Genes and pathways affected by CAG-repeat RNA-based toxicity in Drosophila

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Received August 6, 2011; Revised and Accepted September 9, 2011

Spinocerebellar ataxia type 3 is one of the polyglutamine (polyQ) diseases, which are caused by a CAG-repeat expansion within the coding region of the associated genes. The CAG repeat specifies glutamine, and the expanded polyQ domain mutation confers dominant toxicity on the protein. Traditionally, studies have focused on protein toxicity in polyQ disease mechanisms. Recent findings, however, demonstrate that the CAG-repeat RNA, which encodes the toxic polyQ protein, also contributes to the disease in Drosophila. To provide insights into the nature of the RNA toxicity, we extracted brain-enriched RNA from flies expressing a toxic CAG-repeat mRNA (CAG100) and a non-toxic interrupted CAA/G mRNA repeat (CAA/G105) for microarray analysis. This approach identified 160 genes that are differentially expressed specifically in CAG100 flies. Functional annotation clustering analysis revealed several broad ontologies enriched in the CAG100 gene list, including iron ion binding and nucleotide binding. Intriguingly, transcripts for the Hsp70 genes, a powerful suppressor of polyQ and other human neurodegenerative diseases, were also upregulated. We therefore tested and showed that upregulation of heat shock protein 70 mitigates CAG-repeat RNA toxicity. We then assessed whether other modifiers of the pathogenic, expanded Ataxin-3 polyQ protein could also modify the CAG-repeat RNA toxicity. This approach identified the co-chaperone Tpr2, the transcriptional regulator Dpld, and the RNA-binding protein Orb2 as modifiers of both polyQ protein toxicity and CAG-repeat RNA-based toxicity. These findings suggest an overlap in the mechanisms of RNA and protein-based toxicity, providing insights into the pathogenicity of the RNA in polyQ disease.

INTRODUCTION

A large number of neurodegenerative diseases are caused by unstable trinucleotide repeat expansions in the associated genes (1). Based on pathogenic mechanisms, these disorders are categorized into different groups, including loss of protein function, RNA toxicity and dominant protein-based toxicity. Myotonic dystrophy type 1 (DM1) and myotonic dystrophy type 2 (DM2) are thought to be caused primarily by a gain-of-function RNA toxicity. CUG and CCUG RNA expansions in the non-coding regions of the dystrophia myotonica-protein kinase (DMPK) gene and the zinc finger 9 (ZNF9) gene cause DM1 and DM2, respectively (2). Polyglutamine (polyQ) diseases are dominantly inherited disorders expanded CAG repeats within the coding region of the associated genes, such as Huntington’s disease (HD) and the spinocerebellar ataxias (SCA1, 2, 3, 6, 7 and 17) (3). A pathological hallmark of these diseases is ubiquitinated nuclear inclusions (NI) containing chaperones and subunits of the proteasome (3).

The prevailing hypothesis for polyQ disease mechanisms is a protein effect due to the polyQ. Upregulating chaperones suppresses toxicity and supports the hypothesis that protein misfolding or aberrant interactions of the mutant protein cause disease (4). Therefore, studies have traditionally focused on protein-based mechanisms of toxicity conferred by the expanded polyQ domain. Recent studies, however, suggest that RNA toxicity is a component of degeneration (5). The toxicity of a pathogenic, expanded polyQ Ataxin-3 protein in Drosophila is significantly reduced by interrupting the typically pure CAG-repeat sequence encoding the polyQ domain, with a CAA-interrupted repeat which also encodes glutamine. CAG-repeat RNA forms a hairpin structure which is thought
to confer toxicity (6,7). Interruptions of the CAG-repeat RNA by CAA codons are predicted to disrupt the hairpin structure (8). Furthermore, evidence suggests that pure or interrupted CAG-repeat expansions in ATAXIN-2 (ATXN2) are associated with distinct effects (9–12). Expanded pure CAG repeat in ATXN2 typifies SCA2. CAA interruptions in ATXN2 are found in CAG-repeat expansions that present with parkinsonism or amyotrophic lateral sclerosis (ALS). Taken together, these findings suggest a role of the CAG-repeat RNA in toxicity and effects. Additional studies indicate that simply a non-coding RNA bearing only an abnormally long CAG repeat but not coding for a protein can induce progressive neuronal dysfunction, including shortened lifespan and locomotor defects (5). Interestingly, muscleblind (mbl), an RNA-binding protein implicated as a modifier in DM1, can dramatically enhance the toxicity of both the Ataxin-3 protein and non-coding CAG-repeat RNA in Drosophila (13,14). These findings suggest that the CAG-repeat RNA, in the absence of coding for a protein, may be a component of toxicity in polyQ disease. However, mechanisms of CAG-repeat RNA toxicity remain unclear.

