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Isolation of gene sets affected specifically by polyglutamine expression: implication of the TOR signaling pathway in neurodegeneration

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Abstract

Transcriptional dysregulation as a result of sequestration of essential transcription factors into protein aggregates formed by polyglutamine (polyQ) expansions can lead to late-onset progressive neurodegeneration. DNA microarray analysis of *Drosophila* expressing polyQ in the compound eye over time revealed large numbers of transcriptional changes at the earliest stages of the disease including repression of the transient receptor potential calcium channels in a polyQ-induced cell death specific manner. While significant differences in expression profiles were found between the *Drosophila* compound eye and polyQ-sensitive neural cells, a number of possible key overlapping regulators were extracted. Among these, PDK1 was shown to act as a mediator for polyQ-toxicity, suggesting the involvement of the TOR pathway in polyQ-induced neurodegeneration.

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Abbreviations: polyQ, polyglutamine; Htt, Huntingtins

Introduction

The underlying mechanisms leading to polyglutamine (polyQ)-induced neurodegeneration of diseases such as Huntington's disease and the spinocerebeller ataxias remain controversial. Expression of proteins containing an expansion of a CAG trinucleotide repeat leads to a clear accumulation of unfolded proteins in the form of aggregates appearing in diseased tissue; however, it is still uncertain whether these inclusion bodies cause cellular disruption or play a protective role.[1–3] A further complication stems from tissue expression patterns of polyQ disease causing proteins; despite their ubiquitous expression, only postmitotic neurons are affected.

Models for polyQ-induced diseases, including *Caenorhabditis elegans*, *Drosophila*, and mice take advantage of easily amendable genetics to investigate neurodegeneration.[4–9] From these studies, numerous insights have come to light including a role for molecular chaperones in delaying the onset of neurodegeneration.[6,10] The neurodegeneration requires a functional translocon able to transport, as yet unknown, toxic protein(s) into the cytoplasm.[11,12] Proteasome inhibition also likely contributes to the accumulation of toxic proteins.[13,14]

Another model to explain the mechanisms behind neurodegeneration suggests sequestration of essential proteins including transcription factors.[15–18] Expanded polyQ-containing proteins have been shown to bind the transcriptional coactivator and acetyltransferase, CREB-binding protein (CBP) resulting in its depletion from the nucleus.[15,19] In agreement with this model, both addition of histone deacetylases as well as overexpression of CBP have been shown to reduce polyQ pathology.[20,21]

DNA microarrays provide an excellent means by which to monitor global changes in gene expression and have been used successfully to study cell signaling events.[22–24] Therefore, in order to examine possible transcriptional dysregulation during the progression of polyQ pathogenesis in *Drosophila,* polyQ-specific gene sets were isolated in this study. Identification of a gene set that is specifically affected by polyQ before the onset of neuronal degeneration is especially important because these genes must be early markers of disease progression as well as potential targets of the therapeutic drug. We could successfully isolate a common set of gene induction and repression before and after the onset of neuronal degeneration in a polyQ-induced cell death specific manner. These gene changes may be involved in polyQ-induced neural dysfunction that precedes the degeneration. In addition to measuring transcript levels in a late-onset neurodegeneration model of flies, we measured gene profiles of an *in vitro* model of polyQ-induced cell death (rapid cell death model) and through comparison of these profiles several potential regulators of polyQ-induced cell death were extracted. Among these, the PKD1/S6K pathway was shown to be required for the degeneration thereby implicating TOR signaling in polyQ-induced neurodegeneration.

Results

Isolation of polyglutamine-specific gene sets

PolyQ-induced cell death is a complex process that cannot be blocked by caspase inhibition; however, firm connections between polyQ-induced disease progression and transcriptional dysregulation have been established. We therefore used DNA microarrays to monitor global changes in transcription in tissues expressing a polyQ expansion protein. Figure 1 shows a schematic of the experimental design enabling early transcriptional changes to be compared with those derived from late stages of neurodegeneration. The UAS-GAL4 system was used to express a 78 polyQ repeat expansion under control of the eye-specific promoter *GMR*. Under this system, neurons undergo a late-onset progressive degeneration leading to the deterioration of the eye without affecting fly viability (Figure 1). Total RNA was isolated from the heads of adult flies harvested at day 1 whereas *GMR-GAL4* flies crossed to wild-type flies lacking the polyQ protein were used as a control. Set at a 99% confidence level, by day 1 nearly 800 genes showed altered transcription including the heat shock family of proteins (Figure 2a). Considering very little degeneration of eye neural tissue can be seen by day 1 (Figure 1), these transcripts represent putative indicators

