Polyglutamine expansion causes neurodegeneration by altering the neuronal differentiation program

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Huntington’s disease (HD) and spinocerebellar ataxia type 7 (SCA7) belong to a group of inherited neurodegenerative diseases caused by polyglutamine (polyQ) expansion in corresponding proteins. Transcriptional alteration is a unifying feature of polyQ disorders; however, the relationship between polyQ-induced gene expression deregulation and degenerative processes remains unclear. R6/2 and R7E mouse models of HD and SCA7, respectively, present a comparable retinal degeneration characterized by progressive reduction of electroretinograph activity and important morphological changes of rod photoreceptors. The retina, which is a simple central nervous system tissue, allows correlating functional, morphological and molecular defects. Taking advantage of comparing polyQ-induced degeneration in two retina models, we combined gene expression profiling and molecular biology techniques to decipher the molecular pathways underlying polyQ expansion toxicity. We show that R7E and R6/2 retinal phenotype strongly correlates with loss of expression of a large cohort of genes specifically involved in phototransduction function and morphogenesis of differentiated rod photoreceptors. Accordingly, three key transcription factors (Nrl, Crx and Nr2e3) controlling rod differentiation genes, hence expression of photoreceptor specific traits, are down-regulated. Interestingly, other transcription factors known to cause inhibitory effects on photoreceptor differentiation when mis-expressed, such as Stat3, are aberrantly re-activated. Thus, our results suggest that independently from the protein context, polyQ expansion overrides the control of neuronal differentiation and maintenance, thereby causing dysfunction and degeneration.

INTRODUCTION

Huntington’s disease (HD) and spinocerebellar ataxia type 7 (SCA7) are inherited neurodegenerative diseases that belong to polyglutamine (polyQ) disorders, which also include spino-bulbar muscular atrophy, dentatorubro-pallidoluysian atrophy and the SCA1, SCA2, SCA3, SCA6 and SCA17 (1). The causal mutation is a CAG repeat expansion in the corresponding genes encoding an expanded polyQ tract in the proteins. PolyQ expansions confer a gain of toxic properties to mutant proteins, which display aberrant interactions with normal protein partners and accumulate into neurons to form intranuclear inclusions (NIs), a microscopic hallmark of these diseases (2).

Although polyQ disorders share common genetic features, they generate distinct pattern of neuronal degeneration despite overlapping protein expression areas (1). For instance,
the primary target in HD is the striatum, whereas SCA7 causes degeneration of the cerebellum and brain stem and is the only polyQ disorder affecting the retina. The mechanism underlying selective neurodegeneration is unknown. When compared with adult onset cases, juvenile patients who carry very large expansions often present a more severe phenotype resulting from broader brain degeneration, suggesting that neurons present different sensitivity depending on polyQ expansion length. In mouse models for polyQ disorders, expression of truncated mutant proteins bearing the polyQ expansion is more harmful than full-length proteins and can cause toxicity in neuronal cell types normally spared by the disease process, supporting the idea that polyQ expansion itself is toxic, while the protein context modulates the toxicity and contributes to the selectivity of neuronal degeneration (3).

Studies of mouse models for polyQ diseases revealed that mutant proteins induce neuronal dysfunction prior to causing cell death. Several non-exclusive mechanisms underlying polyQ toxicity have been proposed, such as impairment of protein folding and degradation, calcium homeostasis, axonal transport or synaptic transmission (2). Many of these defects are known to trigger and maintain neuronal stress conditions. Cumulative evidences indicate that deregulation of gene expression also occurs during polyQ pathogenesis (4,5). This might be the result of sequestration of transcriptional regulators into polyQ aggregates or of aberrant interactions between mutant proteins and nuclear factors regulating gene expression. For instance, mutant huntingtin (htt), the protein involved in HD, interacts with and impairs the function of various transcriptional activators such as SP1, TAF4 and CBP, which may contribute to neuronal degeneration in HD (5). Interestingly, ATXN7, the SCA7 gene product, was shown to be a subunit of the TFTC/STAGA transcription co-activator complex (6). It was recently proposed that mutant ATXN7 impairs the histone acetyltransferase activity of the complex (7,8). Gene deregulation might also result from a programmed cellular response to polyQ-induced stress (9).

Other evidences of gene deregulation are provided by DNA microarray analyses comparing gene expression profiles of polyQ disorder models versus control. These studies revealed that polyQ toxicity affects expression of genes involved in multiple cellular functions such as neuronal signaling, calcium regulation, stress and inflammation (4,10,11). Although these expression changes could have serious consequences for neuronal function, how they relate to the disease process remains unclear (4). Correlations between phenotypic features of mouse models and gene deregulation were difficult to establish. These issues are crucial to dissociate primary from secondary effects of polyQ toxicity and to draw a comprehensive scheme of the pathomechanism involved in polyQ diseases.

The retina provides several advantages over other CNS regions to study the mechanism of neuronal degeneration. Indeed, many of the molecular events regulating retina development, differentiation and function are known and correlations between functional, morphological and molecular defects underlying the retinopathy can often be made (12,13). The retina is composed of six neuronal cell types spatially subdivided into laminated layers. Photoreceptors, which constitute 70% of retinal neurons, comprise 97% of rods and 3% of cones in mouse retina. Differentiated photoreceptors display a characteristic morphology that defines the retinal outer nuclear and segment layers composed, respectively, of their small cell bodies, surrounding very compact nuclei, and of their protuberant cytoplasm.

