Ayurvedic Amalaki Rasayana and Rasa-Sindoor suppress neurodegeneration in fly models of Huntington's and Alzheimer's diseases

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We examined two Ayurvedic Rasayana formulations, claimed to facilitate 'healthy ageing', for their role in neuroprotection in fly models of polyQ (127Q and Huntington's) and Alzheimer's disorders. Our earlier findings showed that dietary supplement of Amalaki Rasayana, a preparation derived from Indian gooseberry fruits, and Rasa-Sindoor, an organo-metallic Bhasma prepared from mercury and sulphur, improves general well-being of fruit flies. Here we show that dietary supplement of either of these formulations during larval period substantially suppressed neurodegeneration in fly models of polyQ and Alzheimer's disorders without any side-effects. Dietary Amalaki Rasayana or Rasa-Sindoor prevented accumulation of inclusion bodies and heat shock proteins, suppressed apoptosis, elevated the levels of heterogeneous nuclear ribonucleoproteins and cAMP response element binding protein and at the same time improved the ubiquitin–proteasomal system for better protein clearance in affected cells. Our studies suggest, the potential of these Ayurvedic formulations in providing a holistic relief from the increasingly common neurodegenerative disorders.

Keywords: Ayurvedic formulations, dietary supplement, fruit fly, neurodegenerative disorders.

WITH improved healthcare and general hygiene and consequent longer life, the societal burden of the various late onset neurodegenerative diseases has substantially increased in recent times. Some of the inherited neurodegenerative diseases, known as codon reiteration disorders, are associated with a unique class of dynamic mutations which increase the number of trinucleotide repeats in certain genes beyond the gene-specific normal and stable threshold[1–3]. Several of these codon reiteration neurodegenerative disorders, which include Huntington's disease (HD) and several spinocerebellar ataxias (SCA), are grouped together as polyQ expansion disorders since they result from expansion of CAG repeats coding for polyglutamine (polyQ) tracts. Alzheimer's disease (AD), the other common form of senile dementia in humans, is associated with truncated Aβ peptides produced by aberrant proteolytic cleavage of the transmembrane receptor amyloid precursor protein (APP)[3–5]. A characteristic feature of these neurodegenerative diseases is the accumulation of protein aggregates, formed either by the repeat expanded or the truncated protein. AD patients[3–5] show presence of amyloid plaques formed by Aβ peptides and tau protein filament tangles in affected neuronal cells, while polyQ inclusion bodies (IB) are seen in polyQ disorders like HD and many of the SCAs[1–6]. The amyloid plaques or the polyQ IBs disrupt cellular homeostasis because a variety of critical cellular proteins like molecular chaperones, transcription factors, proteasome subunits and cytoskeletal components get sequestered with the IBs, which directly or indirectly cause cellular damage and consequent death of the target neuronal cells[1–6]. Therefore, these diseases are proteinopathies resulting primarily from a failure of the protein quality control mechanisms of the cell.

With a view to understand the molecular and cellular pathophysiology of neurodegeneration and to discover potential drug targets for therapeutic applications, several human neurodegenerative diseases like HD, different SCAs, AD, etc. have been modelled in yeast, *Caenorhabditis*, *Drosophila* and mouse model systems[1,2,5–7]. The *Drosophila* models offer many advantages because of the powerful genetic resources available in this organism[1]. Such studies have indeed helped in a better understanding of these neurodegenerative disorders and suggested several therapeutic approaches, although therapies that provide holistic relief with little side-effects continue to remain elusive.

Several traditional Ayurvedic formulations claim to facilitate 'healthy ageing'[8] and thus have the potential to mitigate the suffering from neurodegenerative diseases[9]. Although Ayurveda, the traditional medicine system of India, has been widely practised for several thousand years, very few systematic studies have been carried out to understand Ayurvedic formulations and practices in terms of contemporary science. With a view to fill this gap, we established *Drosophila* as a powerful model for

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studying the cellular and molecular biological bases of Ayurvedic formulations like Amalaki Rasayana (AR) and Rasa-Sindoor (RS)[10]. This study indeed showed that the biological effects of dietary supplement of AR or RS in flies were generally similar to those traditionally claimed for humans. The present study examines the effects of these two formulations on fly models of neurodegenerative disorders.

AR and RS are used as part of the rejuvenating Rasayana therapy, which is one of the eight major branches described in classical Ayurvedic texts like *Sushruta Samhita*[8,11]. Rasayana therapy is believed to promote long life with enhanced physical and mental strength so that the old age associated ailments are minimized. The Rasayana therapy involves, along with adoption of a certain lifestyle, oral administration of formulations based on plant and/or animal or mineral/metal sources. AR, a *kasthoushadhy*, is prepared from fruits of amla or Indian gooseberry (*Phyllanthus emblica*, synonym *Emblica officinalis*), while RS is a *Rasaoushadhi bhasma* in the form of mercuric sulphide with crystal size ranging from 25 to 50 nm, close to the nanocrystalline materials[12,13]. In our earlier study[10], we found that feeding of larvae and flies on food supplemented with 0.5% (weight/volume) of AR or RS significantly improved tolerance to thermal or starvation stresses and enhanced cellular levels of various heterogeneous RNA-binding proteins (hnRNPs), which have key roles in gene expression and RNA processing/transport[14,15]. Recently, we found that either of these formulations also enhances the levels of cAMP-response-element-binding protein (CBP/p300), a histone-acetyltransferase[16], in wild type *Drosophila* larval tissues (V. Dwivedi and S. C. Lakhotia, unpublished). Several earlier studies in different model systems[1,14,17–19] have shown that elevated levels of hnRNPs, CBP and better tolerance to thermal and/or oxidative stress suppress neurodegeneration. Therefore, we examined if dietary supplement of AR or RS, which enhances the levels of hnRNPs, CBP and stress tolerance, affects neurodegeneration in fly models of polyQ disorders or AD.