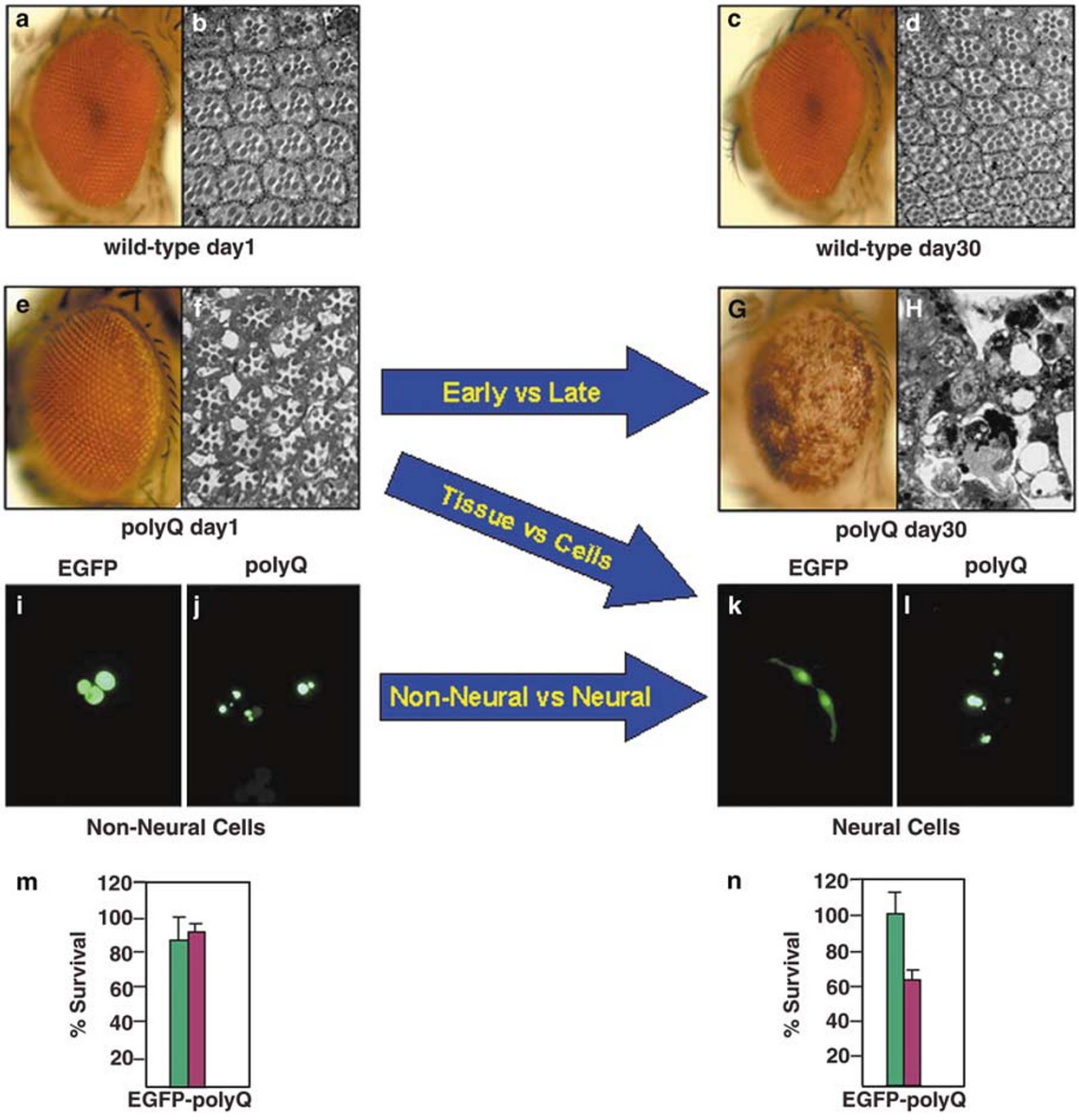

Figure 1 Schematic of experimental design to derive polyQ-specific transcripts. Briefly, comparison of expression profiles derived from day 1 flies (both wild-type and polyQ-expressing) to day 30 flies allowed early (day 1) *versus* late (day 30) gene changes to be elucidated. Similarly, expression profiles derived from neural and non-neural stable cell lines expressing EGFP and polyQ were used to compare neural and non-neural polyQ transcriptional changes. Overlap of tissue and cell line polyQ-specific transcripts were then elucidated. (**a**, **c**, **e**, and **g**) show the exterior of fly eyes. (**b**, **d**, **f**, and **h**) show the interior of fly eyes. (**i** and **k**) show EGFP-expressing cells while (**j** and **l**) show polyQ-expressing cells. (**m** and **n**) show cell death assays 24 h after heat shock