The SCA7 retinal dystrophy was reproduced in one knockin and two transgenic mouse models (14–16). Despite different level and cell type specificity of mutated gene expression, these models display similar phenotypes characterized by intranuclear accumulation of mutant ATXN7 correlating with progressive electrotoretinogram (ERG) dysfunction and shortening of segment layers. Retinal dysfunction occurs prior to loss of photoreceptors. Opposite to SCA7 knockin mice that die from severe neurological phenotype at 14–19 weeks of age (16), SCA7 transgenic mice R7E, which express the mutant ATXN7 harboring 90Q in rod photoreceptors, have a normal life span (14). Strikingly, analysis of aged R7E revealed that rod ERG activity decreases to flat response despite limited loss of rod cells (17). Instead, the ERG defect appeared to result from morphological change of rods, which entirely lose their segments (17). Reduction of ERG activity is also accompanied by down-regulation of the rhodopsin (Rho) gene expression, suggesting that early transcriptional impairment underlies R7E rod dysfunction (17).

Interestingly, the HD mouse model, R6/2, also develops a progressive retinal degeneration comparable to the R7E retinopathy (18). This transgenic model ubiquitously expresses the mutated HD gene exon-1, which encodes the N-terminal 90 amino acids of htt protein with 150 glutamines (representing only 3% of htt) (19). R6/2 has a broader spectrum of brain degeneration than typically seen in HD. Similitudes of R7E and R6/2 phenotypes demonstrate that, independently of the protein context, polyQ expansion is sufficient to trigger retinal degeneration in mouse.

In the present study, we aimed at elucidating the molecular and cellular events underlying retinal degeneration in R7E and R6/2 mice. To this end, we performed gene expression profiling analysis of R7E and R6/2 retina. To correlate gene deregulation along the progression of R7E retinal degeneration, we examined R7E and the control R7N mice at onset and moderate stage of pathology. We also compared gene expression changes in R7E and R6/2 retina to highlight common deregulated molecular pathways, which have a high probability of being relevant to polyQ-induced retina degeneration. Our study reveals a strong correlation between the progressive dysfunction and morphological change of photoreceptors and loss of expression of mature rod genes in both R7E and R6/2 retina. More interestingly, we provide evidence that polyQ expansion, regardless of the protein context, compromises the genetic program maintaining photoreceptor differentiation, hence the expression of photoreceptor specific traits.

RESULTS

To get insight into molecular and cellular pathways involved in polyQ-induced retinal degeneration, we used genome-wide oligonucleotide microarrays (MOE430A Affymetrix) to
characterize gene expression profiles in retina of R7E versus R7N mice [which express wild-type (wt) ATXN7 with 10Q] and of R6/2 versus wt littermates. Retina RNA was prepared from four to six animals from each mouse line. Each RNA sample was independently hybridized on a different DNA microarray. Hybridization of control RNA samples (R7N or wt) indicated that nearly 50% of the array probe sets (about 11,500 out of 22,690) display a positive signal based on absent/present detection calls (MAS analysis). To select the differentially expressed genes, three consecutive filters were applied as detailed in Materials and Methods. A list of all deregulated transcripts identified in this study is available as Supplementary Material, Table S1.

Gene expression changes during SCA7 retinopathy progression

We first examined gene expression profiles in R7E compared with R7N at 3 and 9 weeks of age, which correspond to onset and moderate stages of disease, respectively (14,17). Until 3 weeks of age, R7E retina develops normally and displays no obvious phenotype. At 3 weeks, NIs are detected in some rod photoreceptors and the first functional abnormalities detected by scotopic ERG occur between 3 and 4 weeks of age. At 9 weeks, moderate stage is characterized by the presence of NIs in most rod cells and a marked reduction of ERG response. Nine-week-old retina also displays morphological alterations of rod photoreceptors characterized by loss of segments and enlarged nuclei with atypical decondensed chromatin (17,60). However, there is no significant neuronal loss at this stage. Later on, R7E retinopathy worsens towards flattening of ERG recording, complete loss of segment layers and thinning of the outer nuclear layer.

Comparison of gene expression of R7E versus R7N at retinopathy onset showed that the level of 106 transcripts was changed by 1.5-fold or more. Of these, 42% were over-expressed and 58% under-expressed in R7E versus R7N. At moderate stage of disease, a substantially higher number of transcripts (486) showed a change in expression level with a conserved ratio between over- (38%) and under-expressed (62%) transcripts. Venn diagram (Fig. 1A) intersecting gene expression changes at onset and moderate disease progression shows that 50% of differentially expressed transcripts at 3 weeks of age were specific to the onset of disease. The other 50% of early altered transcripts also displayed expression changes at moderate stage, the majority of which being under-expressed (73%).

Gene expression changes primarily due to polyQ expansion

Retinas of 9-week-old R7E and R6/2 mice display very similar retinopathy according to their rod ERG dysfunction and morphological alterations, which result in outer nuclear layer disorganization and segment layers reduction (18). R6/2 ERG dysfunction also involves cones and light-induced post-synaptic neurons, consistent with the ubiquitous expression of mutant htt-exon-1. We compared gene expression changes in 9-week-old R6/2 versus R7E retina, assuming that common changes are likely to concern rod photoreceptor expressed genes and to be primarily due to polyQ expansion independent of protein context. To allow comparison of these two models of different genetic background, we first identified modulated transcripts in R6/2 versus wt littermates. Out of 311 differentially represented transcripts, 74% were under-expressed and 26% over-expressed. Intersecting differentially expressed transcripts of R6/2 and R7E revealed 113 common deregulated transcripts, most of which (87 transcripts) were under-expressed (Fig. 1B). Of these 113 transcripts, 24 were altered at disease onset in R7E retina.