We used two well-established fly models for polyQ disorders, one expressing HA-tagged 127Q polypeptide (*UAS-127Q*)[20]; and the other expressing HA-tagged mutant human Huntingtin protein with a stretch of 93 glutamine residues (*UAS-httex1p Q93*)[21]. For AD, we used a fly stock carrying four copies of *GMR-Aβ42* transgene, which synthesizes the truncated Aβ polypeptide in developing eyes resulting in the formation of the amyloid plaques characteristic of AD[22]. Following the well-established practice in fly models of neurodegenerative disorders, the desired pathogenic polyQ transgene was expressed in developing eyes using the *GAL4-UAS* binary system of targeted gene expression[23] and the damage in the neuronal cells of eyes was assessed in the differentiating larval eye discs and/or adult eyes. We show that dietary supplement of 0.5% (weight/volume) of AR or RS results in significant suppression of neurodegeneration in parallel with greatly reduced accumulation of polyQ IBs in 127Q or the amyloid deposits in AD model. Levels of hnRNPs (like Hrp36 and Bancal, homologs of human hnRNP A1 and hnRNP K respectively), the CBP and the ubiquitin–proteasome (UPS) activity were significantly elevated in the 127Q transgene expressing eye discs in larvae reared on AR or RS supplemented food. Since these Ayurvedic formulations had no adverse side-effects in any of these fly models, further studies to examine the therapeutic potential of AR and RS in human neurodegenerative disorders like HD and AD would be rewarding.

Materials and methods

AR and RS, prepared by Arya Vaidya Sala (Kottakkal, Kerala, India), were separately mixed in fly-food (0.5% w/v) for rearing of experimental larvae and/or flies at 24° ± 1°C as described earlier[10], keeping controls on the standard agar–cornmeal–sugar–yeast food. Wild type (*Oregon R*$^+$), *w/w*; *UAS-127Q* (ref. 20), *w/w*; *UAS-httex1p Q93/CyO* (ref. 21), *w*; *GMR-GAL4* (ref. 24), w^{1118} *elav-GAL4* (ref. 25), *w; UAS-Ub*^{G76V}*-GFP* (ref. 26), w^{1118}*; GMR-Aβ42*^{K52}; *GMR-Aβ42*^{K53} (ref. 22) fly stocks were used. Appropriate crosses were carried out to obtain progenies of desired genotypes.

The surface organization of ommatidia in adult eyes was examined using nail-polish imprints[27], while the retinal rhabdomeres in adult eyes were visualized by pseudopupil technique[28] or phalloidin-TRITC staining[29]. Vision of 1, 5 or 10 days old wild type or *GMR-GAL4 > UAS-httex1p Q93* expressing flies, reared on different feeding regimes, was measured by the phototaxis assay as described earlier[29]. Apoptosis in eye discs was assayed by AO staining[30]. The total number of flies or larval eye discs of different genotypes and feeding regimes that were examined in each case is noted in the 'Results' section.

For assessing survival of individuals expressing *UAS-127Q* in the entire central nervous system under the *elav-GAL4* driver[29] on different feeding regimes, freshly hatched *elav-GAL4 > UAS-127Q* larvae were transferred to formulation-supplemented or standard food and the mean proportions (%) of larvae reaching pharate and adult stages were calculated from eight replicates of 25 larvae each.

Late third instar larval eye discs of desired genotypes were immunostained as described earlier[29,30] using P11 anti-Hrp36 (ref. 31), Q18 anti-Hrb57A or Bancal[31], 6E10 anti-amyloid plaque, mab22C10 anti-neuornal cells (DSHB, Iowa), SPA806 anti-Hsp60 (Stressgen), anti-CBP[32], SC-805 anti-haemagglutinin (Santa Cruz) for polyQ IBs, or 7Fb anti-Hsp70 (ref. 33) primary antibody. In many cases, the discs were also co-immunostained for HA-tagged polyQ IBs (SC-805 anti-haemagglutinin, Santa Cruz). Appropriate secondary antibodies conju-

gated with Cy3 (Sigma-Aldrich) or Alexa Fluor 488 (Molecular Probes) were used. Chromatin was counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI). The immunostained discs were examined with Zeiss LSM 510 Meta confocal microscope.

For inhibiting the UPS activity, eye discs were dissected out from late third instar *GMR-GAL4 > UAS-127Q* expressing larvae and incubated in Poels' salt solution (PSS)[34] containing 1 μM proteasome inhibitor (clastolactacystin β-lactone, Sigma-Aldrich, India) for 2 h following which they were processed for immunostaining with the SC-805 anti-haemagglutinin Ab and confocal microscopy.

At least 20 eye discs were examined by confocal microscopy for each immunostaining. Four consecutive mid-level (along the *Z*-axis) optical sections, which distinctly showed the morphogenetic furrow, were used to generate the projection images of immunostained discs. These projection images were used to compare the levels of a given protein (expressed in arbitrary fluorescence units) in eye discs from differently fed larvae using the Histo option of Zeiss LSM 510 Meta software.

Western blots[10] of total proteins from eye discs (three replicates) were challenged with anti-Hrp36 or anti-β-tubulin and signals were detected using HRP conjugated anti-mouse or anti-rabbit IgG (Bangalore Genei, India) secondary antibodies respectively.

