synaptic function.

Several other mutants have been reported to show reduced ERG amplitudes that disproportionately affect the b-wave, including laminin β2 (*Lamb2*), neurotrophic tyrosine kinase receptor type 2 (*Ntrk2*) and bassoon (*Bsn*) mouse mutants and the *no optokinetic response c* (*nrc*) zebrafish mutant (Allwardt et al., 2001; Dick et al., 2003; Libby et al., 1999; Rohrer et al., 1999). The *Ntrk2*-null mouse showed retarded development of the retina (Rohrer et al., 1999), whereas we saw no evidence of a general retardation in development in dilute lethal retinas. *Bsn*, *nrc* and *Lamb2* mutants all have electroretinographic responses – similar to those found in dilute lethal – that suggest involvement with photoreceptor synaptic function (Allwardt et al., 2001; Dick et al., 2003; Libby et al., 1999). All three of these mutants have floating ribbons, suggesting that these proteins are required (either directly or indirectly) for anchoring the ribbon to the synapse and that this defect leads to synaptic failure. The dilute lethal mice have a different morphological defect. The ribbon in dilute lethal photoreceptors is properly localized but it often has an abnormal club-like shape. Furthermore, in dilute lethal mice, synaptic vesicles often do not associate with the ribbon, which they do in the *Bsn*, *nrc* and *Lamb2* mutants. These data suggest that myosin