Validation of gene expression data

Expression of the Rho gene was previously shown to be drastically reduced in R7E and R6/2 retina (17). Quantitative RT–PCR (Q-PCR) in 3.5- and 9-week-old R7E measured, respectively, a 3-fold and 10-fold decrease in mRNA level. Similarly, in 10-week-old R6/2, Rho gene expression was decreased by 4.9-fold. Our microarray data corroborate these expression changes, showing reduction of Rho expression by 1.6- and 2.3-fold, respectively, at onset and moderate stage in R7E and by 2.0-fold in R6/2. To further test the reliability of microarray data, expression profiles of a subset of 15 genes were examined by Q-PCR (Table 1). These genes were selected because they displayed different expression profiles and broad variation spectra ranging from the minimal 1.5 cut-off value and up to 26-fold variation. Many of them are discussed further. For all genes tested, Q-PCR data confirmed the altered mRNA expression and changed orientation detected by microarray analysis, even for variations near the 1.5-fold cut-off value. Expression changes measured by Q-PCR were
in general more important, indicating that the microarray data under-estimated the level at which genes were deregulated. Q-PCR performed on several genes at onset and moderate disease stages in R7E mice demonstrated that sensitivity of microarray was sufficient to detect progressive decrease or increase of gene expression (e.g. Rho, Hes5, etc.) that correlated with aggravation of phenotype. Consequently, we are confident that transcriptional alterations identified in our microarray analysis are likely to reflect not only changes in trend but also progressive modifications in gene expression occurring during disease aggravation in mutant mice retina.

Deregulated genes gather into specific functional pathways over time

Given the large number of transcripts showing altered level of expression in R7E and R6/2 retina, we used a systematic approach to interpret the biological significance of these gene deregulations. First, we re-annotated each transcript by analyzing the corresponding Affymetrix probe sets sequences with RetScope platform (Chamel F. and Poch O. unpublished data). Briefly, RetScope, based on the analysis of the BLAST (20) homology searches in the GenBank (21), UniProt (22) and Human genome (23) public databases, associates with high confidence each probe set sequence with existing full-length transcript and protein accession numbers. Then, GOAnno (24) was used to predict Gene Ontology (25) annotations for each identified protein. Finally, we identified over-represented GO Biological Process (GOBP) terms by calculating a z-score using the whole human proteome as reference (26). We reasoned that genes over-represented in a given GOBP term have a higher probability of being relevant to polyQ-induced retinopathy. Among the 727 deregulated transcripts (Supplementary Material, Table S1), 541 were assigned to GOBP terms, and 50% of them gather in enriched GOBP terms presenting a z-score of 3 or greater (corresponding to a probability of less than 0.00135).

Deregulated genes gather into specific functional pathways over time

For each gene are shown the AFC and real-time Q-PCR fold change between mutant and control mice. The genes tested displayed significant AFC (≥1.5-fold change, P < 0.015) in at least one comparison group (R7E versus R7N or R6/2 versus wt); non-significant AFC (NS); AFC values inferior to the 1.5 cut-off but presenting P < 0.015 (in brackets) are also indicated. (−) and (+), respectively, indicate lower and higher expression in the mutant versus control animals. Some of the listed genes were tested at the onset stage in the R6/2 mice. The real time Q-PCR fold change between mutant and control mice, for Rho, Bcp, Gnat2 and Arr3 were −1.6, −4.2, −1.7 and −3, respectively.

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<th>Gene name</th>
<th>R7E (onset) AFC</th>
<th>R7E (onset) Q-PCR</th>
<th>R7E (moderate) AFC</th>
<th>R7E (moderate) Q-PCR</th>
<th>R6/2 (moderate) AFC</th>
<th>R6/2 (moderate) Q-PCR</th>
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AFC Q-PCR AFC Q-PCR AFC Q-PCR

For each gene are shown the AFC and real-time Q-PCR fold change between mutant and control mice. The genes tested displayed significant AFC (≥1.5-fold change, P < 0.015) in at least one comparison group (R7E versus R7N or R6/2 versus wt); non-significant AFC (NS); AFC values inferior to the 1.5 cut-off but presenting P < 0.015 (in brackets) are also indicated. (−) and (+), respectively, indicate lower and higher expression in the mutant versus control animals. Some of the listed genes were tested at the onset stage in the R6/2 mice. The real time Q-PCR fold change between mutant and control mice, for Rho, Bcp, Gnat2 and Arr3 were −1.6, −4.2, −1.7 and −3, respectively.

*Fold change was measured by densitometry analysis of northern blot.
retinal degeneration and dysfunction in the two models. Expectedly, a cohort of genes was enriched in categories unique to R6/2. Under-expressed genes involved in synaptic transmission, transport and cell adhesion are likely to underlie pan-retina dysfunction, consistent with the ubiquitous expression of mutated htt-exon-1 in R6/2 retina.

Down-regulation of genes essential for photoreceptor light transduction

The most compelling finding of the above analysis is the pre-eminent deregulation of genes associated with visual function in R7E and R6/2 retina, which occurs in the absence of overt cell death. In 9-week-old R7E, this concerned 27 genes, most of which were under-expressed (Table 2). Of these, eight genes displayed expression changes already at the onset of R7E retinopathy. A first group of 14 R7E deregulated genes are directly involved in phototransduction cascade of rod photoreceptors: Rho, transducin subunits (Gngt1, Gnbl, Gnat1 and Gnb3), cGMP phosphodiesterase subunits (Pde6a and Pde6b), genes involved in ion channel structure and regulation (Cnga, Guca1b and Slc24a1) and in other phototransduction functions (Sag, Rhok, Rdh12, Rcv and Rbp). A second group of genes are implicated in morphogenesis of photoreceptor segments (Rom1 and Rds). A third group is composed of genes having diverse but essential functions in photoreceptor, because they cause retinopathy when mutated in human and mouse (Rp1, Tulp1, Impdh1 or Rs1h) or because their orthologs are involved in retinal degeneration in flies (Pitpm1 and Ppef2).