To further understand mechanisms of CAG-repeat RNA toxicity, we assessed transcriptional changes in flies expressing toxic expanded CAG-repeat RNA using microarray analysis in Drosophila. This approach identified transcriptional changes in 160 genes specifically in the brain of flies expressing toxic CAG repeats, suggesting a robust component of transcriptional alterations. These findings led us to test the overlap between modifiers defined for protein-based polyQ changes in 160 genes specifically in the brain of flies expressing toxic expanded CAG-repeat RNA using microarray analysis (Fig. 1C). To assess whether the brain dissection enriched for brain RNAs, the dissection quality was assessed by determining the relative level of expression of an eye gene, chaoptin (chp), which is selectively expressed in photoreceptor cells, and the neural gene marker amyloid precursor protein like (appl), which is selectively expressed in neurons thus enriched in brain compared with eye tissue (15,16). Quantitative real-time polymerase chain reaction (qRT-PCR) analysis indicated that the relative expression level of chp was significantly low (less than 10-fold compared to head RNA) in all samples, whereas the level of appl was enriched more than 12-fold compared with head RNA (Fig. 1D and E). This analysis thus indicated that dissection of the brain from the eyes successfully enriched for RNA expressed in the brain among the four independent replicates of the microarray analysis.

The expression levels of each trinucleotide repeat transgene were also controlled. Transcript levels were determined by assessing the level of the DsRed portion of the transgenes by qRT-PCR. The transgenes were adjusted to match at the mRNA expression level, such that CAG0 flies had one copy of the transgene and the CAG100 flies had five copies. The CAA/G105 line expressed the transgene at levels lower than those of the CAG100. Thus, we used two copies of this transgene in order to express this RNA at a level at least as high as that of the CAG100 flies (Fig. 1F).

### RESULTS

#### Defining gene expression changes in response to a toxic non-coding CAG-repeat RNA

To identify gene pathways involved in response to a toxic non-coding CAG-repeat RNA, we performed microarray analysis on Drosophila brains using the Affymetrix Drosophila Genome 2.0 Array. In order to assess early transcriptional changes and to avoid detecting secondary responses, presymptomatic adult flies expressing a CAG repeat in the 3′ untranslated region (3′-UTR) of a transgene encoding the control protein DsRed were used (Fig. 1A). The control CAG0 flies express a DsRed reporter gene with no CAG repeat in the 3′-UTR. The CAG100 and CAG250 flies have a CAG repeat of about 100 and 250 CAG trinucleotides in the 3′-UTR, as previously described (5). The CAA/G105 flies are a non-toxic trinucleotide control and express DsRed with an interrupted CAA/CAG-repeat sequence in the 3′-UTR.

The experimental design is illustrated in Figure 1B. To define genes whose expression is altered in response to a toxic CAG-repeat RNA, we compared CAG100 flies with age-matched flies expressing CAG0. To exclude transcriptional changes in response to a non-toxic trinucleotide repeat, a second gene list was generated by comparing CAA/G105 flies with age-matched CAG0 flies. To then focus on the list of genes specific to the toxic CAG-repeat RNA, we eliminated genes that were differentially expressed in common to both CAG100 and CAA/G105 flies. At 3 days, flies expressing CAG100 in all neurons of the brain with elav-GAL4 driver show normal climbing ability and normal survival compared with age-matched control flies expressing CAG0 (5). Fly brains were thus dissected from 3-day animals, and RNA was isolated for microarray analysis (Fig. 1C). To assess whether the brain dissection enriched for brain RNAs, the dissection quality was assessed by determining the relative level of expression of an eye gene, chaoptin (chp), which is selectively expressed in photoreceptor cells, and the neural gene marker amyloid precursor protein like (appl), which is selectively expressed in neurons thus enriched in brain compared with eye tissue (15,16). Quantitative real-time polymerase chain reaction (qRT-PCR) analysis indicated that the relative expression level of chp was significantly low (less than 10-fold compared to head RNA) in all samples, whereas the level of appl was enriched more than 12-fold compared with head RNA (Fig. 1D and E). This analysis thus indicated that dissection of the brain from the eyes successfully enriched for RNA expressed in the brain among the four independent replicates of the microarray analysis.

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#### Global analysis of genes differentially expressed in flies expressing a toxic CAG RNA

Genes differentially expressed in CAG100 and/or CAA/G105 were defined by comparing RNA from brains of flies expressing CAG100 versus CAG0 and CAA/G105 versus CAG0. Pearson’s correlation coefficient showed a strong correlation between the biological replicates of any one transgene (r > 0.99). We predicted that the transcriptional profile of CAA/G105 flies would be more similar to CAG0 flies than to CAG100 flies, because the interrupted CAA/G105 repeat is not toxic. Hierarchical clustering confirmed a higher similarity between the transcriptional profiles of CAG0- and CAA/G105-expressing flies than between CAG0- and CAG100-expressing flies (Fig. 2A). In addition, it further supported our experimental design to exclude genes differentially expressed in response to a long trinucleotide repeat by using the interrupted CAA/G105 RNA.