Levels of 127Q and G3PDH transcripts in *GMR-GAL4 > UAS-127Q* expressing eye discs from larvae reared on different feeding regimes were measured by semi-quantitative RT–PCR as described earlier[29].

Sigma Plot 11.0 software was used for statistical analyses. All percentage data were subjected to arcsine square-root transformation. One-way ANOVA was performed for comparison between the control and formulation-fed samples. Data are expressed as mean ± SE of mean of several replicates.

Results

Feeding on AR or RS supplemented (0.5%) food suppressed polyQ toxicity

The compound eye of the adult fly is a highly ordered array of nearly 800 ommatidial units, each having its own lens and 8 neuronal photoreceptor cells or rhabdomeres. The undifferentiated precursor cells of adult eyes are present in larvae as a pair of eye imaginal discs, which begin to differentiate and form the ommatidial units in an orderly manner during late larval and pupal stages.

It is known that *GMR-GAL4 > UAS-127Q* transgene expression in eye cells severely disrupts the regular arrays of ommatidia due to the polyQ toxicity-induced neurodegeneration[20,28]. We examined the organization of ommatidial units in eyes of one-day-old *GMR-GAL4 > UAS-127Q* flies, fed from the beginning of their larval life on normal or 0.5% AR or 0.5% RS supplemented food, through nail-polish imprints of eye surface or phalloidin staining of the retinal rhabdomeres. Compared to the regular arrays of ommatidia in eyes of wild-type flies reared on regular (Figure 1 *a*) or formulation supplemented food (Figure 1 *b*, *c*), nail-polish imprints of eyes of 75% flies ($N = 67$) expressing *GMR-GAL4 > UAS-127Q* and reared since larval stage on normal food showed near complete absence of the ommatidial arrays (Figure 1 *g*); eyes in the remaining 25% flies appeared slightly better, but the ommatidial pattern was still highly disorganized (not shown). Interestingly, a majority of *GMR-GAL4 > UAS-127Q* expressing flies reared during the larval and adult period on food supplemented with AR (58.34%, $N = 72$) or RS (71.6%, $N = 81$) showed some indication of arrays of ommatidia (Figure 1 *h*, *i*), more so in RS-fed flies. The ommatidial organization in the remaining formulation-fed flies was also better than in any of those reared on normal food.

Phalloidin staining of eyes of wild type flies reared on normal (Figure 1 *d*), or AR or RS supplemented food (Figure 1 *e*, *f*) showed the seven characteristically arranged rhabdomeres (photoreceptors) in each ommatidial unit. However, like the above noted complete disruption of ommatidial arrays, there was a near complete loss of photoreceptor neurons in each rhabdomeric unit in the eyes of all ($N = 17$) freshly emerged *GMR-GAL4 > UAS-127Q* expressing flies reared since the larval life on normal food, so that the phalloidin-positive F-actin formed irregular scattered aggregates (Figure 1 *j*). On the other hand, among those reared on formulation supplemented food since larval period, 43% ($N = 16$) of the AR and 67% ($N = 23$) of the RS-fed flies showed improvement in development of the photoreceptor neurons (Figure 1 *k*, *l*). The presence of at least some phalloidin-positive photoreceptor elements in ordered rows in AR or RS-fed 127Q expressing eyes (Figure 1 *k*, *l*) compared to the randomly distributed phalloidin-positive fragments in flies reared on normal food (Figure 1 *j*) and the presence of 2–3 more intense phalloidin-positive bodies in each cluster in their eyes clearly suggest an improvement in F-actin organization in rhabdomeres following the formulation feeding.

The overall structure of the eye surface and retina appeared significantly better in RS-fed flies (Figure 1 *i*, *l*).

As reported earlier[29], a pan-neuronal expression of the *127Q* transgene using the *elav-GAL4* driver resulted in substantial organismal lethality, mostly during pupal differentiation, so that on normal food only about 8% of eggs ($N = 200$ from 8 replicates of 25 larvae each) reached the pharate stage, none of which enclosed. Significantly, AR or RS supplemented food ($N = 200$ from 8 replicates of 25 larvae in each case) allowed 23.5% and 14% respectively, to reach pharate stage and 14.5% and 8% respectively, to actually eclose as flies with normal lifespan.