In R6/2, six rod phototransduction genes (Rho, Gngt1, Gnbl, Sag, Rhok and Rdh12) were as well down-regulated. Moreover, in R6/2, six cone-specific genes involved in vision (Bcp, Gcp, Gnat2, Pde6c, Pde6h and Arr3) were under-expressed, consistent with the ubiquitous expression of the mutant htt-exon-1 transgene. It is noteworthy that the expression of three cone-specific genes (Bcp and Gnat2 and Pde6c) and the retinal pigmented epithelium (RPE)-specific gene Myo7a were also altered in R7E. Up-regulation of Myo7a, which is involved in phagocytosis of photoreceptor outer segment disks by the RPE, suggests an increased phagocytic activity of these cells in response to structural disorganization of the R7E photoreceptor segment layers.

As shown in Table 1, Q-PCR analysis validated the deregulation of eight tested genes (Rho, Bcp, Rhok, Rom1, Gnat1, Gnat2, Arr3 and Pde6b) in R7E and R6/2 retina. Furthermore, Q-PCR analysis of some of these genes at onset and moderate disease stages revealed that deregulation progresses along phenotype aggravation in both R7E and R6/2 retina. Together, these data indicate that the abnormal ERGs and structural disorganization of segment layers in R7E and R6/2 retina result from a very specific down-regulation of genes involved in function and morphogenesis of photoreceptors.

Altered expression of genes controlling photoreceptor differentiation

To specify the unique properties of photoreceptor cells, the developmental pathway in vertebrate retina is regulated by a series of transcription factors acting before and during the terminal differentiation of photoreceptors (12,13). Enrichment of a large cohort of deregulated genes involved in visual perception as well as in development, morphogenesis, organogenesis and cell growth, maintenance and organization suggested that the genetic controls maintaining photoreceptor differentiation are impaired in R7E and R6/2 retina. Among the deregulated transcripts belonging to these pathways, we found a number of transcription factors controlling various
aspects of neuronal cell fate specification and differentiation (Table 3).

Under-expressed transcription factor genes. Among this group, Crx, Nrl and Nr2e3 present a special interest, as they are key regulators of the terminal differentiation and maintenance of rod photoreceptors (27–29). These genes are expressed in immature rod cells and interplay together to ensure the proper expression of rod- and repression of cone-specific genes during rod terminal differentiation. Crx is also required for terminal differentiation of cones (27). Two other genes, Errβ and Mef2C, are markers of differentiation fate, as their expression increases in maturing photoreceptors (30,31). Mef2C is also known to be involved in neuronal differentiation process. For these five genes, the expression level in R7E retina progressively decreased from 1.4 to 2.9-fold between onset and moderate stage of pathology. Although the 1.4-fold variation seen at onset is inferior to the 1.5-fold used as a confident cut-off for our microarray analysis, the data are consistent with the progressive aggravation of the R7E phenotype. In R6/2 retina, Nrl, Errβ and Mef2c expressions were also significantly decreased, and Crx was reduced by 1.4-fold. We performed Q-PCR analysis of Crx, Nrl and Nr2e3 at R7E moderate stage and of Crx in R6/2 and confirmed their reduced level of expression (Table 1).

Reduced expression of the late differentiation regulators Crx, Nrl and Nr2e3 is expected to cause deregulation of a large subset of their target genes in both mouse models. To evaluate the extent to which dysfunction of these transcription factors contributes to polyQ-induced retinopathy, we compared our data sets with recent expression profiling studies, which revealed the transcriptional networks regulated by Nrl and Crx in mouse (32–35). Using Affymetrix microarray analysis, Yoshida et al. (32) identified 164 differentially expressed genes in Nrl−/− versus wt mature retina, which are likely candidate Nrl-regulated genes. We found 15 Nrl-regulated genes presenting altered expression level at onset and 45 at moderate stage of R7E retinopathy (Supplementary Material, Table S2), an increasing number inversely correlating with the progressive down-regulation of Nrl gene along disease phenotype (Table 3). Similarly, 24 Nrl-regulated genes were also altered in R6/2 retina. Many of the Nrl-regulated genes showing expression changes in R7E and R6/2 retina function in phototransduction cascade. Expression profiling data also revealed that Nr2e3 and Mef2c are regulated by Nrl (32,33). Nr2e3 inhibits expression of cone-specific genes in rod cells. Evidence for Nr2e3 dysfunction

### Table 2. Deregulation of genes essential for photoreceptor function

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<th>Gene name</th>
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<td>Rhok</td>
<td>−3.5</td>
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Together, these results indicate that reduced level of paired box gene 6 (Pax6), signal transducer and activator of transcription 3 (Stat3), LIM homeobox protein 2 (Lhx2), hairy and enhancer of split 5 (Hes5), and MEF2C (Mef2c) in part responsible for the loss of expression of many rod and cone-specific genes (Gnat2 and Pde6c) (Table 2). As for Nrl, we compared our data set with candidate Pax6-regulated genes revealing by SAGE analysis of Pax6+/− versus wt P10 retina (34,35). Again, the number of Pax6-regulated genes showing alteration in R7E increased from onset (9 genes) to moderate stage (16 genes) in correlation with the progressive down-regulation of Pax6 gene (Table 3 and Supplementary Material, Table S3). Similarly, nine Pax6-regulated genes were altered in R6/2. In addition, change trends of genes deregulated in R7E and R6/2 were generally in agreement with the expected transcriptional consequence of Pax6 and Nrl inactivation. Together, these results indicate that reduced level of Pax6, Nrl and Nr2e3 expression in R7E and R6/2 retina is in part responsible for the loss of expression of many rod differentiation genes.

Other transcription factors under-expressed in R7E and R6/2 retina might also contribute to the loss of differentiated state of retinal neurons (Table 3). Pax6, Mab21l1 and Lhx2 genes are essential for early eye development and remain expressed in adult retina (36). Nurr codes for a master regulator of neuronal differentiation and maintenance in other brain regions (37) and might display a similar role in the retina.