Va is not necessary for proper ribbon localization but might be important in establishing or maintaining the function of the ribbon, in the fast and continuous synaptic vesicle release that occurs at the photoreceptor synapse.

Myosin Va is present in conventional presynaptic terminals in the CNS and localizes with synaptic vesicle markers (Bridgman, 1999; Evans et al., 1998; Prekeris and Terrian,
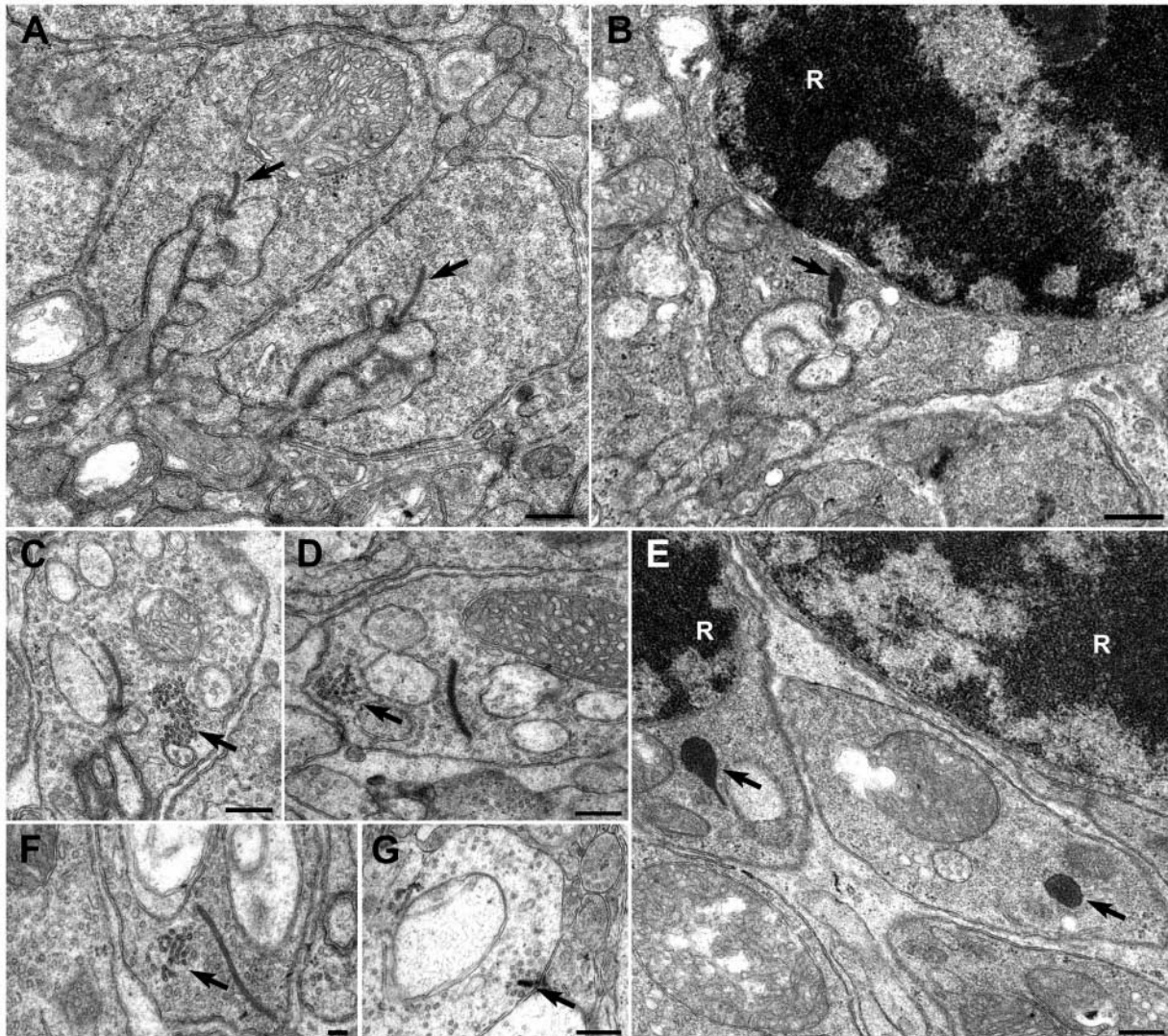
Fig. 3. Dilute lethal mutants have abnormal photoreceptor synapses. Transmission electron micrographs of synaptic regions. Ribbon synapses (arrows) look normal in the control mice (A). In $Myo5a^{d-l}/Myo5a^{d-l}$ mice, the ribbons of photoreceptor synapses appear club-like (B,E), although the ribbons of bipolar cell synapses appear normal (G). Photoreceptor synapses of $Myo5a^{d-l}/Myo5a^{d-l}$ mutants also possess clusters of presumed synaptic vesicles that are ectopically located (C,D,F, arrows). At least three sections were examined from each of four $Myo5a^{d-l}/Myo5a^{d-l}$ mice (abnormalities were consistently observed in the mutant mice) and two control mice ($Myo5a^{d}/Myo5a^{d \ or \ d-l}$; the synaptic morphology of these mice is similar to all wild-type mice we have previously examined, this dilute genotype does not affect synaptic morphology, which would be expect from their ERGs). Scale bars, 100 nm (F), 300 nm (others).