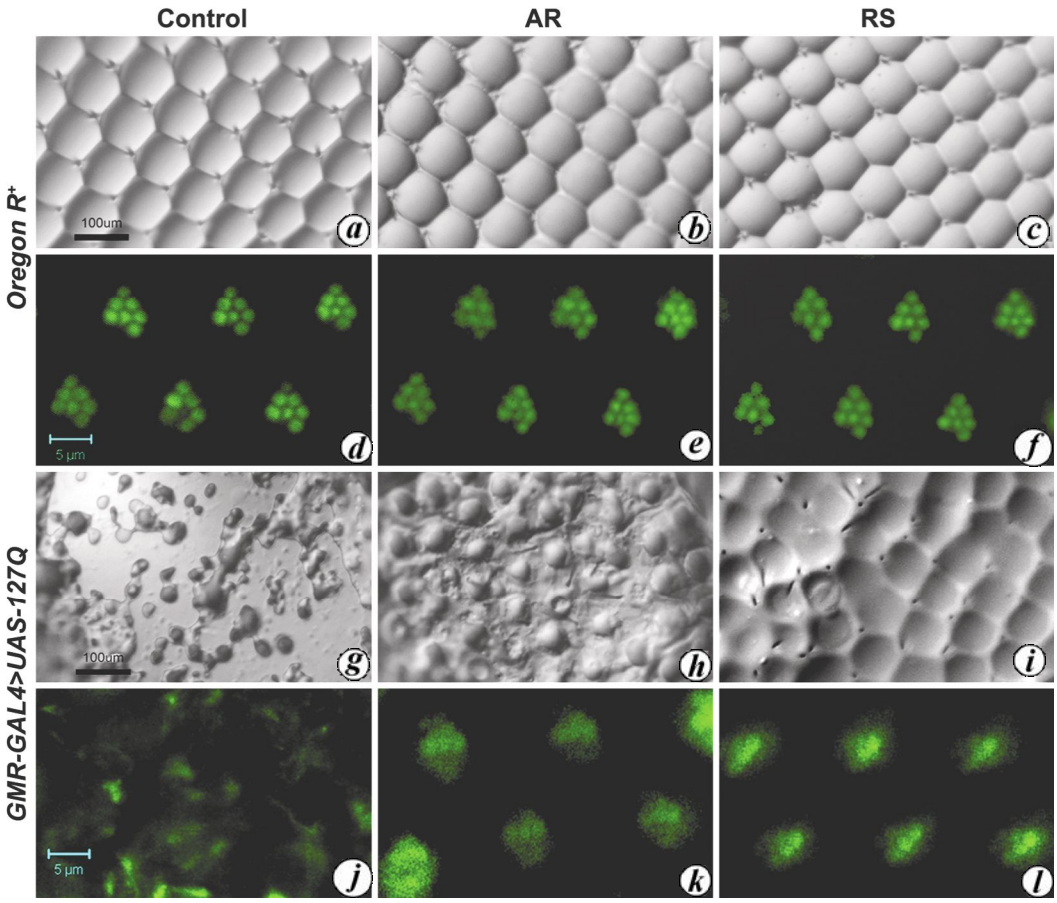

Figure 1. Dietary supplement of AR or RS substantially improved the ommatidial arrays damaged due to expression of *UAS-127Q*. Images of nail-polish imprints (***a***–***c*** and ***g***–***i***) and phalloidin-stained eyes (***d***–***f*** and ***j***–***l***) of one-day-old wild type *Oregon R*$^{+}$ (***a***–***f***) or *GMR-GAL4 > UAS127Q* adults (***g***–***l***) fed on normal (control, ***a***, ***d***, ***g***, ***j***) or AR (***b***, ***e***, ***h***, ***k***) or RS (***c***, ***f***, ***i***, ***l***) supplemented food since the beginning of larval life. Note the improved ommatidial arrays (***h*** and ***i***) and rhabdomeric units (***k*** and ***l***) in formulation-fed flies compared to those in (***g***) and (***j***) respectively. Scale bars in (***a***) and (***g***) correspond to 100 µm, while those in (***d***) and (***j***) correspond to 5 µm and each applies to all the images in that row.

To see if the above suppressive effects of AR or RS are applicable to other polyQ disorders, we used *GMR-GAL4* driver to express the *UAS-httex1p Q93* transgene which mimics the HD phenotype in flies[1,21,29,30]. It is known[1,29] that the *GMR-GAL4 > UAS-httex1p Q93* expressing freshly eclosed flies have near normal eyes and vision but show a progressive age-dependent degeneration, becoming almost completely blind by 10 days, even though the external eye surface of *GMR-GAL4 > UAS-httex1p Q93* expressing flies does not show any appreciable deterioration with age. Pseudopupil images of the rhabdomeres of 5 or 10-day-old *GMR-GAL4 > UAS-httex1p Q93* expressing flies revealed that while all the flies reared on normal food showed severely damaged retina with no detectable rhabdomere-like structures (Figure 2 *a*, *d*), those reared since larval stage on AR or RS supplemented food displayed at least some organized rhabdomere-like structures in 40–50% of 5-day-old flies (Figure 2 *b*, *c*) and 30–40% of 10-day-old flies (Figure 2 *e*, *f*). Interestingly, feeding on formulation-supplemented food during only the larval period also resulted in restoration of rhabdomere organization (Figure 2 *g*–*l*) comparable to that seen after larval as well as adult feeding. Significantly, however, when larvae were reared on normal food and AR or RS-supplemented food was provided after the flies emerged from pupal case, the retinal organization was as disrupted as in flies reared on normal food during larval as well as adult stages (Figure 2 *m*–*r*). This shows that these formulations can suppress neurodegeneration when it is taking place during development, but cannot restore the damage that has already occurred.

Assay for phototaxis, based on their preferential movement to the illuminated arm of a *Y*-maze, revealed, as reported earlier[1,29], that the *GMR-GAL4 > UAS-httex1p Q93* expressing 1-day-old flies reared on normal or formulation-supplemented food showed the expected near normal positive movement towards light (not shown here). However, with age, unlike the continuing positive phototactic behaviour of visually normal wild-type flies, which almost always moved to the lighted chamber, the *GMR-GAL4 > UAS-httex1p Q93* expressing flies reared on normal food became completely blind by day 10 so that