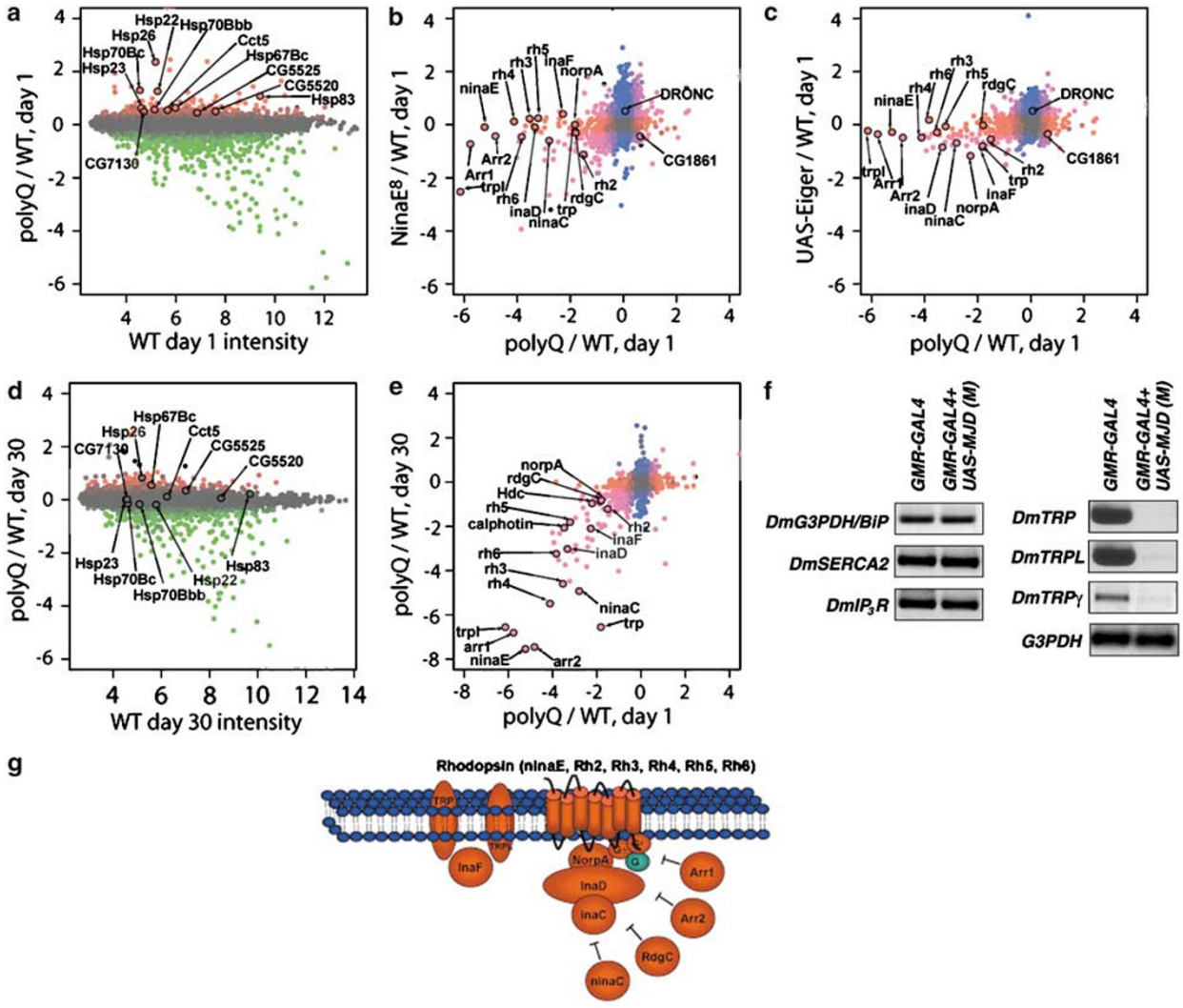

Figure 2 DNA microarray analysis of *Drosophila* heads expressing a 78 polyQ repeat in post mitotic neurons reveals widespread transcriptional changes. (**a**) Expression profile derived from polyQ-expressing flies compared to control flies both harvested at day 1 after eclosion. Data are displayed as intensity *versus* polyQ-expressing/control ratio (log scale). Red dots represent gene induction, green dots gene repression. Color intensity of each spot represents the degree of confidence from gray ($P>0.1$) to red/green ($P<10^{-5}$). (**b**) Correlation plot of *ninaE*^{8} mutants (expression profile derived from control and *ninaE*^{8} homozygotes) and polyQ-expressing flies (expression profile derived from control and polyQ-expressing flies) both harvested at day 1. Genes whose expression increased or decreased in both experiments are plotted as pink stars. Blue dots correspond to genes whose expression changed only in the transcriptional profile plotted on the *y*-axis whereas red dots correspond to genes whose expression changed only in the transcriptional profile plotted on the *x*-axis. Decreasing color intensity represents decreasing statistical significance with gray dots representing genes whose expression did not change significantly in either experiment at a 99% confidence level. (**c**) Correlation plot of *eiger*-expressing (expression profile derived from control and *eiger*-expressing flies) and polyQ-expressing flies (expression profile derived from control and polyQ-expressing flies) both harvested at day 1. (**d**) Expression profile derived from polyQ-expressing flies compared to control flies both harvested at day 30 after eclosion. Data are displayed as intensity *versus* polyQ-expressing/control ratio (log scale). (**e**) Correlation plot of day 1 (expression profile derived from control and polyQ expressing flies) and day 30 (expression profile derived from control and polyQ expressing flies) harvested flies. (**f**) Specific reduction of TRP channels in polyQ-expressing flies. The indicated genotypes of adult fly eyes were used for RT-PCR analysis. (**g**) Schematic of the inositide signaling phototransduction pathway in flies. Genes in red represent transcriptionally repressed genes in response to polyQ expression

