Changes in Retinal Synaptic Proteins in the Transgenic Model Expressing a Mutant HRG4 (UNC119)

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PURPOSE. HRG4 (UNC119) is a photoreceptor synaptic protein, a truncation mutant of which has been shown to cause late-onset cone–rod dystrophy in a patient and retinal degeneration with marked synaptic degeneration in a transgenic model. To investigate the mechanism of the retinal degeneration, the effect of the mutant protein expression on the other synaptic proteins was examined.

METHODS. The status of 12 known synaptic proteins in the retinas of 5-month- and 13-month-old HRG4 transgenic and control mice was examined by Western blot analysis. Three selected proteins were analyzed by immunohistochemistry in the 13-month-old retinas. The 12 proteins were tested for binding to HRG4 by a direct-binding assay and Western blot analysis.

RESULTS. A decrease in three synaptic vesicle proteins and an increase in five cytoplasmic and plasma membrane proteins was detected by Western blot analysis in the older but not the younger transgenic retinas. These changes were demonstrated in both the outer and inner plexiform layers of the retina by immunofluorescence, along with a significant reduction in the thickness of the inner plexiform layer. A 23-kDa specie was found to bind to HRG4, but none of the 12 synaptic proteins matched it, according to immunoblot analysis.

CONCLUSIONS. The expression of a mutant HRG4 protein in the photoreceptor synapses of the transgenic model had an intrasynaptic and transsynaptic effect, resulting in a decrease in three synaptic vesicle proteins, an increase in five cytoplasmic and plasma membrane proteins, and a significant reduction in the thickness of the inner plexiform layer. These changes were age dependent, similar to the pathologic phenotype of the transgenic model and the patient, and supported a close relationship of HRG4 with other participants in synaptic vesicle function. This interaction was not mediated by a direct coupling of HRG4 with any of the tested synaptic proteins but possibly through interaction with a 23-kDa protein. (Invest Ophthalmol Vis Sci. 2002;43:308–313)

To discover novel retina-expressed genes that may be important in retinal degeneration, we used a PCR-enhanced subtractive cDNA cloning strategy to isolate a number of retina-enriched genes,1,2 including HRG4.3 HRG4 is a novel 35-kDa synaptic protein found in abundance in rod and cone photoreceptor ribbon synapses, primarily in association with synaptic vesicles.4 HRG4 is homologous to UNC119, a Caenorhabditis elegans neuroprotein found to cause defects in coordination, feeding, and chemosensation when mutated.5 HRG4 was also found to be homologous to cGMP phosphodiesterase δ which has been demonstrated to solubilize the membrane-bound form of rod phosphodiesterase6 and to bind to the retinitis pigmentosa 3 gene, the retinitis pigmentosa guanosine triphosphatase (GTPase) regulator, RPGR.7 The HRG4 gene was mapped to 17q11.2 and shown to consist of 5 exons with a promoter containing GC boxes.8 Screening of the HRG4 gene in patients with retinopathies uncovered a heterozygous premature termination codon mutation in a patient with late-onset cone–rod dystrophy.9 A transgenic mouse model carrying the identical mutation in HRG4 showed a late-onset retinal degeneration characterized by electroretinogram (ERG) b-wave depression, marked photoreceptor synaptic degeneration, and probable transsynaptic degeneration affecting the inner retina.10 The association of HRG4 with synaptic vesicles and the phenotype of the transgenic model expressing the mutant HRG4, consisting of depressed b-wave reflecting a problem in synaptic transmission and synaptic degeneration, pointed to a role of HRG4 in synaptic vesicle function. To pursue this possibility, the effect of the mutant HRG4 on the known participants in synaptic function was examined in the transgenic model. The other synaptic proteins examined included those involved in the function of synaptic vesicles (synapsin IIa, synaptotagmin, synaptogyrin, doc2), docking and exocytosis (syntaxin 4 and 6, SNAP25, complexin-2, and munc-18), and endocytosis (rabphilin 3A, rabaptin 5, and rsec8; Fig. 1). A reduction in proteins associated with synaptic vesicles and a possible compensatory increase in proteins involved in docking-exocytosis and endocytosis were observed in both the outer and inner plexiform layers (OPL, IPL) of the retina, consistent with a synaptic and transsynaptic effect, respectively, of the mutant HRG4. None of the synaptic proteins analyzed, however, interacted directly with HRG4. Evidence of marked transsynaptic degeneration was observed in the inner retina with a significant reduction in the size of the IPL.