About 129 transcripts were identified that were 2-fold or greater upregulated and 81 that were 2-fold or greater downregulated in CAG100 flies compared with CAG0 flies (Fig. 2B). Among these, 19% (25 transcripts) upregulated and 31% (25 transcripts) downregulated transcripts were also differentially up- or downregulated in CAA/G105 flies (Supplementary Material, Table S1a). These transcripts were not pursued at this time; these transcripts might reflect a general response to expressing a long trinucleotide repeat RNA,
whether toxic or not. About 160 transcripts (104 up and 56 down) were altered selectively in CAG100 flies (Supplementary Material, Table S1b). About 122 transcripts (48 up and 74 down) were altered in CAA/G105 flies, although not in CAG100 flies (Supplementary Material, Table S1c). To verify the microarray analysis, we selected 12 genes of a range of gene expression alterations to assess for expression changes by qRT-PCR in CAG100 flies (Supplementary Material, Table S2). Five out of 12 genes expression changes, or 42%, were positively validated in CAG100 (P, 0.05).

Gene Ontology (GO) analysis based on the Database of Annotation, Visualization and Integrated Discovery was performed to provide insights into the biological processes affected by the genes differentially expressed selectively in CAG100 flies (Supplementary Material, Table S1b) (17,18). We used the GO_FAT category which filters out very broad GO terms to identify statistically enriched functional groups. Several broad GO terms were enriched, including iron ion binding, nucleotide binding and oxidation reduction (Fig. 3). Genes related to ion channels and receptors were also affected in response to CAG100. *Trypgamma* of the transient receptor potential (TRP) classic channel family and CG34123 of the TRP melastatin-like channel family were upregulated in CAG100 flies (2.4-fold change). TRP channels are essential for the control of extracellular magnesium levels (19).

RNA-binding proteins are especially interesting targets for potential mechanisms of CAG-repeat RNA toxicity. The transcripts of two RNA-binding protein genes were downregulated in CAG100 flies: *RNA-binding protein 4* (2.4-fold change), which has an RNA recognition motif (RRM), and *CHKov1* (2.3-fold change), predicted to have an RNA-binding motif.
and a domain of RNA-directed DNA polymerase activity. In the list of upregulated transcripts, expression of the RNA-binding protein eIF4E-5 was upregulated 4.9-fold in CAG100 flies.

The cytochrome P450 superfamily (Cyp) is a large and diverse group of enzymes that catalyse oxidation. Of these, four cytochrome P450-related genes were downregulated and four were upregulated. Their differential expression might reflect a detoxification response in CAG100 flies at the pre-symptomatic stage.

Significant upregulation of cytochrome P450-related genes has also been observed in the microarray analysis of a Drosophila model of Parkinson’s disease (PD) (20).

**Hsp70, a genetic modifier of polyQ toxicity, is upregulated in microarray analysis**

Intriguingly, in the microarray analysis, we found that some of the Hsp70 genes were significantly upregulated in brains of animals expressing the toxic CAG100 RNA (Supplementary Material, Table S1b). Heat shock protein 70 (Hsp70) is a powerful modulator of the pathogenic polyQ protein, such that in Drosophila models for SCA3, Hsp70 becomes localized to the NIs (4). Furthermore, upregulation of Hsp70 suppresses polyQ-mediated neurodegeneration. There are multiple copies of the Hsp70 genes in Drosophila at distinct loci, including Hsp70Aa and Hsp70Ab at chromosomal position 87A and Hsp70Ba, Hsp70Bb, Hsp70Bhh and Hsp70Bc at 87B. We found that probe sets of transcripts for Hsp70Ba, Hsp70Bb and Hsp70Bhh genes were upregulated 4.06-fold.

Given this upregulation of Hsp70 transcripts, we used a second technique to determine whether abnormal localization or levels of Hsp70 protein were detectable in flies expressing CAG100. Tissue immunostaining of fly brains using an antibody that detects the Hsc70/Hsp70 protein family was performed on flies expressing the toxic RNA repeat (Fig. 4A). At 3 days, when an Hsp70 transcriptional change was detectable by the microarray analysis, we did not see an alteration of the Hsc70/Hsp70 protein in CAG100 flies compared with controls (Fig. 4C and D). By 28 days, the brain normally shows a...
modest degree of Hsc70/Hsp70 accumulations (Fig. 4F). Strikingly, immunostaining of CAG100-expressing animals revealed abundant Hsc70/Hsp70 accumulations in the cortical region of the brain at 28 days (Fig. 4E). Quantification of the accumulations (Fig. 4E’ and F’) showed that the number of Hsc70/Hsp70-positive particles within the area shown in (E’) and (F’) was quantified. Error bars represent SD (\( P < 0.05\), Student’s t-test; \( n = 3–4\)). (C and D) At 3 days, Hsc70/Hsp70 accumulation is not detectable in CAG0 and CAG100 fly brains. Enlarged images of the dashed outline area are shown in (C’) and (D’) (green: anti-Hsc70/Hsp70, blue: DAPI). (E and F) At 28 days, there are prominent Hsc70/Hsp70 accumulations in CAG100 flies. Enlarged images of the dashed outline area are shown in (E’) and (F’). Genotypes of flies: elav-GAL4 in trans to (CAG0) UAS-DsRed-CAG0 and (CAG100) UAS-DsRed-CAG100 (5×). DAPI images are shown next to (C’–F’) for orientation.