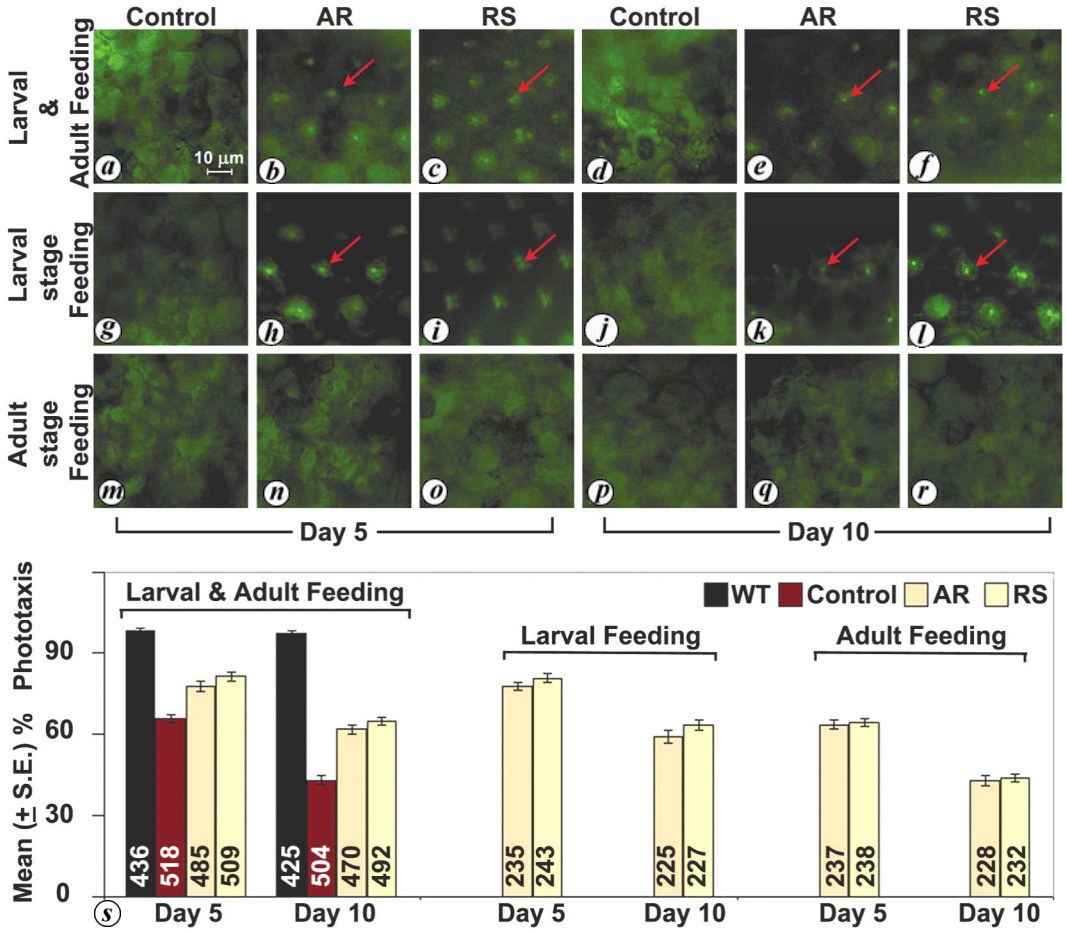

Figure 2. AR or RS feeding suppressed the progressive degeneration of eyes of adult flies expressing *GMR-GAL4 > UAS-httex1p Q93* as seen in the pseudopupil images of eyes (***a–f***, green pseudocolour) on day 5 (***a–c***) or day 10 (***d–f***) following rearing on normal food (control, ***a***, ***d***; *N* = 40 and 32 respectively) or AR (***b***, ***e***; *N* = 38 and 30 respectively) or RS (***c***, ***f***; *N* = 43 and 34, respectively) supplemented food since first instar larval stage; those reared on normal food showed severe disruption of rhabdomeres already on day 5, while those reared on AR or RS-supplemented food showed some rhabdomere-like structures (red arrows) even on day 10. A comparable improvement of ommatidial organization was seen in flies that received the AR (***h*** and ***k***) or RS (***i*** and ***l***) supplemented food only during larval period (*N* for control = 27, AR = 22 and RS = 29 for day 5 and control = 28, AR = 24 and RS = 21 for day-10 samples). Rearing of larvae on normal food and providing AR (***n*** and ***q***) or RS (***o*** and ***r***) supplemented food only from the day of fly emergence did not bring about any improvement in rhabdomere organization (*N* for control = 27, AR = 21 and RS = 25 on day 5 and control = 27, AR = 24 and RS = 28 on day 10). ***s***, Age-dependent loss of phototaxis in *GMR-GAL4 > UAS-httex1p Q93* flies is partially suppressed by larval feeding on formulation-supplemented food as seen in histograms of mean frequencies (± SE) of positively phototactic flies (*Y*-axis) fed on normal (control) or formulation supplemented food during larval and/or adult stages (feeding regime is shown above the histogram bars) on day 5 or day 10 (*X*-axis); frequencies of positively phototactic wild type (WT) flies reared on normal food are also shown for comparison. The number of flies examined for each data point is indicated within the frequency bars. The same set of flies was examined on day 5 and day 10 in each case, but since a few flies died in between, the numbers on day 10 were lesser than on day 5.

they moved randomly between the illuminated and dark chambers of the *Y*-maze (Figure 2 *s*). In contrast, more than 60% of the *GMR-GAL4 > UAS-httex1p Q93* expressing flies reared from first instar stage onwards on AR or RS supplemented food moved to the illuminated arm (Figure 2 *s*), indicating retention of some degree of functional rhabdomeres. The phototactic response of *GMR-GAL4 > UAS-httex1p Q93* flies reared on AR or RS-supplemented food only during the larval period was comparable to that of *GMR-GAL4 > UAS-httex1p Q93* flies that received the formulation-supplemented food during larval as well as adult stages. However, *GMR-GAL4 > UAS-httex1p Q93* flies that were fed on the supplemented food only after emergence from pupal case behaved like those that were reared all through on normal food (Figure 2 *s*).