Over-expressed transcription factor genes. Three R7E over-expressed genes, Optx2, Hes5 and Stat3, display crucial functions in the retinal development (Table 3). Optx2 is required for early eye development (38), whereas the Notch effector Hes5 is expressed in retinal progenitors and modulates glial cell fate specification (39). Stat3 is thought to play a key role in retinal development by maintaining retinal precursors in an undifferentiated state (40). Interestingly, persistent Optx2, Hes5 or Stat3 expression in developing retina caused inhibitory effects on photoreceptor differentiation (38,39,41). Our microarray data indicated that Hes5 transcript was not detected (‘absent call’) in control retina, consistent with previous in situ hybridization study on mature retina (39). Q-PCR analysis confirmed that Hes5 was re-expressed in R7E (Table 1). Immunofluorescence analysis of wt retina revealed a faint Hes5 staining restricted to ganglion cell and inner nuclear layers (Fig. 3) and no staining in photoreceptor layer. In R7E retina, anti-Hes5 antibody stained rod photoreceptors containing NIs, but not those in which NIs were absent (presumably due to the absence of transgene expression) (Fig. 3). Our data suggest that Hes5 gene was aberrantly re-expressed in R7E photoreceptors.

We also confirmed by western blot analysis that increased Stat3 gene expression correlated with increased level of Stat3 protein and its phosphorylated (tyrosine 727) form in 9-week-old R7E and R6/2 (Fig. 4A). Interestingly, phosphorylation of Stat3 was detected as early as 3 weeks of age, preceding the increase in Stat3 protein, and was sustained over time in R7E retina, whereas it remained undetected in R7N controls. Another study found that phosphorylated Stat3 precedes the increase of Stat3 protein level, suggesting that Stat3 regulates its own promoter (42). We also found that phospho-Stat3 was enriched in the nuclear fraction when compared with the cytoplasmic one in the R7E (Fig. 4B), consistent with the known process of Stat3 activation.

Stat proteins are terminal transcription factors of a well-documented signaling pathway, which involves external cytokine stimuli, membrane receptors, Janus tyrosine kinases (Jak) and several negative regulators such as protein inhibitor of activated Stat (Pias) and suppressor of cytokine signaling (Socs) (43). Affymetrix microarray MOE430A contained probe sets to assess expression of several of these genes controlling Stat3 activation. We found that only Pias3 gene displayed altered expression in both R7E and R6/2 retina when compared with their controls (Table1). Decreased expression of Pias3, which inhibits Stat3 binding to the DNA regulatory elements, is consistent with Stat3 protein activation. We confirmed by Q-PCR analysis that Pias3 gene expression was decreased in both R7E and R6/2 retina.

Altogether, these results indicate that transcription factors associated to early steps of retinal development and able to cause negative effect on photoreceptor differentiation are aberrantly over-expressed in polyQ retina.

Table 3. Deregulation of transcription factors implicated in retinal development

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>R7E (onset)</th>
<th>R7E (moderate)</th>
<th>R612 (moderate)</th>
<th>Function</th>
<th>Reference</th>
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<tr>
<td>Cone-rod homebox containing gene (Crx)</td>
<td>(−1.4)</td>
<td>−1.6</td>
<td>(−1.4)</td>
<td>Differentiation and maintenance of cones and rods</td>
<td>(27)</td>
</tr>
<tr>
<td>Neural retina leucine zipper protein (Nrl)</td>
<td>(−1.4)</td>
<td>−2.2</td>
<td>−1.5</td>
<td>Differentiation and maintenance of rods</td>
<td>(24)</td>
</tr>
<tr>
<td>Nuclear receptor (Nr2e3)</td>
<td>NS</td>
<td>−2.1</td>
<td>NS</td>
<td>Differentiation and maintenance of rods</td>
<td>(29)</td>
</tr>
<tr>
<td>Estrogen-related receptor beta</td>
<td>(−1.4)</td>
<td>−2.9</td>
<td>−1.8</td>
<td>Expression increases in differentiating photoreceptors</td>
<td>(30)</td>
</tr>
<tr>
<td>Myocyte enhancer factor 2C</td>
<td>−1.5</td>
<td>−2.9</td>
<td>−2.2</td>
<td>Expression increases in differentiating retina</td>
<td>(31)</td>
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<tr>
<td>Paired box gene 6 (Pax6)</td>
<td>NS</td>
<td>NS</td>
<td>−1.8</td>
<td>Early eye development</td>
<td>(36)</td>
</tr>
<tr>
<td>Mab-21-like 1 (Mab21l1)</td>
<td>(−1.4)</td>
<td>−1.8</td>
<td>NS</td>
<td>Early eye development</td>
<td>(36)</td>
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<tr>
<td>LIM homeobox protein 2 (Lhx2)</td>
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<td>NS</td>
<td>NS</td>
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<td>(36)</td>
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<td>Nuclear receptor (Nurr)</td>
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<tr>
<td>Signal transducer and activator of transcription 3 (Stat3)</td>
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<td>+1.7</td>
<td>+2.1</td>
<td>Retinal progenitor proliferation</td>
<td>(40)</td>
</tr>
<tr>
<td>Hairy and enhancer of split 5 (Hes5)</td>
<td>NS</td>
<td>+2.4</td>
<td>NS</td>
<td>Retinal gliogenesis, inhibits neurogenesis</td>
<td>(39)</td>
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The AFC is shown and (−) and (+) indicate, respectively, lower and higher expression in the mutant versus control mice. Values inferior to 1.5 cut-off but presenting P < 0.015 are also indicated in brackets. NS, non-significant.
DISCUSSION

Mature rod photoreceptors are issued from sequential development of retinal progenitor cells (RPC) during vertebrate retinogenesis. In the initial phase, a pool of dividing RPC becomes post-mitotic precursors committed to rod fate. Then, following a period of specification, immature rods terminally differentiate into mature rods, which express specific proteins essential to establish a characteristic cell shape and to execute light transduction. Commitment and differentiation of rod photoreceptors require transcriptional programs that are being uncovered (12,13). In the present study, we have used gene expression profiling to build a comprehensive scheme of the pathomechanism involved in two mouse models of rod photoreceptor degeneration induced by different polyQ expansion proteins. Our data show that polyQ expansion, regardless of the protein context, causes a progressive loss of expression of mature rod genes by compromising the genetic programs involved in the maintenance of rod differentiation state, resulting in rod dysfunction and degeneration.