Figure 4. Hsc70/Hsp70 accumulated in aged CAG100 flies. (A) Cryosection of a CAG100 fly head at 28 days, immunostained for Hsc70/Hsp70. The dashed outline represents the area enlarged in (C–F). (B) Graph of the mean number of Hsc70/Hsp70 particles in CAG0- and CAG100-expressing animals at 28 days. The average number of Hsc70/Hsp70-positive particles within the area shown in (E’) and (F’) was quantified. Error bars represent SD (\( P < 0.05\), Student’s t-test; \( n = 3–4\)). (C and D) At 3 days, Hsc70/Hsp70 accumulation is not detectable in CAG0 and CAG100 fly brains. Enlarged images of the dashed outline area are shown in (C’) and (D’) (green: anti-Hsc70/Hsp70, blue: DAPI). (E and F) At 28 days, there are prominent Hsc70/Hsp70 accumulations in CAG100 flies. Enlarged images of the dashed outline area are shown in (E’) and (F’). Genotypes of flies: elav-GAL4 in trans to (CAG0) UAS-DsRed-CAG0 and (CAG100) UAS-DsRed-CAG100 (5×). DAPI images are shown next to (C’–F’) for orientation.

Hsp70 is a modifier of Ataxin-3 protein-based neurodegeneration and of CAG-repeat RNA-based toxicity

To determine whether upregulation of Hsp70 could modify the toxicity of the CAG-repeat RNA, we co-expressed human Hsp70 together with a non-coding CAG250 RNA ubiquitously in the animal with daughterless-GAL4 (da-GAL4). CAG250 flies express a sequence of 250 CAG trinucleotide repeats in the 3’-UTR of the DsRed reporter. Flies expressing CAG250 showed a stronger toxicity than animals with tissue-specific expression of the RNA, thus facilitating the analysis of modifier genes. When expressed in this manner, the CAG250 repeat caused compromised climbing ability at day 1 that progressively deteriorated over 14 days (Fig. 5A). Upregulation of Hsp70 mitigated the toxic effects of the CAG250 repeat, such that now 61 ± 5% of the animals could climb at day 1, compared with 38 ± 7% in the absence of added Hsp70 (Fig. 5B). The effect of Hsp70 persisted with time such that by 14 days, whereas normally only 12 ± 7% of CAG250 flies could climb, with co-expressed Hsp70, now 27 ± 5% of CAG250 flies could climb. We confirmed that Hsp70 had no effect on the level of expression of the CAG250 repeat (Fig. 5C); rather, added Hsp70 mitigated the toxic effects of the CAG250 RNA. These data indicate that Hsp70 is a modulator of both pathogenic polyQ protein-based toxicity and toxicity conferred by a non-coding expanded CAG-repeat RNA.
enhancer–promoter (EP)-element insertion lines for ability to modulate the CAG-repeat RNA toxicity. To assess effects, we examined several indicators of RNA-based toxicity when a CAG250 repeat is expressed in the animal: wing posture and motility deficits. Normally, flies hold their wings flat to the back, rarely showing an abnormal upward wing posture (Fig. 6A). However, 70% of CAG250 flies displayed an abnormal wing posture, holding one or both wings up (Fig. 6A and B). This abnormality may be due to muscle- and/or neural-based effects. Previous findings also show that flies display defects in climbing ability when expressing the toxic CAG-repeat RNA in neurons with elav-GAL4 (5) (Fig. 5A). We used these two indicators of the toxicity of the CAG250 RNA (held-up wings and loss of climbing ability) to assess whether additional previously defined modifiers of Ataxin-3 protein-based toxicity could affect RNA toxicity.

Twenty-five different modifiers were previously isolated in a genome-wide screen for suppressors or enhancers of pathogenic polyQ Ataxin-3 protein toxicity (21). Of these, three mitigated both the wing posture and locomotor defects of CAG250 flies: the co-chaperone Tpr2\(^{EB7.14}\), the lin-41 homologue dpld\(^{EP546}\) and RNA-binding protein orb2\(^{lin-41}\) (Fig. 6D–I). While only 26 ± 4% of CAG250 flies had normal wing posture, now 66 ± 10% of flies with upregulated Tpr2 showed normal wing position (Fig. 6D). Tpr2\(^{EB7.14}\) also functionally rescued the climbing defects of CAG250 flies (58 ± 8% of Tpr2\(^{EB7.14}\) flies could climb, whereas only 36 ± 5% of CAG250 flies could climb in a similar manner in parallel control experiments) (Fig. 6E). Upregulation of dpld with the dpld\(^{EP546}\) allele rescued both wing posture and locomotor dysfunction, such that 58 ± 9% of CAG250 flies with upregulated dpld\(^{EP546}\) had normal wing posture, and climbing was restored such that 62 ± 9% of animals could climb (Fig. 6F and G). Upregulation of orb2 with the orb2\(^{lin-41}\) allele resulted in 80 ± 14% of CAG250 flies now showing normal wing posture and 62 ± 8% of animals climbing normally (Fig. 6H and I). By qRT-PCR, we confirmed that these modifiers did not affect the expression levels of the CAG-repeat RNA (Fig. 6C). Taken together, these data indicated that these modifiers, originally identified as genes that when upregulated can mitigate Ataxin-3 pathogenic protein-based toxicity, also rescue CAG-repeat RNA-based toxicity.