Dietary AR or RS improved retinal organization, suppressed inclusion bodies and apoptosis

It is known[3,29,30] that *GMR-GAL4 > UAS-127Q* or the *GMR-GAL4 > UAS-httex1p Q93* transgene expression leads to accumulation of polyQ IBs posterior to the

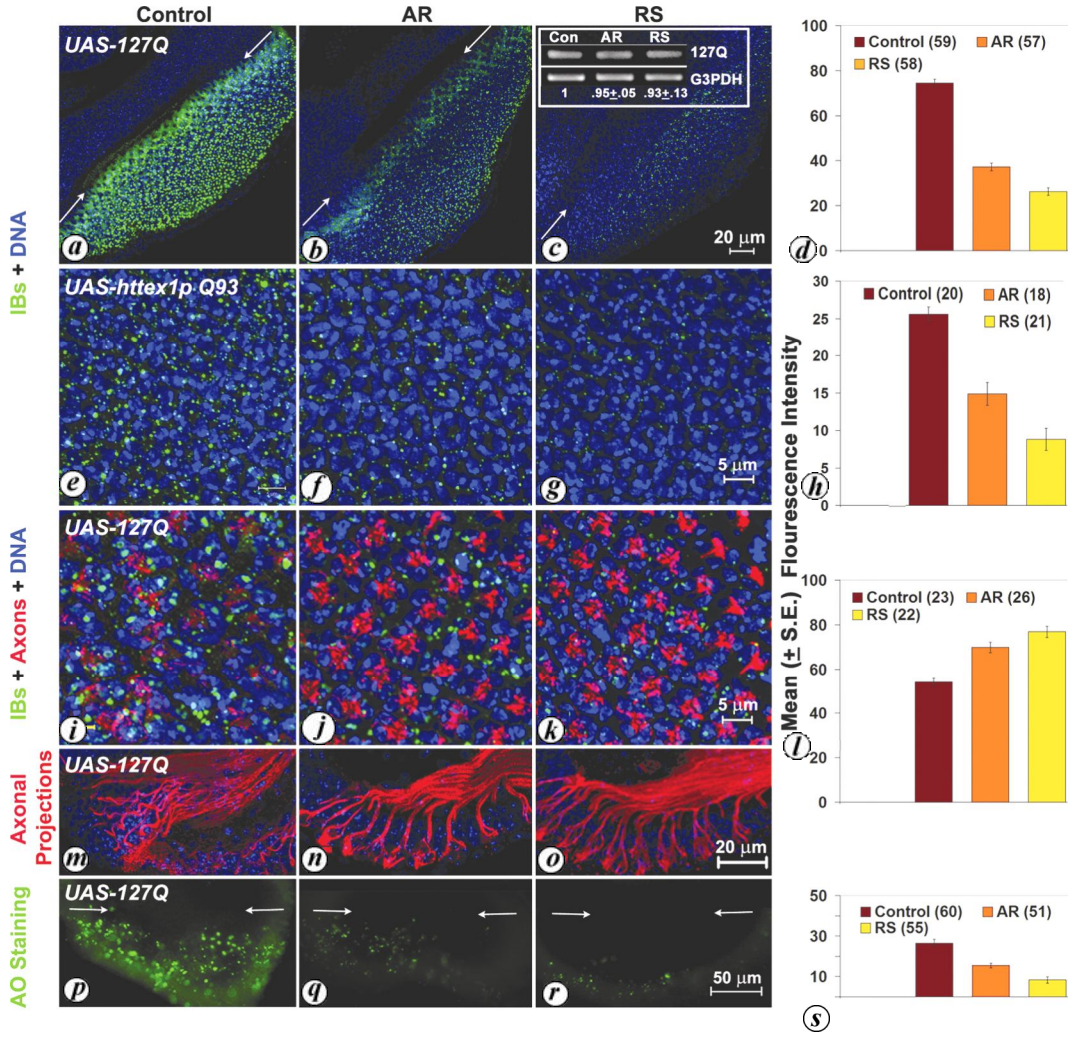

Figure 3. Formulation feeding reduced the accumulation of polyQ IBs (green, ***a***–***c***, ***e***–***g***), damage to rhabdomeric axons (red, ***i***–***o***) and cell death (***p***–***r***) in poly Q expressing eye discs. The specific polyQ transgene expressing in each case (*UAS-127Q* or *UAS-httex1p Q93*) is noted on the top left corner in column 1 of the row. Compared to normal food (***a***, ***e***), feeding on AR (***b***, ***f***) or RS (***c***, ***g***) substantially reduced the polyQ aggregates (***a***–***c***, ***e***–***g***) and improved the disarrayed axonal projections (immunostained with mab22C10, red) in the optic stalk (***i***–***o***). Images ***i***–***k*** show higher magnification confocal projections of four middle *z*-axis optical sections of eye discs immunostained for polyQ IBs (green) and axons (mab22C10 in red). Images ***m***–***o*** show axonal projection (mab22C10 staining, red) in optic nerve coming out of the *GMR-GAL4 > UAS-127Q* expressing eye discs from larvae reared on normal (***m***), AR (***n***) or RS (***o***) supplemented food. The nuclei were counterstained with DAPI (blue). Scale bars in ***c***, ***g***, ***k***, ***o*** and ***r*** apply to all images in the given row. White arrows in ***a***–***c*** and ***p***–***r*** indicate position of the morphogenetic furrow in eye discs. The inset in (***c***) shows the 127Q (upper row) and G3PDH (lower row) amplicons generated by semi-quantitative RT–PCR with total RNA from larval eye discs from larvae reared on Control, AR and RS-supplemented food (indicated on top of the columns). The values below each column indicate the mean (± SE, $N = 3$) levels of polyQ transcripts relative to that in control sample, which was taken as 1.0. ***p***–***r***, Compared to normal food (***p***), rearing on AR (***q***) or RS (***r***) significantly reduced apoptotic cell death as revealed by AO-stained live eye discs expressing *GMR-GAL4 > UAS-127Q*.. The scale bar (50 µm) in (***r***) applies to ***q***–***s***. Histograms in (***d***), (***h***) and (***s***) represent the mean (± SE) fluorescence intensities (measured in arbitrary fluorescence units) of polyQ IB (***d***), mab22C10 (***h***) and AO (***l***) staining in *GMR-GAL4 > UAS-127Q* expressing eye imaginal discs of late third instar larvae reared on different feeding regimes; numbers in parentheses after the bar legends indicate the figures of eye discs examined for each data point.