Correlating rod dysfunction and morphological change with polyQ-induced transcriptional alterations

In SCA7 R7E mouse model, rod-specific expression of ATXN7 with expanded polyQ causes a progressive rod degeneration timely defined by molecular (NI formation), functional (scotopic ERG loss), histological (segment layer reduction) and cell shape anomalies, occurring prior overt cell death (17). Using DNA microarrays and Q-PCR, we show that R7E rod phenotype was attributable to reduced expression of a large subset of genes specifically involved in phototransduction and segment morphogenesis of mature rods. Previous studies (15,16) reported similar findings in other SCA7 mouse models by analyzing a restrained number of selected photoreceptor genes. Our genomewide analysis gives now an exhaustive view over time of the loss of expression of genes essential to mature rod features. Likewise, expression of mutant htt-exon-1 in R6/2 mouse retina, which causes a retinopathy comparable to R7E (18), alters the regulation of a similar subset of mature rod genes. Mutant htt-exon-1, which is expressed in cones as well and causes photopic ERG dysfunction of R6/2 retina, also leads to reduced expression of cone phototransduction genes. Down-regulation of mature photoreceptor genes is progressive and

Figure 3. Aberrant photoreceptor expression of Hes5 in R7E retina. Hes5 staining is observed in ganglion cells (GC) and inner nuclear layer (INL) of wt and R7E retina, but not in wt photoreceptors (ONL) or in R7E photoreceptors that do not contain NIs presumably due to the absence of transgene expression (arrowhead). In contrast, R7E photoreceptors containing NIs displayed Hes5 staining. The animals are 13 weeks of age. Scale bar: 50 μm.

Figure 4. Aberrant activation of Stat3. (A) Stat3 expression is increased in R7E retina when compared with controls at different stages of the pathology. Phospho-Stat3, the activated form of Stat3, is detected as early as 3 weeks of age (arrow). This activation persists and increases at later stages. Stat3 and phospho-Stat3 are also increased in R6/2 when compared with control’s retina. Anti-TFIIFα antibody is used to control the loaded protein amounts. (B) Phospho-Stat3 is enriched in the nuclear fraction compared with the cytoplasmic one in the R7E mice.
affects an increasing number of genes along phenotype aggra-
vation in R7E and R6/2. Together, these results indicate that
photoreceptors expressing polyQ expansion proteins progress-
ively lose their differentiation features due to transcriptional
alterations of mature photoreceptor genes. Apart from genes
directly implicated in rod maturation state, a large number of
genes involved in development, morphogenesis, cell growth,
maintenance and organization are also significantly deregu-
lated in 9-week-old retina, a disease time point when
segment layer is severely reduced in both polyQ models
and R7E rod nuclei lose their chromatin organization (60).
This indicates that important reprogramming of gene
expression accompanied reshaping of rod cells to neurons
being non-functional and showing immature traits.

Down-regulation of rod differentiation program
Consistent with the loss of rod photoreceptor differentiation
features, three key transcription factors (Crx, Nrl and Nr2e3)
that control terminal differentiation and maintenance of
mature rod are down-regulated in R7E and R6/2 retina.
Mice lacking the homeobox Crx develop precursor neurons
committed to rod fate, but fail to develop proper outer seg-
ments and to express phototransduction proteins (27,34).
The bZIP Nrl is essential for expression of rod-specific and
repression of cone-specific genes in rods. Nrl and Crx proteins
can interact to exhibit transcriptional synergy in gene acti-
vation. Inactivation of Nrl in mice results in a complete loss
of rods at the expense of supernumerary S-cones (28). One
Nrl repression mechanism of cone genes proceeds via induc-
ton of the orphan nuclear receptor Nr2e3 expression. Rdl7
mutant mouse, which carries a spontaneous Nr2e3 deletion,
develops aberrant hybrid cone–rod photoreceptors (29,44).

The reduced Crx and Nrl expressions have a direct impact
on R7E and R6/2 photoreceptor expression profiles, as a
large number of Crx- and Nrl-target genes show deregulation
in polyQ retina models, even though our microarray analyses
are performed at early stages (onset and moderate phenotype)
of retinopathy. Strikingly, the number of Crx- and Nrl-target
genes deregulated in R7E retina increases from onset to
moderate stage, inversely correlating with the progressive
down-regulation of Crx and Nrl genes along phenotype aggra-
vation. Moreover, Nr2e3 dysfunction in R7E retina is
suggested by increased level of cone-specific gene expression.
Thus, we conclude that progressive loss of Crx, Nr2e3 and Nrl
functions in R7E and R6/2 retina results in the failure of
rod photoreceptors to maintain the expression of mature rod
gen genes.

La Spada et al. (15) reported that mutant ATXN7 can
interfere with CRX and NRL transactivation function in vitro.
On the basis of direct interaction between ATXN7 and CRX
and on the cone and rod CRX expression, the authors
avored a model in which interference with transactivation
function of CRX, rather than NRL, would be the major
defect leading to cone and rod dystrophy in SCA7. Our
result suggests another mechanism whereby mutant ATXN7
and mutant htt-exon-1 cause Crx, Nrl and Nr2e3 dysfunction
by repressing their expression.