The genetic screen for modifiers of Ataxin-3 protein toxicity was performed with a transgene bearing a pure CAG-repeat RNA. As these four modifiers could modulate both the toxic effect of this transgene and the toxic effect of a non-coding CAG-repeat RNA, we were interested in whether the mitigation of the Ataxin-3 toxicity was due to a selective effect on the RNA versus an effect on both the protein and the RNA. To address this, we determined whether interrupting the RNA sequence of the Ataxin-3 pathogenic protein affected the interaction with these modulators. If these modifiers were no longer effective, then this would suggest that these modifiers were directed solely to the CAG-repeat RNA. We therefore co-expressed Hsp70, Tpr2\(^{EB7.14}\), dpld\(^{EP546}\) and orb2\(^{lin-41}\), with an Ataxin-3-expressing transgene in which the polyQ domain is encoded by a CAA/G-interrupted repeat sequence. The SCA3trQ78CAA/G transgene encodes a toxic polyQ protein identical in sequence to the CAG-repeat RNA.
encoding transgene SCA3trQ78CAG, differing only in whether the RNA is a pure CAG or interrupted by CAA codons. Expression of SCA3tr Q78CAA/G in the eye by gmr-GAL4 gave a disrupted eye effect (Fig. 7A), and co-expression of these modifiers mitigated the toxicity (Fig. 7B–E). These data indicate that these modifiers can modulate both Ataxin-3 pathogenic protein-based toxicity and CAG-repeat RNA-based toxicity, highlighting the overlap between RNA-based and polyQ protein-based toxicity mechanisms.

**DISCUSSION**

PolyQ diseases have been considered toxic polyQ-based disorders, although recent data suggest that the expanded CAG
trinucleotide repeat RNA encoding the polyQ domain may contribute to the disease (5). Given that mechanisms of RNA toxicity remain unclear, we provided insights into the nature of the CAG-repeat RNA toxicity by performing a microarray analysis to define the global transcriptional profile of the fly brain upon expression of toxic trinucleotide repeat RNA. These studies revealed that 160 genes were profiled of the fly brain upon expression of toxic trinucleotide repeat RNA. These data indicate an overlap between genes, as modifiers of both the polyQ protein and toxic CAG-repeat RNA. These data indicate an overlap between mechanisms of the protein-based and RNA-based toxicities that may contribute to polyQ disease.

**Microarray analysis identifies altered Hsp70: a modifier of CAG-repeat RNA-based toxicity**

Our microarray analysis identified Hsp70 as upregulated in CAG100 flies. To confirm these findings, we performed immunostaining analysis for the Hsc70/Hsp70 protein in brains of animals expressing the toxic RNA. This revealed age-dependent accumulations. These data indicate that upregulated Hsp70 induced by the toxic CAG repeat occurs at both transcriptional and translational levels. Given that Hsc70/Hsp70 accumulations are induced by expressing CAG100, and Hsp70 is a powerful suppressor of several human neurodegenerative diseases, we then performed functional analysis. These findings showed that upregulation of Hsp70 rescues the locomotor dysfunction caused by the toxic CAG repeat. Similarly, in a *Drosophila* model of fragile X syndrome, which is also an RNA-mediated neurodegenerative situation, upregulation of Hsc70 modifies the CGG-repeat RNA-induced effects (22). Taken together, these data suggest that Hsc70/Hsp70 chaperones can modify toxicity conferred by both pathogenic RNA and proteins. Hsp70 is thought to prevent aggregation or to assist in the refolding of misfolded protein. Possible mechanisms of suppression by Hsp70 in RNA-mediated toxicity might involve a misfolded protein response triggered by deleterious consequence of the pathogenic repeat RNA.

Other ways in which Hsp70 may suppress include modulation of death pathways (23). Notably, several genes associated with the regulation of apoptosis were in the list of genes affected in the CAG100 flies. Upregulation of cytchrome c transcript is a general early apoptotic response, and studies show that Hsp70 suppresses apoptosis by acting downstream of cytochrome c release in mammalian cells (24,25). Intriguingly, cytchrome c distal (Cyt-c-d), one of the *Drosophila* cytchrome c genes, was upregulated pre-symptomatically in CAG100 flies in the microarray analysis (Cyt-c-d, 2.0-fold change). Moreover, upregulation of Hsp70 suppresses the symptomatic effects of toxicity of CAG250 flies. Potentially, Hsp70 may suppress RNA-based toxicity through modulation of apoptosis processes as an additional mechanism.