morphogenetic furrow in late third instar larval eye discs (Figure 3 *a*, *d*). The polyQ IBs were significantly reduced in eye discs from *GMR-GAL4 > UAS-127Q* (Figure 3 *b*, *c*) or *GMR-GAL4 > UAS-httex1p Q93* (Figure 3 *f*, *g*) expressing larvae that were reared on AR or RS-supplemented food, more so in RS-fed larvae (Figure 3 *c*, *g*). A quantitation of the polyQ immunofluorescence intensity in eye discs of each genotype confirmed that the accumulation of IBs in AR and RS-fed larvae was significantly reduced when compared to those reared on regular food (Figure 3 *d*, *h*).

In order to know if the greatly reduced polyQ IBs in formulation-fed samples were due to reduced transcription, levels of 127Q transcripts were measured through semi-quantitative RT–PCR using RNA isolated from *GMR-GAL4 > UAS-127Q* expressing larval eye discs of

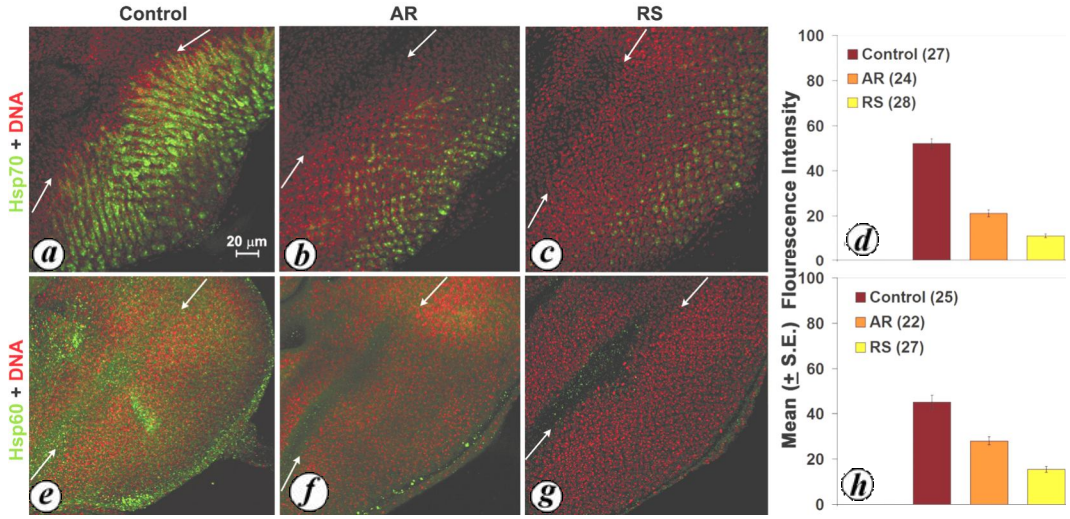

Figure 4. Compared to normal food (***a***, ***e***), feeding on AR (***b***, ***f***) or RS (***c***, ***g***) resulted in reduced levels of Hsp70 (***a–c***, green) and Hsp60 (***e–g***, green) in *GMR-GAL4 > 127Q* expressing larval eye discs. DAPI stained nuclei are shown in red (***a–g***). White arrows indicate position of the morphogenetic furrow in eye discs. The scale bar in (***a***) corresponds to 20 μm and applies to all the confocal projection image panels. Histograms in (***d***) and (***h***) show the mean (± SE) fluorescence intensities (in arbitrary fluorescence units) of Hsp70 and Hsp60 respectively, in *127Q* expressing larval eye imaginal discs under different feeding regimes; numbers in parentheses after the bar legends indicate the number of eye discs examined for each data point.

late third instar larvae reared on different feeding regimes. It was seen (inset, Figure 3 *c*) that the transcriptional activity of *GMR-GAL4 > UAS-127Q* transgene was not affected by feeding on any of the formulations since levels of polyQ transcripts in formulation-fed and normally fed samples remained similar (inset, Figure 3 *c*). This shows that the reduced load of polyQ IBs following AR or RS feeding is not due to reduced transcriptional activity of the *UAS-127Q* transgene.