of early disease state in polyQ-induced neurodegeneration in addition to containing possible key regulators of the disease. Furthermore, since less than half the tissue from which RNA was extracted expressed GAL4, these changes likely underestimate the actual transcriptional changes actually occurring. A complete list of all statistically significantly changed transcripts, in addition to raw data, from all experiments can be found at http://www.brain.riken.jp/labs/mns/Drosophila/.

In order to ensure that altered transcription detected in polyQ-expressing flies was not merely a result of nonspecific reductions in eye tissue, polyQ-expressing fly profiles were compared to a polyQ-independent, caspase-dependent progressive degenerative mutant profile. NinaE corresponds to the G-protein coupled photoreceptor in the phototransduction pathway of *Drosophila* eyes; *ninaE*^{8} homozygotes contain very little rhodopsin thereby leading to a late-onset eye degeneration and large numbers of transcriptional changes

(data not shown). By comparing profiles derived from *ninaE*^{8} homozygote heads with polyQ-expressing flies at day 1, a large overlap of gene transcription could be seen (Figure 2b). However, despite this overlap, a unique set of genes induced/repressed specifically in polyQ-expressing flies could be identified. By comparing expression profiles derived from polyQ-expressing flies with polyQ-independent cell death activators, gene sets containing putative key regulators of polyQ-induced degeneration can be elucidated; here, genes in red belong to this set (Figure 2b, Supplementary information Table 1).

As another control, flies expressing a caspase-independent but nonprogressive, non-late-onset cell death activator were profiled. Eiger, a tumor necrosis factor (TNF) ligand, driven by a weak *GMR-GAL4* line shows weak eye degeneration and large numbers of gene transcription changes by day 1[25] (data not shown). Little overlap was seen between transcriptional profiles of Eiger-expressing and polyQ-expressing flies, further suggesting that many transcriptional changes in polyQ-expressing flies were a direct result of polyQ expression rather than mere loss of tissue (Figure 2c). Again, genes in red represent repressed/induced transcripts specific for polyQ-expression (Supplementary information Table 2).

Polyglutamine-specific changes are not affected by ageing: reduction of TRP channels prior to the onset of degeneration

In order to ascertain whether the aging process affected any polyQ-specific changes, polyQ-expressing flies were harvested at day 30 when easily discernible and large-scale measurable changes in transcription would be expected (Figure 1). Indeed, many transcripts showed increases and decreases; however, the number was reduced compared to day 1 to 483, the majority still repressed (Figure 2d). Interestingly, many of the heat shock proteins were no longer induced. Despite the increased degeneration seen at day 30, a large overlap in gene expression was seen when transcriptional profiles were compared to day 1 (Figure 2e). Many of the genes found to change transcription specifically in response to polyQ expression were found in this overlapping group suggesting that aging does not affect polyQ-induced gene changes.

An interesting group of genes found repressed specifically during polyQ expression was the phosphoinositide signaling phototransduction pathway (Supplementary information Table 3). The phototransduction pathway in *Drosophila* is complexed into a signaling unit referred to as the signalplex comprised of a G-protein coupled receptor, Rhodopsin1 (Rh1/NinaE) leading to a canonical phosphoinositide cascade[26] (Figure 2g). Among these genes, *Rhodopsin (Rh)1/ninaE* together with *Rh2,3,4,5, and 6*, *inaD* (a scaffold for subcellular compartmentalization of the phototransduction machinery), *ninaC* (calmodulin binding protein), and *norpA* (GTPase activator), were all transcriptionally repressed. Genes associated with the inositide signaling phototransduction pathway, including the light-activated calcium channels, transient receptor potential (*TRP)*, and *TRPL*, *inaF*, and the negative regulators, *arr1*, *arr2*, *and rdgC* (seine/threonine phosphatase), were all transcriptionally downregulated. Interestingly, spinocerebellar ataxia type 1 (SCA1) model mice have shown a transcriptional reduction in the *TRP* ortholog, *TRP3* suggesting a potential conserved mechanism for polyQ-induced neurodegeneration.[27,28] To confirm the reduction of these genes, we performed semiquantitative RT-PCR and a dramatic reduction of *trp*, *trpl* and *trp*$_{\gamma}$ was observed (Figure 2f).