METHODS

Preparation of Mouse Retinal Proteins

Age-matched transgenic and nontransgenic control mice were examined at the ages of 5 and 13 months. All procedures using animals were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Fresh mouse retinas (six retinas per age group) were homogenized in a buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 100 μg/mL phenylmethylsulfonyl fluoride (PMSF), 1 μg/mL aprotinin, and 1% Triton X-100, centrifuged at 15,000 g for 5 minutes at 4°C to remove cellular debris, and used in the experiments. The protein concentration of the supernatant was measured by the modified Lowry method.
Preparation of Frozen Sections

Age-matched transgenic and nontransgenic control mice were examined at 13 months of age. Mouse eyes were enucleated, fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 2 hours, and soaked in 20% sucrose in 0.1 M phosphate buffer overnight. Before sectioning, the eyes were embedded in optimal cutting temperature compound (OCT; Miles Laboratories, Inc., Elkhart, IN) and frozen in liquid nitrogen. Six-micrometer sections were cut on a cryostat and stored at −20°C until used.

Immunofluorescence

Frozen sections were analyzed by immunofluorescence with polyclonal antibodies to three known synaptic proteins, because the original monoclonal antibodies used for the Western blot analysis did not work well for immunofluorescence. The three proteins examined were syntaxin-1B, synaptotagmin, and synapsin (Synaptic Systems GmbH, Göttingen, Germany). The frozen sections were dried at room temperature for 20 minutes, blocked overnight with blocking buffer (10% goat serum, 1% BSA, 0.1% Triton X-100 in PBS [pH 7.4]), and incubated with the primary antibody in PBS at a dilution of 1:50 for 90 minutes. After three washes in PBS, the sections were incubated with the secondary antibody (FITC-labeled goat anti-rabbit IgG; Life Technologies, Grand Island, NY) at a dilution of 1:50 for 30 minutes, followed by three final washes in PBS. The sections were mounted (Fluoromount-G; Southern Biotechnology Associates, Inc, Birmingham, AL) and photographed with a photomicroscope (Carl Zeiss, Oberkochen, Germany). The fluorescence signals were semiquantitated through photoimaging and analysis of the pixel histograms. Three different sections were obtained from each group and examined.

Direct Binding Analysis of HRG4

Rat retinas were homogenized in PBS containing 1 μg/mL aprotinin and 100 μg/mL PMSF and centrifuged at 15,000g. The supernatant was recovered and measured for protein concentration. Two hundred microliters (~600 μg) of glutathione-Sepharose-complexed with glutathione-Transferase (GST)-HRG4 was prepared as described, incubated with 1 μg of rat retinal supernatant overnight at 4°C, washed five times with PBS, and analyzed by SDS-PAGE and Coomassie blue staining.

RESULTS

Twelve synaptic proteins were examined by Western blot analysis of transgenic and age-matched nontransgenic mouse retinas. The 12 proteins included 4 associated with the synaptic vesicle membrane either integrally (synaptogyrin) or peripherally (synapsin IIa, synaptotagmin, and doc2), 4 associated with the plasma membrane and involved in vesicle docking and fusion (syntaxin 4 and 6, SNAP-25, munc-18, and complexin-2), and 3 found in the cytoplasm that reversibly associate with synaptic vesicle or plasma membrane protein (rabphilin-3A, rabaptin-5, and rsec8; Fig. 1). Because the appearance of the pathologic phenotype in the HRG4 transgenic model was age dependent, present only in mice aged 1 year or more, young (5 months) and older (13 months) transgenic mice were studied.