1997). It has been hypothesized that myosin Va is important in recycling and/or replenishing synaptic vesicles (Bridgman, 1999; Evans et al., 1998; Prekeris and Terrian, 1997; Schnell and Nicoll, 2001). In fact, Bridgman reported abnormal accumulation of synaptic vesicles in the presynaptic terminals of cerebellar granule cells of dilute lethal mice. In chromaffin cells, myosin Va has been shown to be important in replenishing neurosecretory vesicles (Rose et al., 2003). These data, coupled with our results, support the hypothesis that myosin Va has an important role in maintaining an adequate number of vesicles available for release. This might be particularly important at the photoreceptor synapse because there can be long periods of sustained, continuous synaptic vesicle release. If myosin Va has a direct role in synaptic

vesicle availability, it could explain why some ribbons are found without synaptic vesicles in the dilute lethal photoreceptors. It could also explain why so many ribbons have an abnormal morphology. It is now well established that the length and gross morphology of the mouse photoreceptor ribbons can change and that this change is directly related to the light cycle (or potentially synaptic activity) (Adly et al., 1999; Balkema et al., 2001; Spiwoks-Becker et al., 2004). Our results suggest that myosin Va might play a role in this dynamic regulation of synaptic ribbon morphology.

Although myosin Va is found in many CNS presynaptic terminals, no defects in synaptic transmission have yet been reported. Because of the continuous vesicle release from the photoreceptor synapses, requiring greater synaptic vesicle
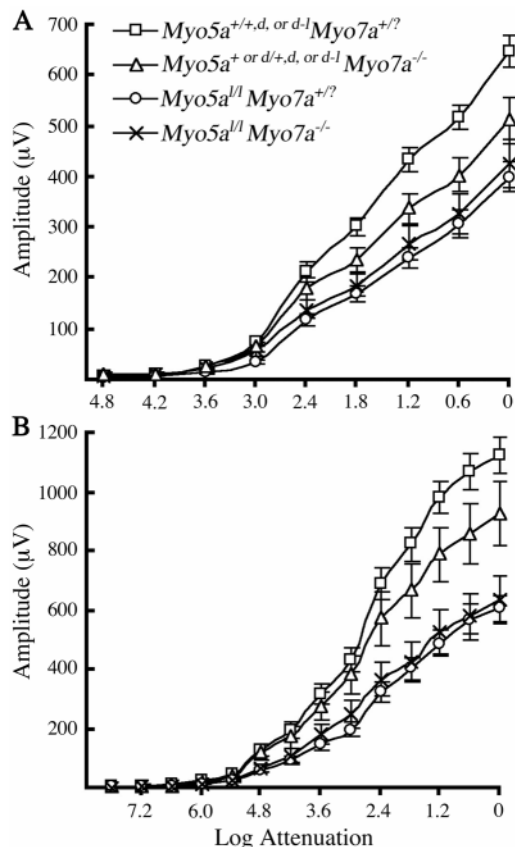
Fig. 4. Myosin Va and Myosin VIIa have no additive impact on visual function. Response amplitudes ± standard error plotted as a function of light intensity, from mice from the intercross between the *Myo5a* and *Myo7a* mutants (a presumptive null allele of myosin VIIa, *Myo7a^{4626SB}*, was used and is referred to as –): (A) a-waves; (B) b-waves. Four groups of mice are shown: the control group of mice that were heterozygous or wild type at both loci (*Myo5a$^{+/+,d or d-l}$*, *Myo7a$^{+/?}$*; all had normal pigmentation); mice homozygous for the *Myo7a* mutation and carrying a non-lethal genotype for *Myo5a* (*Myo5$^{+ or d/+,d or d-l}$*, *Myo7a$^{-/-}$*); mice homozygous for dilute lethal and heterozygous or wild type at the *Myo7a* locus (*Myo5a$^{d-l/d-l}$*, *Myo7a$^{+/?}$*); and mice homozygous for both dilute lethal and *Myo7a* mutations (*Myo5a^{d-l}/Myo5a^{d-l}*, *Myo7a$^{-/-}$*). There is no evidence of additivity of the of the 50% reduction in amplitude in dilute lethals and the 20% reduction with *Myo7a* mutants. Number of mice analyzed for each genotype: *Myo5a$^{+/+,d or d-l}$*, *Myo7a$^{+/?}$*, 9; *Myo5$^{+ or d/+,d or d-l}$*, *Myo7$^{-/-}$*, 8; *Myo5a$^{d-l/d-l}$*, *Myo7a$^{+/?}$*, 13; *Myo5a$^{d-l/d-l}$*, *Myo7a$^{-/-}$*, 7.

replenishment, defects in the absence of myosin Va might be more readily evident at this synapse. Thus, our data might support a general role for myosin Va in CNS synaptic function in general. Interestingly, Miyata et al. (Miyata et al., 2000) have shown that myosin Va is important in establishing long-term synaptic depression in cerebellar Purkinje cells. Abnormal Purkinje-cell smooth endoplasmic reticulum and a lack of inositol-triphosphate-receptor-laden reticular tubules in the postsynaptic spine of cerebellar neurons have been reported in dilute lethal mice (Dekker-Ohno et al., 1993; Petralia et al., 2002; Takagishi et al., 1996) and these abnormalities could result in the loss of long-term synaptic depression in Purkinje

cells (Miyata et al., 2000). This post-synaptic physiological phenotype might be caused by myosin Va being necessary for proper organelle localization (its more traditional role) and not directly in synaptic transmission. Importantly, Miyata et al. (Miyata et al., 2000) did not find any abnormalities in synaptic transmission or morphology. Therefore, there are at least two distinct physiological roles for myosin Va in the mammalian CNS. One involves postsynaptic processing that might or might not be a secondary consequence of myosin Va's normal function (Miyata et al., 2000). The other, described here, is a role of myosin Va in presynaptic physiology. This is supported by the fact that myosin Va is localized to the synapse. Our work suggests the possibility that myosin Va mutations lead to synaptic dysfunction at certain CNS synapses and/or under certain conditions. This possibility should be considered as a potential cause of the more generalized neurological defects observed in the dilute lethal mouse mutant and patients with Griscelli syndrome type 1 and Elejalde syndrome.

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