Dysregulation of cellular Ca^{2+}-homeostasis has been postulated to be a component of Huntington's disease and a polyQ-induced downregulation of other Ca^{2+}-homeostasis and mobilization genes including inositol triphosphate receptor 1 (IP3R1) and sarcoplasmic endoplasmic reticulum calcium ATPase type 2 (SERCA2) in mouse models of Huntington's disease and SCA1 pathogenesis has been seen.[27–29] No endoplasmic reticulum stress could be observed as measured by BiP levels nor could any changes in the expression of *SERCA2* and *IP3R* be seen by RT-PCR in *Drosophila* (Figure 2f), further suggesting the importance of *trp* reduction in polyQ-induced neurodegeneration.

Different neural cells yield different polyglutamine-induced gene changes: involvement of PDK1/S6K as an early determinant of severity of degeneration

In order to determine whether polyQ-induced gene changes occurred commonly *in vitro* between different types of neural cells, the first exon of human Huntingin (Htt) containing a 150-glutamine expansion was fused to EGFP and driven by the heat shock promoter in both *Drosophila* embryonic derived Schneider's S2 cells and *Drosophila* neural BG2 cells. Stable clones were isolated and heat shocked for 1 h at 37°C. Both cell types formed protein aggregates, however, only the neural BG2 cells showed measurable cell death 24 h after heat shock (Figure 1). Global transcriptional changes were monitored by DNA microarrays in both S2 and BG2 clones before and 24 h after heat shock. Stable S2 and BG2 clones expressing EGFP alone were simultaneously profiled. Sets of repressed and induced transcripts belonging specifically to neural BG2 cells can be seen in red (Figure 3a, Supplementary information Table 4).

When comparing the fly eye transcriptional profile with that derived from BG2 cells there is little overlap between gene sets (Figure 3b, Supplementary information Table 5). Unlike fly eyes, BG2 cell death is rapidly induced by polyQ expression (Figure 1) possibly accounting for the differences in gene sets. Furthermore, *reaper* was transcriptionally induced specifically in polyQ-expressing neural BG2 cells (and not in S2 cells) and may be responsible for the initiation of this rapid polyQ-induced cell death (Figure 3a).

Genes with commonly altered transcription in response to polyQ expression, both in fly eyes and neural cell lines, may act as key regulators of neurodegeneration. Table 1 lists polyQ-specific genes in common that are polyQ-specific; that is, not changed in *ninaE*^{8} mutants or *Eiger*-expressing flies. From this table, only nine transcripts were commonly transcriptionally induced including *appl*, encoding a beta amyloid protein precursor ortholog implicated in Alzheimer's disease (Figure 3b, Table 1 and Supplementary information Table 5). A total of 45 repressed transcripts were shared