The molecular chaperone Hsp70 responds to several neurodegenerative disease situations in animal models. In the *Drosophila* model of SCA3, Hsp70 is localized to the nuclear accumulations, whereas normally there is a low level of Hsp70 in the cytoplasm (4). Upregulation of Hsp70 suppresses the toxicity caused by the pathogenic Ataxin-3 protein. In mouse models of spinal bulbular muscular atrophy (SBMA), due to polyQ expansions in the androgen receptor gene, Hsp70 levels are increased in the muscle, and upregulation of Hsp70 ameliorates pathogenicity of the SBMA model (26). In the *Drosophila* model of fragile X-associated tremor/ataxia syndrome, Hsp70-containing inclusions are induced by expressing long CGG repeats in the 5'UTR of an enhanced green fluorescent protein (EGFP) reporter gene. Upregulation of Hsc70 modifies CGG-repeat-induced degeneration (22). *RnrL*, which suppresses non-coding CTG112 RNA-induced neurodegeneration in a *Drosophila* model for SCA8, was also identified in our present study (*RnrL*, 7.4-fold change) (27). These data suggest that candidate genetic modifiers can be identified through microarray analysis, defining altered genes in the disease-associated condition.

A recent study shows that modifier of *mdg4* ([mod(*mdg4*)]) transcript levels is altered in *Drosophila* heads upon expression of CAG-, CUG- and AUUCU-repeat RNA and that *mod(*mdg4*)* is an enhancer of CAG- and CUG-repeat toxicity (28). We did not find that *mod(*mdg4*)* met our criteria in generating gene lists; however, we used only brain tissue and not heads with eye tissue included. Transcriptional profiles may differ in distinct cell types in response to toxic elements, and brain-enriched RNA may reveal distinct or more subtle changes in gene expression that are selective to neurons.

**Candidate targets for further investigation of RNA-based toxicity mechanisms**

Several functional classes of genes were differentially expressed in CAG100 flies (Fig. 3). These classes give insights...
into molecular pathways that respond to the toxic CAG-repeat RNA. For example, microglial activation and innate immune system activation are recognized as a disease feature in Alzheimer’s (AD), PD, HD and ALS. Anti-inflammatory treatments may reduce susceptibility to AD and PD (29). Proteins involved in the innate immune response could be used as molecular biomarkers to identify pre-symptomatic stage of AD (30). Beyond changes in the cytochrome P450 superfamily, there were three upregulated transcripts associated with innate immune response, including *attacin*, *Immune-induced molecule 2* and *metchnikowin*. These gene lists not only provide candidate targets for further genetic analysis, but might also provide insights into potential CAG-repeat RNA pathogenesis and the common characteristic of neurodegenerative diseases. Furthermore, a set of genes was selectively affected in CAA/G105 and not in CAG100 or CAG0 (Supplementary Material, Table S1c). These transcripts may be relevant to situations in which the polyQ repeat sequence in the disease gene is interrupted, as in ATXN2 polyQ expansions that present with parkinsonism or ALS rather than SCA2. Future studies may reveal insights with regard to potential pathways modulated.

### Expanded CAG-repeat RNA and pathogenic polyQ protein trigger common pathways

Beyond *Hsp70*, three genetic modifiers identified in the present study, *Tpr2*Δ27-1A, *dpld*EP546 and *orb2*Δ68-3, also suppress both CAG-repeat RNA-based toxicity and polyQ protein-based toxicity. Previous studies show that these modifiers do not alter the Hsp70 expression level, thus function in a distinct manner (21). *dTPr2* is homologous to the human *tetratricopeptide repeat protein 2*. Tpr2 is a co-chaperone containing two chaperone-binding TPR domains and a DnaJ homologous J domain (31,32). In previous studies, *Hsp70* and five other modifiers of Ataxin-3 protein-associated neurodegeneration were categorized in the chaperone class (21). Surprisingly, two of these six also suppressed CAG-repeat RNA toxicity (*Hsp70* and *Tpr2*Δ27-1A) (Fig. 8). This raises a role for chaperones and ubiquitin-associated pathway components in CAG-repeat toxicity, although likely specific genes involved reflect specific processes primarily detected.

*dpld*EP546 was previously identified as a strong suppressor of Ataxin-3 toxicity and could suppress tau toxicity as well as a cell-death gene (*hid*). Intriguingly, data indicate that the mouse homologue of *dpld* (lin-41) is localized to P-granules, which are sites of RNA processing and degradation (33). This overlap between *dpld* as a modifier of polyQ protein toxicity and the CAG-RNA-based toxicity, coupled with the implication of P-granule involvement, supports the idea of RNA pathways going awry in both situations.

*Drosophila orb2* is also known as *Drosophila cytoplasmic polyadenylation element-binding protein* (CPEB). CPEB belongs to a family of RNA-binding proteins which have two RRM domains and a Zn-finger domain (34). CPEBs regulate translation of target mRNAs (35) and function in many processes, including synaptic plasticity, learning and memory (36–38). Recent studies show that *Drosophila* Orb2 binds directly to transcripts associated with long-term memory, and Orb2 targets are associated with neuronal growth, synapse formation and protein turnover (39). Taken together, these studies indicate that Orb2 is broadly involved in various neuronal functions. Moreover, Orb2 is co-localized in dFMR1-containing (fragile X mental retardation 1) foci in cultured *Drosophila* primary neurons (40). Fragile X mental retardation protein has been implicated in the translational regulation of target mRNAs in neurons and is the protein affected in fragile X syndrome (41–43). The pathogenic role of neuronal RNA granules in neurological disease suggests the possibility that CAG-repeat RNA might interfere with the normal function of Orb2 in neuronal RNA granules to cause toxicity. Homologues of *mbl*, which modifies Ataxin-3 protein toxicity, CAG-repeat RNA toxicity and CUG-repeat RNA toxicity, co-localize with RNA stress granules in cell culture (44,45). Thus, *mbl* may be broadly involved in other aspects of RNA metabolism that contribute to neurodegenerative disease. Future investigation to examine effects of the CAG-repeat RNA, and neural granules and P-bodies, together with studies of Orb2 will reveal further insight.