We also noted the reduced expression of other transcription
factors (Table 3), which are involved either in early eye
development (Pax6, Mahb2l1 and Lhx2) or in neuronal differ-
entiation (Mef2c, Erbbeta; and Nurr). Although the role of
these factors in mature retina is currently unknown, their
deregulation might also contribute to the RE7 and R6/2
retinal phenotype. Whatever is their contribution, down-
regulation of these factors reveals that polyQ expansion
toxicity has a broad effect on the transcriptional program of
mature retina.

Aberrant activation of transcription factors that inhibit
photoreceptor differentiation
Another important finding of our study is the increased level
of Optx2, Hes5 and Stat3 expression in polyQ retina models.
It has already been shown using several experimental con-
ditions that persistent expression of these transcription
factors in developing vertebrate retina causes inhibitory
effect on rod photoreceptor differentiation (38,39,41). It is
notable that Stat3 protein is activated in the two polyQ
models, and in the case of R7E, activation is triggered at
onset and sustained along the pathogenic process. During
retinal development, Stat3 inactivation is required for rod
photoreceptor precursors to differentiate into mature rod and
for Crx and Rho gene expression (40,41). The cytokines
CNTF and LIF, which activate Stat3 signaling pathway, also
prevent photoreceptor differentiation in rodents (45,46).
In addition, LIF effect on photoreceptor differentiation is
due to inhibition of Crx and Nrl expression (46). The consequence
of activating Stat3 in adult retina is currently unknown.
However, in preclinical studies on animal models for photo-
receptor degeneration, prolonged CNTF treatment increased
photoreceptor survival without rescuing their function
(47,48). These rod photoreceptors displayed enlarged nuclei
with less compacted chromatin, similar to photoreceptor
nuclei of aged R7E mice (60). On the basis of the current
knowledge of CNTF, LIF and Stat3 effects on photoreceptor
differentiation, aberrant Stat3 activation in R7E and R6/2
retina might be one of the early events leading to loss of rod
differentiation by causing repression of Crx and Nrl
gene expression.

Our study also points out on several pathways that could
contribute to Stat3 activation. Pias3 gene expression is
reduced in both R7E and R6/2 retina. Down-regulation of
Pias3 protein, which interacts with and inhibits DNA-
binding activity of Stat3, could strengthen Stat3 activation
and its transcriptional activity. In Drosophila, proper
expression level of both Pias and Stat ortholog genes are
 crucial for eye development (49). Interestingly, deletion of
dpias inhibits retinal cell differentiation. Besides the canonical
cytokine/receptor/Jak pathway, other routes of Stat activation
have been identified. A recent study shed light on a cross-talk
between Notch–Hes and Jak–Stat signaling pathways via
direct interaction of Hes1 or Hes5 with Jak2 and Stat3,
which promotes Stat3 phosphorylation and activation. Con-
sequently, aberrant Hes5 expression in R7E retina may
contribute to the sustained Stat3 activation. Moreover, Stat
proteins can also be activated by diverse cellular stresses
(50). For instance, oxidative stress, which is associated with
polyQ-induced toxicity in several model systems, can activate
Stat3 pathway (51). We previously showed that neuronal stress involving the activation of JNK/Jun/AP1 pathway occurred in R7E and R6/2 mouse retina (9). Our microarray data also reveal over-expression of regulators of oxidative and endoplasmic reticulum stress response, such as Bach2 and Xbp1 genes, respectively (Table 1 and Supplementary Material, Table S1), warranting further investigation of the role of stress in the loss of neuronal differentiation induced by polyQ expansion.

PolyQ toxicity and loss of neuronal identity

The detailed mechanisms whereby polyQ expansion causes loss of neuronal differentiation remain to be characterized. However, it is worth to note that deregulation of the genetic program maintaining photoreceptor differentiation by polyQ expansion seems unique, because such gene deregulation specificities were not reported in transcriptome analyses of other retinal degenerations associated with rd1, Rho or Rp1 mutations (52,53) (Chalmel, F. and Poch, O., manuscript in preparation). Our conclusions that polyQ induces loss of neuronal differentiation are based on the study of a highly organized and well-characterized neuronal tissue, the retina. However, failure of neurons to maintain their differentiated state might be a common feature to polyQ disorders. Early studies on gene deregulation occurring in HD showed that genes encoding proteins essential for striatal neuron functions such as neurotransmitters, receptors and other neuronal signaling proteins were down-regulated (10,54). Although the mechanism remains to be identified, recent studies showed that one critical regulator of neuronal terminal differentiation, neuron-restrictive silencer factor (NRSF), was altered in HD. Indeed, NRSF protein was abnormally localized in the neuronal nucleus in HD, resulting in repression of NRSF-target genes (55). Another study showed that polyQ expansion caused up-regulation of NRSF gene expression and prevented neuronal differentiation of embryonic stem cells (56). Over-expression of NRSF was shown to inhibit neurite outgrowth (57). Morphological abnormalities of dendrites and defects of neurite outgrowth were reported in HD and in in vitro and in vivo HD models (58,59). In regard to our findings in R7E and R6/2 retina, it is thus conceivable that mutant htt compromises neuronal identity of striatal neurons by deregulating the genetic program controlling their differentiation.

One puzzling aspect of polyQ disorders is that long-term neuronal dysfunction precedes neuronal cell loss. Little is known about this gradual degenerative process or on the mechanism of neuronal death. Our finding suggests that dysfunction occurred because neurons lose their neuronal features. PolyQ expansion might directly affect the key proteins orchestrating the program of neuronal differentiation. Alternatively, in response to permanent stress caused by polyQ expansion, vulnerable neurons might slowly progress to an immature state. By being in conflict with the mature environment of the nervous system, these immature neurons might not receive stimuli promoting their survival. Studies on how polyQ expansion causes loss of neuronal differentiation and how neurons loosing neuronal traits are condemned to death will thus be of major relevance for the development of therapeutic approaches in polyQ disorders.