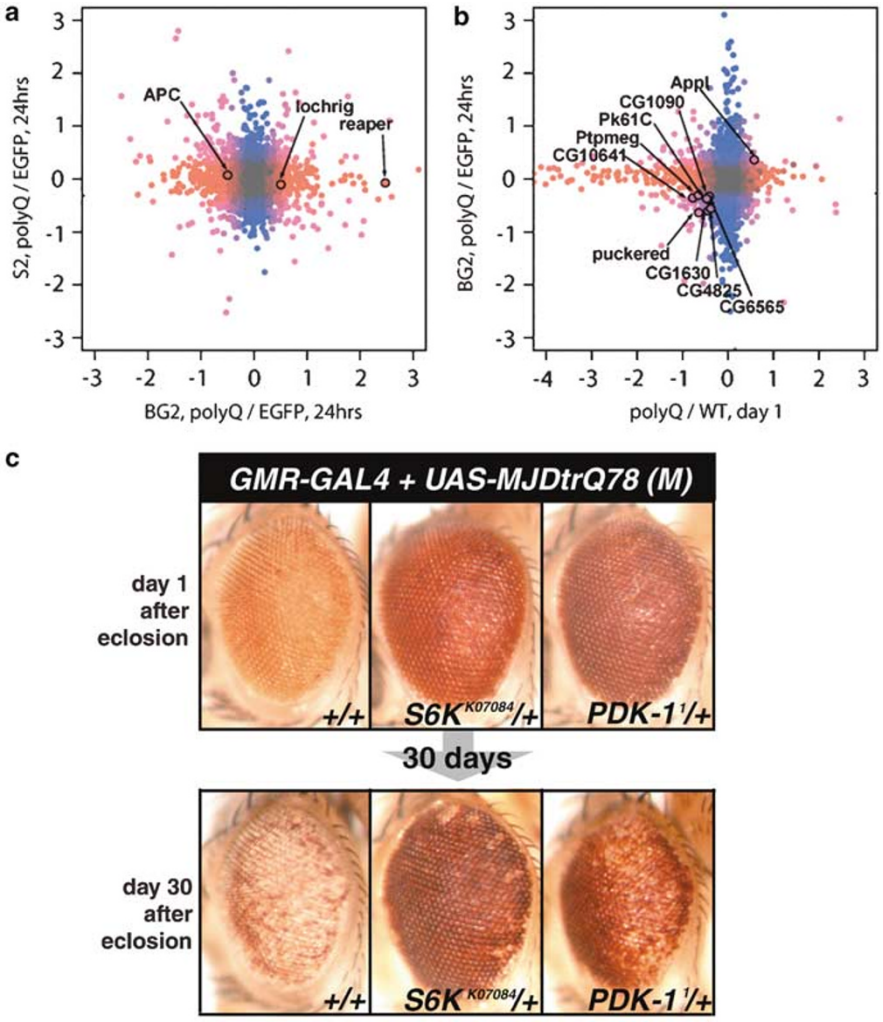

Figure 3 Downregulation of PDK1 and S6K alleviate neurodegeneration. (**a**) Correlation plot of non-neural S2 cells (expression profile derived from control EGFP and polyQ-expressing stable cells 24hrs after heat shock) and neural BG2 cells (expression profile derived from control EGFP and polyQ-expressing stable cells 24hrs after heat shock). Colors are as indicated in Figure 2. (**b**) Correlation plot of neural BG2 cells (expression profile derived from control EGFP and polyQ-expressing stable cells 24hrs after heat shock) and day 1 (expression profile derived from control and polyQ-expressing flies) harvested flies. (**c**) Eye morphology of wt, *S6K* heterozygoutes and *PDK-1* heterozygoutes all expressing polyQ

among gene sets, many of which are uncharacterized. *puckered*, a *JUN* kinase phosphatase and a JNK signal target gene,[30] and *ptpmeg*, a protein tyrosine phosphatase, were transcriptionally downregulated in both gene sets. Phospholipid bilayer synthesis may be affected as *CG4825*, a putative CDP-diacylglycerol-serine-*O*-phosphatidyltransferase, and *CG6565* encoding a phosphatidylcholine transporter were both transcriptionally repressed. Lastly, both calcium regulation and inositol signaling were further implicated as *CG1090, CG10641, CG1630*, and *Pk61C* (*dPDK-1*) were all transcriptionally downregulated.

We tested for genetic interactions with available mutants and found that reduction of *Drosophila* phosphoinositide-dependent protein kinase-1 (dPDK1) significantly suppressed polyQ-induced eye degeneration (Figure 3c). PDK1 acts as an activator of the AGC family protein kinases, including AKT, p70 ribosomal S6 kinase (S6K), serum- and glucocorticoide-induced protein kinase (SGK) and protein kinase C (PKC). Reduction of the PDK1 effecter, S6K, also suppressed the progression of polyQ-induced eye degeneration (Figure 3c). The finding of common transcriptional changes in both cell lines and flies expressing polyQ suggests PDK1 and S6K are affected directly by polyQ expression and determining their role in polyQ-induced neurodegeneration may shed light on mechanisms underlying the degeneration.

Discussion

Compare plots provide an easy, attractive method of displaying large amounts of data generated from genomic experiments and through such manipulation of data sets many potential regulators of polyQ-induced neurodegeneration were identified. Several genes whose products have been implicated in cell death were found to have altered transcription specifically in response to polyQ expression including

Table 1 Genes with altered expession in both flies and cell line specifically in response to polyglutamine expression