With equal amount of proteins being examined, the Western blot analysis of retinal proteins from the young and older HRG4 transgenic and nontransgenic control mice with monoclonal antibodies against the 12 synaptic proteins demonstrated a variable pattern of expression, depending on the age and transgenicity. The results, which represent the expression of each synaptic protein as a fraction of the total protein, are shown in Figure 2 and summarized in Table 1. In the 5-month-old mouse no differences between transgenic and nontransgenic retinas were seen in expression of SNAP-25 (Fig. 2A), syntaxin-6 (Fig. 2C), rsec8 (Fig. 2E), rabaptin-5 (Fig. 2E), munc-18 (Fig. 2F), synaptotagmin (Fig. 2H), synapsin IIa (Fig. 2I), rabphilin-3A (Fig. 2J), doc2 (Fig. 2K), and synaptogyrin.
(Fig. 2L), whereas expression of syntaxin-4 (Fig. 2B) and complexin-2 (Fig. 2G) was absent (Table 1). In the 13-month-old mouse retina, in addition to the same 10 synaptic proteins, syntaxin-4 was expressed (Fig. 2B), but 8 proteins showed a difference between the transgenic and nontransgenic retinas (Table 1). Three proteins associated with the synaptic vesicle—synapsin IIa (Fig. 2I), synaptotagmin (Fig. 2H), and doc2 (Fig. 2K)—showed a decrease of varying degrees in the transgenic animal compared with normal control (the size of the downward arrows in Table 1 approximates the degree of decrease). In contrast, three cytoplasmic proteins—rabphilin-3A (Fig. 2J), rabaptin-5 (Fig. 2E), and rsec8 (Fig. 2D) and two plasma membrane proteins—syntaxin-4 (Fig. 2B) and syntaxin-6 (Fig. 2C)—showed varying degrees of increase in expression. The expression of synaptogyrin (Fig. 2L), SNAP-25 (Fig. 2A) and munc-18 (Fig. 2F) appeared to remain equal in the transgenic and normal retinas. Unlike syntaxin-4, complexin-2 (Fig. 2G) remained undetectable in the older mouse retinas.

For some of the Western blot analyses, a few cross-reacting bands were observed besides the specific synaptic protein band confirmed by the positive control, probably due to the presence of some cross-reacting epitopes.

To localize within the normal and transgenic retinas the synaptic proteins showing marked differences in level by Western blot analysis of the retinal homogenates, immunofluorescence analyses of synapsin, synaptotagmin, and syntaxin were performed on frozen sections of 13-month-old transgenic and control mouse retinas. Of the two synaptic vesicle-associated

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<thead>
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<th>Protein</th>
<th>Young Tg</th>
<th>Old Tg</th>
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<tr>
<td>Synaptic vesicle</td>
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<td>↓</td>
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<tr>
<td></td>
<td>Synaptotagmin</td>
<td>=</td>
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<td></td>
<td>Doc2</td>
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<tr>
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<td></td>
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The result of the Western blot analysis with the 12 antibodies for the young (5 months) and old (13 months) transgenic (Tg) retina compared with normal control is represented semiquantitatively. Arrows of different size, up or down, represent the approximate amount of increase or decrease, respectively, in the level of the protein in the Tg compared with normal control. =, no difference between Tg and normal; 0, not present.
proteins that showed a marked decrease by Western blot analysis (i.e., synapsin and synaptotagmin) synapsin was detectable in the IPL of the mouse retina. Immunostaining of the transgenic retina revealed a reduction in the thickness of the IPL in animals of this age (Fig. 3A, compare N and Tg). The fluorescence intensity analysis revealed decreased immunofluorescence of synapsin (~40% decrease in intensity) in the IPL of the transgenic retinas compared with control (Fig. 3A). Synapsin was not expressed in the outer plexiform layer (OPL), the site of localization of the HRG4 protein.

Synaptotagmin, by contrast, was expressed in both the IPL and OPL of the normal and transgenic retinas (Fig. 3B). The IPL was thinned by ~50% at this age, and the inner nuclear layer (INL) was also reduced. No reduction in the thickness of the OPL was apparent by immunofluorescence with the synaptotagmin antibodies (Fig. 3B, lower images). Quantitative analysis of the fluorescent intensity revealed an ~30% decrease in staining of both the IPL and OPL in the transgenic retinas.