Intriguing recent data raise the possibility that expanded repeat RNAs may support protein translation in a manner not dependent on an AUG, as with classical protein-coding genes. That is, the hairpin repeat structure of triplet-repeat RNAs of a variety of sequences can lead to protein translation of the repeat region in all three reading frames. This so-called repeat-associated non-ATG translation (RAN translation) is reported to occur with SCA8 and DM1 CAG expansion transcripts (46). In the fly, previous studies failed to detect a protein generated by the non-coding CAG-repeat RNA (5); however, these studies were performed on young animals. Similarly, in a mouse model of CAG-repeat RNA toxicity, a
polyQ-containing protein was not detected upon expression of CAG repeats in the 3′-UTR of the EGFP gene (47). RAN, however, may be selective to specific cell subtypes. Our finding of accumulation of Hsc70/Hsp70 with age in CAG-repeat expressing flies, suggestive of late-onset accumulation of misfolded protein, may be indicative of such mechanisms, as is the overlap in modifiers of the protein-based toxicity and RNA-based toxicity. Our studies provide a foundation to address the extent to which this mechanism, as well as others, contribution to the toxicity conferred by the expanded repeat CAG-RNA, and its integration with protein-based toxicity mechanisms.

MATERIALS AND METHODS

Drosophila stocks and crosses

General fly lines were from Bloomington Drosophila Stock Center and maintained at 25°C on standard medium, unless otherwise indicated. Transgenic lines UAS-DsRed-CAG0, UAS-DsRed-CAG100, UAS-DsRed-CAG250, UAS-DsRed-CAA/G105 and genetic modifier EP insertion lines have been described (5,21). Control CAG0 flies express a transgene encoding a DsRed reporter protein with no CAG repeat in the 3′-UTR. CAG100 and CAG250 flies express transgenes encoding the DsRed reporter with repeats of approximately 100 and 250 CAGs in the 3′-UTR, as described previously (5). CAA/G105 flies, a non-toxic trinucleotide control, express a transgene encoding DsRed with a repeat sequence of CAA and CAG of 105 triplet codons in the 3′-UTR, as described (5).

RNA isolation

Four independent replicates of flies expressing CAG0, CAG100 or CAA/G105 by elav-GAL4 were raised at 25°C with 12 h/12 h light–dark cycle (5). Three-day female flies from four independent crosses were collected and brains dissected. Fly brain tissue (about 20 brains per sample, dissected from four independent crosses were collected and brains dissected). Fly brain tissue (about 20 brains per sample, dissected from four independent crosses were collected and brains dissected) was isolated in cold phosphate-buffered saline and stored in Trizol Reagent (Invitrogen, Carlsbad, CA) at −80°C. Total brain RNA was extracted and purified using Trizol Reagent (Invitrogen) and the RNeasy Mini system (Qiagen, Valencia, CA) and treated with RNase-free DNase I (Qiagen). cDNA amplification (Ovation Pico WTA systems, NuGEN, San Carlos, CA), labelling and hybridization (Encore Biotin Module, NuGEN) were carried out by the microarray core facility at University of Pennsylvania.

Microarray analysis

Affymetrix Drosophila Genome 2.0 microarrays (Affymetrix) were used for the microarray analysis. Normalization of probe signal intensity levels across arrays was performed using the GC robust multichip average algorithm implemented in Partek Genomic suite 6.2 beta. Principle component analysis and Pearson’s correlation coefficient were used to reveal outlier samples. Three-way analysis of variance (ANOVA) confirmed that the source of variation was not due to different batches of RNA. To generate lists of genes differentially expressed in different conditions, CAG100 and CAG250 were compared with CAG0 at the corresponding age (step-up P < 0.05). We used one-way ANOVA implemented in Partek Genomic suite 6.2 beta to generate a list of differentially expressed genes under each condition. Unannotated probe sets were discarded. When multiple probe sets were assigned to the same gene, the probe set with the smallest step-up P-value was included. Gene lists were restricted to genes that have at least a 2-fold change and step-up P-value less than 0.05. GEO accession number for the data is GSE31875.