	Fold change			
Rank 1	**Flies**	**Cells**	**Gene title**	**Function**
Induced				
1	1.28	3.56	CG3996	
2	1.27	2.13	CGI 8547	
3	1.29	1.75	CG31847	
4	1.48	1.42	CGI 0353	
5	1.49	1.29	Appl beta amyloid protein precursor-like	Neurogenesis
6	1.41	1.26	CG32611	Nucleic acid binding
7	1.33	1.26	CG2076	
8	1.36	1.20	mus209 mutagen-sensitive 209	Nucleotide-excision repair
9	1.25	1.27	CGI 5097	Actin binding
Repressed				
1	1.46	3.94	CG6113	Triacylglycerol lipase activity
2	3.69	1.46	CG722O	Ubiquitin conjugating enzyme activity
3	1.91	1.81	CG5287	Catalytic activity
4	1.80	1.96	CAP	Focal adhesion
5	1.74	1.83	CG8468	Monocarboxylate porter activity
6	1.44	1.82	CGI 3784	Signal transduction
7	1.56	1.56	puc puckered	JNK phosphatase
8	1.50	1.56	CGI 0960	Glucose transporter activity
9	1.65	1.48	didum dilute class unconventional myosin	Motor activity
10	1.96	1.31	CG10311	Transport
11	1.47	1.60	CG4196	
12	1.53	1.48	CG18316	
13	1.48	1.55	Kap-alphal karyopherin alpha 1	Protein-nucleus import
14	1.42	1.47	CG32428	
15	1.83	1.31	Dnzl DNZDHHC/NEW1 zinc-finger protein 11	
16	1.95	1.43	CG31116	Voltage-gated chloride channel activity
17	1.29	1.46	CG15917	
18	2.55	1.27	Fatp Fatty acid (long chain) transport protein	Catalytic activity
19	1.70	1.26	vri vrille	RNA polymerase II transcription factor activity
20	1.38	1.44	CG1939	ATP binding
21	1.35	1.52	IP3K2 Inositol 1,4,5-triphosphate kinase 2	1D-myo-inositol-trisphosphate 3-kinase activity
22	1.30	1.48	CG4825	Phosphatidylserine biosynthesis
23	1.58	1.27	CGI 2292	
24	1.57	1.24	Ptpmeg	Intracellular signaling cascade
25	1.46	1.41	crq croquemort	Scavenger receptor activity
26	1.36	1.32	Olf186-F	NOT serine-type endopeptidase activity
27	1.38	1.30	PDK-1	Positive regulation of cell growth
28	1.36	1.31	CG6565	Phosphatidylcholine transporter activity
29	1.41	1.54	Arc70	Transcription initiation from Pol II promoter
30	1.71	1.28	CGI 0641	Calcium ion binding
31	1.46	1.34	l(2)k09913	
32	1.25	1.34	CGI 1857	Vesicle-mediated transport
33	1.41	1.36	Nrvl Nervana 1	Potassium ion transport
34	1.52	1.34	ctp cut up	Microtubule-based movement
35	1.37	1.45	lace lace	Serine C-palmitoyltransferase activity
36	1.34	1.27	CGI 1537	Sugar porter activity
37	1.33	1.25	CGI 090	Calcium, potassium:sodium antiporter activity
38	1.50	1.31	Nc73EF Neural conserved at 73KF	Oxidoreductase activity
39	1.28	1.25	alphaTub84D Tubulin at 84D	Microtubule-based process
40	1.29	1.35	EG.86E4.2	
41	1.28	1.28	Pfrx fi-phosphofructo-2-kinase	Catalytic activity
42	1.26	1.26	CG10338	
43	1.48	1.26	nes nessy	
44	1.39	1.25	CG8057 antisense construct a of Pan	SNF1 A/AMP-activated protein kinase activity

Ranking is based on joint probability (multiplicaton of) *P*-values for flies (polyQAVT day 1 comparison) and cell line (polyQ/EGFP BG2 cell comparison)

CG1861, *calphotin*, *Hdc, Apc*, and *lochrig*. CG1861 is highly similar to dX11L especially in the PDZ-domain region; overexpression of this region of dX11L is sufficient to induce eye degeneration.[31] Calphotin is a calcium binding protein crucial to both rhabdomere development and photoreceptor cell survival.[32] Hdc functions as a major neurotransmitter in photoreceptor cells and *Hdc* mutants are blind.[33] *Apc* is an ortholog of the adenomatous polyposis coli (*APC*) tumor suppressor gene whose disruption leads to retinal degeneration.[34] *lochrig* mutants display neurodegeneration that is enhanced by mutation to the *amyloid precursor protein-like* (*Appl*) involved in Alzheimer's disease.[35] Many such genes show small but statistically significant changes in expression in response to polyQ expression and it is perhaps an accumulation of such changes that leads to the slow progressive, late-onset neurodegeneration that is the hallmark of the polyQ family of diseases. Without such data representation it may not have been apparent that such genes

displayed transcriptional induction/repression specifically in response to polyQ expression.

One key biological mechanism may have been uncovered when polyQ-expressing flies undergoing neurodegeneration revealed the transcriptional repression of the TRP channels. In fact, mTRP3 was found repressed in polyQ-expressing mice further suggesting a conserved mechanism of neurodegeneration.[27,28] Furthermore, due to the absence of significant neurodegeneration on day 1 it appears that TRPs may serve as markers for the risk of neurodegeneration prior to the onset of the disease.

Phosphoinositide signaling has been implicated in a variety of cellular activities including cell survival, cell growth, and autophagy [36–38] and its downregulation in response to polyQ expression in flies may represent a general mechanism of neurodegeneration. One of the interesting findings of our microarray analysis combined with genetic interaction is the involvement of PDK1/S6K in polyQ-induced neurodegeneration. Recently, it has been shown that mTOR is sequestered in polyQ aggregates thereby inhibiting its kinase activity and inducing autophagy.[39] Furthermore, specific inhibition of mTOR by rapamycin attenuates polyQ-induced aggregation and cell death. Since PDK1/S6K is involved in the TOR pathway, the observed reduction of PDK1 in both polyQ-expressing BG2 cells and day 1 fly eyes may represent a protective cellular response against polyQ toxicity through enhancement of autophagy by inhibition of the TOR pathway.