By Western blot analysis, the presynaptic membrane proteins syntaxin-4 and -6 both showed an increase in the transgenic retinas. These antibodies were not useful for immunofluorescence; however, a polyclonal antibody to syntaxin-1B was suitable for immunostaining the retina and was used to determine the localization of syntaxin in the normal and transgenic retinas. Immunostaining with this antibody revealed staining exclusively in the IPL (Fig. 3C). Quantitative analysis of the fluorescence intensity revealed an increase in immunofluorescence of approximately 25% in the transgenic IPL compared with control, despite the marked reduction in thickness of the IPL in the transgenic retina. Thus syntaxin-1B appeared to be overexpressed in the transgenic retina IPL, despite the ongoing degeneration of the IPL. In all immunostaining experi-
The expression of synaptotagmin, synapsin, rabphilin, and synaptogyrin was demonstrated it to be a photoreceptor ribbon synapse protein, present in both rod and cone photoreceptors and associated mostly with synaptic vesicles. Although its function is unknown, its localization suggests that it may play a role in synaptic vesicle function. The discovery of a premature termination mutation in HRG4 in a patient with late-onset cone–rod dystrophy implicates it in human retinal degeneration. The demonstration of late-onset retinal degeneration in a transgenic model carrying the identical HRG4 mutation confirms the pathogenic role that this protein can play, and the reduced ERG b-wave and significant synaptic degeneration observed in the model supports the postulated role of HRG4 in synaptic–vesicle function. The truncated mutant HRG4 was postulated to act in a dominant negative fashion by either binding ineffectually to the target protein of HRG4 or directly interfering with the action of HRG4.

The specific decrease in three synaptic vesicle–associated proteins in the transgenic retina (synapsin IIa, synaptotagmin, and Doc2) presented in this work go further to support the role of HRG4 in synaptic vesicle function. The time course of appearance of this reduction was consistent with the appearance of pathology in the transgenic model. Transgenic mice less than 1 year old do not manifest significant pathology in retinal function or morphology. The results of the Western blot analysis (Table 1) showed that levels of these synaptic proteins did not differ in the young transgenic and nontransgenic retinas. Functional (reduced ERG b-wave) and structural (retinal disorganization/degeneration) defects begin to appear in the transgenic retina starting at approximately 1 year of age. In this study, reduction in the three synaptic vesicle proteins was demonstrated in the 13-month-old transgenic retina compared with normal control.

One of these proteins, synaptotagmin, is present in the OPL, the site of localization of HRG4. Synaptotagmin, a postulated Ca2+-regulated trigger of exocytosis, binds to the synaptic vesicle through a 27-amino-acid transmembrane domain. HRG4 may somehow facilitate its interaction with the synaptic vesicle in the OPL, and the presence of the mutant HRG4 may negatively affect such interaction, leading to its accelerated degradation. HRG4, however, was not shown to interact directly with synaptotagmin. The effect of HRG4 on synaptotagmin is most likely mediated by another protein with which HRG4 directly interacts. Similar to the result presented herein, an antisense inactivation of synapsin II has been shown to cause a reduction in the level of several other synaptic vesicle proteins, including synaptotagmin and synapto- physin in cultured hippocampal neurons.

Because the result of the Western blot analysis demonstrated only the level of each synaptic protein as a fraction of the total retinal protein, actual visualization of these proteins in the retina by immunofluorescence was required to determine where and how these proteins were changing. The decrease in synaptotagmin and synapsin in the IPL confirms the transsynaptic effect that was demonstrated to be present in this animal model. Evidence of inner retinal abnormality, reflecting a transsynaptic effect, has been reported in outer retinal diseases such as cone–rod and rod–cone dystrophy. In addition to the reduced immunofluorescence of the two proteins, there was a significant reduction in the actual thickness of the IPL in the transgenic retina, reflecting the severity of the transsynaptic degeneration in this model. In contrast to other proteins known to cause human photoreceptor-based retinal degenerations, HRG4 is the only photoreceptor protein localized in the inner end of the photoreceptors—that is, the synaptic termini. The marked degree of transsynaptic degeneration observed in the HRG4 transgenic model might reflect the synaptic site of abnormality in this model.

The decrease in the immunofluorescence of synaptotagmin and synapsin indicates either a decrease in the density of synapses and synaptic vesicles or in the level of the proteins per vesicle. Because severe synaptic degeneration is not seen in the transgenic retina until the animals are much older (24 months), the reduction in the synaptic vesicle proteins at 13 months is most likely not due to actual decrease in the density