Quantitative RT-PCR

qRT-PCR was performed on 3-day adult fly brain RNA. Brain dissections and total brain RNA were prepared and extracted, as described earlier. cDNA was synthesized using the High Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). qRT-PCR was performed using SYBR Green master mix (Applied Biosystems). All RNA samples were analysed in triplicate or quadruplicate using a 7500 Fast Real-Time PCR System (Applied Biosystems). Data were analysed using the relative quantification method. The endogenous control was ribosomal protein 49 (Rp49). Primers identified using Primer Express 2.0 (Applied Biosystems) were synthesized by Integrated DNA Technologies, Inc. Primer sets are as follows:

| Forward Primer | Reverse Primer |
|---------------|---------------|
| 5′-CAACATCGGTATTGAGATCGA-3′ | 5′-AATCCGTTGCGACATCAT-3′ |
| 5′-CCTAGTTTCTAAGAGATCTGTGACTG-3′ | 5′-GGCCAGCTATGGTAGACAAT-3′ |
| 5′-GAACAAGCCACATCGTCA-3′ | 5′-TGTAACACTCTTGCA-3′ |
| 5′-CACGCCCAAG-3′ | 5′-MTK forward: 5′-CACCCGAGCTAAGATGCA-3′ |
| 5′-TCTGCGCAGCTATGATGCAC-3′ | 5′-MK reverse: 5′-TTCTGCATGCA-3′ |
| 5′-GGACCAGTCTGACCTTCGCA-3′ | 5′-CTCF forward: 5′-GCTACACGTTCGGTCTGGA-3′ |
| 5′-CCGTTCTGAATGCGAATTAC-3′ | 5′-CTCF reverse: 5′-CGTCTCTAGGTTGAGCT-3′ |
| 5′-GGACGCATGGAACAGGAA-3′ | 5′-GCCTTTTGAGGTGCACCT-3′ |
| 5′-CAGACGTTCGACACCTTC-3′ | 5′-GCCCTGGTGGTACCA-3′ |
| 5′-GGACACTGGAACACATCA-3′ | 5′-CCGCTGTCGTC-3′ |
| 5′-GGACCAGTTCAGGAAAATACAA-3′ | 5′-CG30104 forward: 5′-CA TGGAATGTAATTTC-3′ |
| 5′-GCCACGTTGCTACAAAGC-3′ | 5′-GA TGCCCTGCTG-3′ |
| 5′-ACCGCAAGACACACAC-3′ | 5′-CA CG12428 GCTTGGATCTC-3′ |
| 5′-GCCGTTAGTGGTTCTCG-3′ | 5′-ACCGCA TGATCTTCGCTTC-3′ |
| 5′-CG18343 forward: 5′-ACGCA ATACCTTTCCTGCTCTC-3′ |
| 5′-GAAGCCGA GAAACCGACATTC-3′ | 5′-CG18343 reverse: 5′-AGCCCGA TTACCCATTTGAG-3′ |
| 5′-CG18343 reverse: 5′-AGCCCGA TTACCCATTTGAG-3′ | 5′-CG12428 forward: 5′-CGCCA GACGACATTTTTC-3′ |
| 5′-CG12428 reverse: 5′-CTGGAA CGGCTGAACATGGAACGAC-3′ | 5′-CG12428 reverse: 5′-CTGGAA CGGCTGAACATGGAACGAC-3′ |
| 5′-GCCTTGGTTCTCG-3′ | 5′-CG18343 reverse: 5′-CTGGAA CGGCTGAACATGGAACGAC-3′ |
| 5′-CG18343 reverse: 5′-CTGGAA CGGCTGAACATGGAACGAC-3′ | 5′-CG12428 reverse: 5′-CTGGAA CGGCTGAACATGGAACGAC-3′ |

Histochemistry and image quantification

Cryosections were performed as described (48). For immunostaining, the primary antibody was anti-Hsc70/Hsp70 (SPA-822, 1:200, Stressgen-Enzo Life Sciences, Farmingdale, NY). The secondary antibody was anti-mouse conjugated to...
Alexa Fluor 488 (1:100, Molecular Probes, Eugene, OR). The number of Hsc70/Hsp70-positive particles was quantitated using the particle analysis method implemented in Image J (NIH). The thresholds were adjusted based on background signals in the central brain region. Images from three to four animals per genotype were analysed.

Climbing assay

Flies expressing the repeat gene by da-GAL4 were raised at 20°C to prevent lethality at pre-adult stages and shifted to 29°C after 1 day for a stronger effect and for time considerations. About 8–20 female flies were anaesthetized by CO2 and placed in a plastic vial. After a 1 h recovery period, flies were banged to the bottom of the vial, and then the number of flies that climbed higher than a 2 cm threshold in 10 s was scored. Three trials were performed at 5 min intervals for each cohort of animals. At least, 100 flies per genotype were examined in each repeat.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

ACKNOWLEDGEMENTS

We thank Drs John Tobias and Shilpa Rao for help with microarray data analysis and interpretation and L.Y. Hao, Y. Fang and L. McGurk for critical comments.

Conflict of Interest statement. None declared.

FUNDING

This work received funding from the NIH (5R01NS043578, 5P01AG090215), the Ellison Medical Foundation, and the MDA. N.M.B. is an investigator of the Howard Hughes Medical Institute. Funding to pay the Open Access publication charges for this article was provided by the Howard Hughes Medical Institute.

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