Materials and Methods

Fly stocks

All general fly stocks and *GAL4* lines were obtained from *Drosophila* stock centers. *white*^{1118} and *GMR-GAL4* were used as a wild-type line for all experiments. The *UAS-MJDtr-Q78* flies were a gift from N Bonini (University of Pennsylvania). The *UAS-eiger* flies were constructed at the same time and same manner as those found in Igaki *et al*.[25] *PDK-1*^{1} was a gift from J Chung.[40] *S6K*^{K07084} was a gift from G Thomas.[41] *ninaE*^{8} homozygote flies were received from the Bloomington Stock Center. Fly cultures and crosses were performed at 22°C following standard protocols. All flies were stored in bottles containing approximately 50 flies and media was changed once every 3 days. Fly histology was performed as described.[11]

Cell culture

Drosophila S2 and BG2 cells were cultured and transfected as reported.[11] To make stable cell lines (both S2 and BG2), cells were cotransfected with 4.5 ng of either *pCaspeR-hs-EGFP* or *pCaspeR-hs-tNhtt-Q150-EGFP* and 0.5 ng of a geneticin-expressing plasmid (*pAct5co-Neomycin*) in a six-well plate. Cultures were grown in the presence of geneticin (1 mg/ml) and diluted in a 10 cm dish. Individual cells were allowed to form colonies that were picked (40 of each), grown, and tested for EGFP expression. Cell death assays were performed as described.[11]

Plasmid construction

The *pCaspeR-hs-tNhtt-Q150-EGFP, pCaspeR-hs-EGFP*, and *pAct5co-Neomycin* (a gift from S Nagata) plasmids have been described.[11,42,43] Original tNhtt constructs were provided by N Nukina.[44]

RT-PCR

The indicated genotypes of adult flies were used for RT-PCR analysis (26 cycles). The compound eyes of anesthetized adult flies were dissected carefully, and the total RNA of the compound eye of adult flies was extracted by TRIzol reagent (GIBCO). To detect mRNA expression, 1 μg of total RNA was reverse-transcribed in a total volume of 30 μl, and 1 μl of the reaction mixture was used to amplify the transcripts by step-down PCR with ExTaq (Takara) using specific primer sets in a total volume of 25 μl. The following primer sets were used: *Drosophila BiP/GRP78*, 5′-CAT GGT TCT GGG CAA GAT GAA GGA AAC CGC-3′ and 5′-CAC AAT TTC GTG CAC GTC CTT CTT GTT CAT-3′; *Drosophila SERCA2* (*Ca-P60A*) [45] 5′-AAG TCC TAC TCG GGT CGT GAA TTC GAC GAC-3′ and 5′-AGC CAT GGT CAT CGC ATG GGG GTC GCT GAA-3′; *Drosophila IP3R* [46] 5′-CTT CGA TTC ATC TTT TTA CTA GGG CCG GAG-3′ and 5′-GGC GAA GGT GTC GAT GAT GAC ACC AAA TAT-3′; *Drosophila TRP*γ,[47] 5′-ATC GAT TTG GAC AGC TTT GAG TTG GAT GGA-3′ and 5′-GTC CAA CGA TGT GGA GAA CTC GGC AAC TGG-3′; *Drosophila TRP*, 5′-GAC CAG GAG AAG GCT TGT ACC ATC TGG CGA-3′ and 5′- GAC GAA GCC GAT TTG GAA GTC CTT AAG GAT-3′; *Drosophila TRPL*, 5′- CTG AAC CAA CTG CTC TGG TAC TTT GCC GCC-3′ and 5′- GTT GGC CGA GGA AAC ATC CAT TCC GCT GTT-3′. The equality of the amount of cDNA samples used for PCR was verified by PCR using the primer set for *Drosophila G3PDH*. The primer sequences used for the detection of *Drosophila G3PDH* have been described.[48,43]

DNA microarrays

Experiments, both fly and cell line, were repeated independently at least two times. Flies were anesthetized with CO_2 and immediately flash frozen in liquid nitrogen. The heads of at least 500 flies for each experiment were isolated using mesh wire, and total RNA was extracted using Trizol and purified using Qiagen Rneasy Kits. Flies were collected at the same time each day to avoid fluctuations in circadian rhythms. Stable cell lines (both S2 and BG2) were seeded in 10 cm dishes at a density of 2×10^5 cells/ml and left overnight at 25°C. At the appropriate time point, media was aspirated and Trizol added directly to the dish, cells were scraped off and lysed with a fine-gauge syringe. Purified total RNA (15 μg) was used to prepare biotinylated probes using standard Affymetrix protocols. Probes were hybridized to Affymetrix arrays using standard protocols.

DNA microarray analysis

Expression values were obtained using the Affy package[49] of Bioconductor software available from http://www.bioconductor.org/. The parameters for the Affy package were set at default. Affy package outputs the referred expression measure as log-scale robust multi-array analysis (RMA). Values from RMA are set at log two therefore a difference of 1.0 is equal to a two-fold change. The comparisons of two groups were done using a Bayesian *t*-test approach,[50] considered more robust than normal *t*-tests in small sampling. The R program scripts for the statistical calculation are downloadable from http://www.brain.riken.jp/labs/mns/Drosophila/.

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