**Figure 4.** SDS-PAGE of HRG4-bound protein. Recombinantly expressed GST-HRG4 hybrid protein was complexed with glutathione-Sepharose, incubated with rat retinal proteins, and analyzed by SDS-PAGE and Coomassie blue staining. Lane A: retinal proteins incubated with glutathione-Sepharose alone; lane B: retinal proteins incubated with HRG4–GST/glutathione-Sepharose; lane C: retinal proteins incubated with GST/glutathione-Sepharose. Arrowhead: ~23 kDa protein bound by HRG4. Left: molecular size markers in kilodaltons.
of synaptic vesicles present. In support of this, the level of an integral synaptic vesicle membrane protein, syntaptogyrin,\(^{16}\) was unchanged in the transgenic retina, suggesting that the number of vesicles had not changed. In fact, it may even have increased, considering the reduced size of the IPL. Thus, the expression of the mutant HRG4 in the OPL appeared to negatively affect, not only the levels of synaptic vesicle proteins in the OPL and IPL, but also the actual thickness of the IPL transsynaptically, all contributing to the decrease in the synaptic vesicle proteins observed by Western blot analysis. The relatively modest decreases in the concentration of synapsin and syntaptotagmin demonstrated by immunofluorescence in the transgenic IPL and OPL indicate that a large part of the decrease shown in the Western blot analysis was due to the reduction in the thickness of the IPL. The specific pattern of up-and-down changes observed in the synaptic proteins and the absence of significant synaptic degeneration at 13 months of age, however, support that these changes are not merely the secondary result of the degenerative process but are most likely specifically induced by the expression of mutant HRG4. The mechanism by which the mutant HRG4 in the OPL affects the synaptic vesicle proteins transsynaptically to reduce their levels in the IPL or to reduce the size of the IPL itself is not clear.

It was interesting that in contrast to the reduction in three synaptic vesicle proteins, an increase in three cytoplasmic (rabaptin-5, rsc8, and rabphilin-3A) and two plasma membrane (syntaxin-4 and syntaxin-6) proteins was observed in the 13-month-old transgenic retina by Western blot analysis. The increase in syntaxin was confirmed by immunostaining in the transgenic IPL, despite its reduced thickness compared with normal control. Thus in this case, the transsynaptic effect was an abnormal increase. The observed increase in the IPL may be a compensatory increase in the presence of ineffective neurotransmission from the OPL in the transgenic retina. The increase, in fact, may not be restricted to the five proteins shown by the Western blot analysis. Because of the reduced thickness of the IPL in the transgenic retina, the two plasma membrane proteins, SNAP-25 and munc-18, which did not show a change in level by Western blot analysis, are probably also increased. For these proteins to show the same fraction of the total protein by Western blot analysis despite the reduction in the thickness of the IPL, their concentrations would have had to increase in the IPL. The mechanism of coordinate expression of synaptic proteins is not known, but the reactive nature of their expression has been demonstrated in the increase of v-SNARE proteins (i.e., cellubrevin and VAMP-2) and a t-SNARE protein (syntaxin-4), all shown to be important in the vesicular translocation of the glucose transporter GLUT4 to the cell membrane. This reactive increase is observed when the translocation cannot be initiated due to insulin resistance.\(^{17}\) The levels of the v-SNARE and t-SNARE proteins returned to normal once the insulin resistance was corrected.

The expression of HRG4 was observed in some of the Western blot analysis (e.g., rsc8 and syntaxin-4) may be a reflection of the relatively common problem observed with monoclonal antibodies generated with small peptides—that is, similarity of the small peptide epitope with other antigens. These cross-reacting bands, however, did not interefere significantly with the interpretation of the results, because the correct protein bands were clearly identified in the positive control lanes.

Precisely how the mutant HRG4 affects the levels of synaptic proteins as described in this report is not clear. The fact that it does, however, suggests that the normal function of HRG4 is related to the function and integrity of these synaptic proteins. The association of HRG4 with synaptic vesicles, documented by immunocytochemistry,\(^{3}\) and the significant negative effect of the mutant HRG4 on the proteins that interact peripherally with synaptic vesicles begins to hint at the function of HRG4. HRG4 may, for example, regulate the insertion and solubilization of proteins, such as syntaptogamin, in the synaptic vesicle membrane. However, HRG4 does not appear to accomplish this function by directly binding to those synaptic vesicle proteins showing a decrease in the transgenic model, because the direct binding experiment did not implicate any of them. Instead, a ~23kDa protein has been identified that appears to be the effector that probably mediates the function of HRG4. Whatever the precise function of HRG4 may be, it is important enough to the synapse that the expression of a mutant HRG4 leads to severe synaptic degeneration and retinal degeneration.\(^{9}\) The demonstration of which synaptic proteins are being affected by the expression of the mutant HRG4 and how opens the way to elucidating the molecular mechanism of the retinal degeneration in this model.

References