MATERIALS AND METHODS

Animals

R7E and R7N transgenic lines were maintained on the inbred C57BL/6 background (14). R6/2 line, purchased from the Jackson Laboratories (Bar Harbor, ME, USA) was originally on C57BL/6:CBA/J background. To avoid the rd1 mutation carried by CBA/J inbred strain, R6/2 mice were backcrossed on C57BL/6 background. The mice used in this study were 75% C57BL/6 and 25% CBAJ. Genotyping of R7N, R7E, R6/2 and rd1 mutations was performed by PCR on tail DNA according to the protocols previously described (17). The experiments were performed in accordance with the National Institutes of Health Guide for Care and use of Laboratory Animals.

RNA isolation

Both retinas from one mouse were isolated, pooled, immediately frozen in liquid nitrogen and stored at −80°C. Retinas were homogenized with an ultraturax homogenizer and total RNA was extracted using Qiagen columns according to manufacturer’s instructions (Qiagen RNeasy kit). RNA quantity and quality were analyzed using 260/280 nm absorbance ratio and Agilent apparatus.

Affymetrix gene profiling and data analysis

For the microarray experiments, we analyzed five R7E versus five R7N mice at 3 weeks of age, six R7E versus six R7N mice at 9 weeks of age as well as four R6/2 versus four wt mice at 9 weeks of age. Biotinylated cRNA were prepared according to the standard Affymetrix protocol (GeneChip Expression Analysis Technical Manual, 2001, 701151 Rev1, Mat No. 1020407, 03/2002; Affymetrix). Ten micrograms of fragmented cRNA were hybridized for 16 h at 45°C on murine MOE 430A GeneChips. These GeneChips contain 22 600 probe sets, about 15 000 of them are against well-annotated full-length genes, whereas the remaining probe sets are against gene clusters containing only EST sequences and some gene clusters with non-EST sequences. GeneChips were washed and stained in the Affymetrix Fluidics Station 400 and further scanned using the Hewlett-Packard GeneArray Scanner G2500A. Initial data preparation was performed by Affymetrix MicroArray Suite Version 5.0 (MAS5) using Affymetrix default analysis settings and global scaling as normalization method. The trimmed mean target intensity of each chip was arbitrarily set to 100. Absolute analyses generate a signal value for each probe set and a detection call of absent, present or marginal. To select differentially expressed genes, three consecutive filters were applied for each of the three comparison groups (3-week-old R7E versus R7N, 9-week-old R7E versus R7N and 9-week-old R6/2 versus wt). First, we performed a Mann–Whitney statistical test called ‘present’ in at least (n−1) mice in a group of n mutant mice for the up-regulated genes, or n control mice for the down-regulated genes.
Q-PCR analysis
Total RNA (1 µg) was subjected to reverse transcription using SuperScript Reverse Transcriptase (Invitrogen). For each gene, primers were designed using Primer 3 software and are available upon request. Real-time PCR was performed with SYBRGreen using the Light Cycler apparatus. Specificity of reactions was confirmed by melting curve analysis. Significant fold change was calculated based on the difference in the calculated concentration between the transgenic and the control mice after normalization to Pitia or Arbp as internal controls. Three to six animals for each genotype, R7E, R6/2 and wt, were analysed.

Western blotting
Retinas were homogenized in lysis buffer containing 50 mM Tris–HCl pH 8.0, 10% glycerol, 5 mM EDTA, 400 mM KCl, 1 mM phenylmethylsulfonyl fluoride and a cocktail of protease inhibitors. Triton was then added to a final concentration of 0.1% to whole retinal homogenates. Retinas were then incubated on ice for 15 min, sonicated and centrifuged for 15 min at 4°C. Supernatants were analyzed on 10–12% SDS–PAGE gel.

Primary antibodies used were mouse monoclonal Stat3 (F-2): sc-8019 (Santa Cruz, USA), rabbit monoclonal Phospho-Stat3 (Tyr705) (58E12) (Cell signaling). They were revealed with appropriate anti-mouse or anti-rabbit peroxidase-conjugated secondary antibodies and the chemiluminescent reaction (Pierce, Rockford, IL, USA).

Immunohistochemistry
Enucleated eyes were dissected to remove lens and cornea and fixed in fresh 4% paraformaldehyde for 2 h at 4°C. Fixed retinas were placed for 1 h in 30% sucrose and fixed in fresh 4% paraformaldehyde for 2 h at 4°C. Crystall sections (10 µm) were mounted on SuperFrost/Plus slides (O. Kindler, Freiburg, Germany). Sections were permeabilized and blocked for 1 h with 10% normal goat serum, 0.5% bovine serum albumin (BSA), 0.1% Tween-20 and 1 x phosphate-buffered saline (PBS). After a washing step in PBS, sections were incubated with primary antibodies diluted in 3% normal goat serum, 0.5% BSA, 0.1% Tween-20 and 1 x PBS. Primary antibodies used were rabbit Anti-Hes5 affinity purified polyclonal antibody AB5708 (Chemicon International) and mouse anti-ATXN7 monoclonal antibody (2A10) (17). Sections were then washed and incubated with secondary antibodies diluted in the same solution as primary antibody. The secondary antibodies used were CY3- and Oregon green-conjugated goat anti-mouse and anti-rabbit IgG at a dilution of 1:200. Nuclei were counterstained with 0.5 µg/ml 4,6-diamidino-2-phenylindole.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG Online.