In order to see if the reduced polyQ accumulation following the AR or RS feeding was also accompanied by restoration of ommatidial integrity and the axons projecting from rhabdomeres to the optic lobe in the brain, we co-immunostained *GMR-GAL4 > UAS-127Q* eye discs from larvae reared on normal or AR or RS-supplemented food with anti-HA (for polyQ IBs) and mab22C10 antibody, which specifically identifies the axons in fly retina (Figure 3 *i–k*). These clearly showed that along with the reduction in levels of polyQ IBs in eye discs from larvae reared on AR or RS-supplemented food, the differentiating arrays of ommatidial units were remarkably better organized, each with intact axons projecting out from the central region of each of the rhabdomeric complexes (Figure 3 *j*, *k*). Together with the disarrayed rhabdomeric complexes, the axons were also irregular or often missing in *GMR-GAL4 > UAS-127Q* eye discs from larvae reared on normal food (Figure 3 *i*). Quantitation of the mab22C10 fluorescence intensity in these discs also confirmed that there were more axons in the formulation-fed larval eye discs than in those from normally fed *GMR-GAL4 > UAS-127Q* larvae (Figure 3 *l*). The axonal projections from the rhabdomeres were also followed in the optic nerve from eye disc to optic lobe in the brain by immunostaining with mab22C10 antibody (Figure 3 *m–o*). In wild-type eyes, the axonal projections from different photoreceptor cells in each ommatidial unit follow a regular order with all the fibres running in a parallel pattern in the optic nerve (not shown, but see refs 27 and 28). It was clear that the axonal projections too were irregularly wavy and disarrayed in *GMR-GAL4 > UAS-127Q* larvae that were reared on normal food (Figure 3 *m*), while those reared on AR or RS-supplemented food showed an orderly arrangement of the axonal projections in the optic nerve (Figure 3 *n*, *o*) similar to that in the wild-type[27,28]. The polyQ IBs were not seen along the length of axons.

Since a high incidence of apoptosis is seen in eye disc cells expressing *GMR-GAL4 > UAS-127Q* transgene[30], we performed acridine orange (AO) staining of live 127Q expressing eye discs from larvae fed on normal or AR or RS-supplemented food to identify the apoptotic cells. This revealed a significant reduction in the incidence of apoptosis in differentiating eye discs from formulation-fed larvae (Figure 3 *q*, *r*) in comparison to control (Figure 3 *p*). Comparison of the fluorescence intensity of AO-stained eye discs confirmed that rearing on AR or RS formulation supplemented food (Figure 3 *s*) resulted in a significant reduction in cell death.

It is notable that RS feeding provided more pronounced suppressive effects in all these cases.

AR or RS feeding reduced the induction of Hsp70 and Hsp60 in 127Q expressing eye disc cells

As reported earlier[28,29], accumulation of IBs is accompanied by elevated levels of Hsp70 (Figure 4 *a*) and Hsp60 (Figure 4 *e*) in the polyQ-expressing eye discs. The

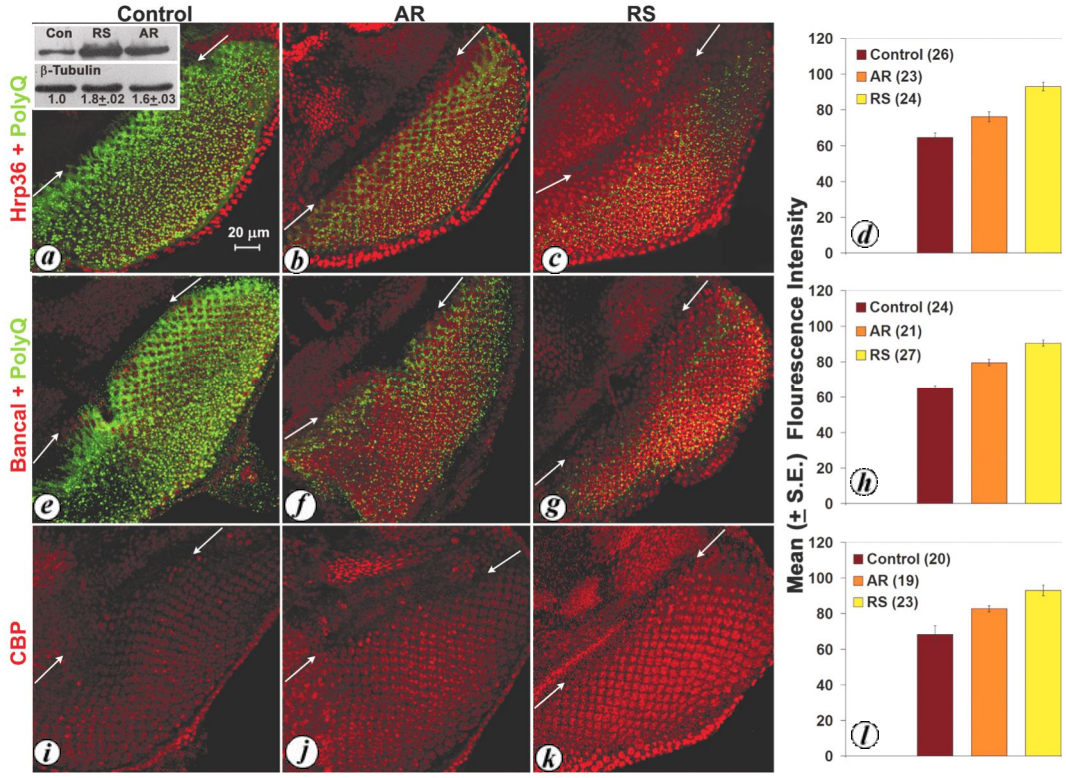

Figure 5. AR or RS-formulation feeding elevated cellular levels of Hrp36 (red, ***a***–***d***), Bancal (red, ***e***–***h***) and CBP (red, ***i***–***l***) along with the reduction in accumulation of polyQ IBs (green, ***a***–***g***) in *GMR-GAL4 > 127Q* expressing larval eye discs. The discs in (***i***–***k***) are immunostained only for CBP. The feeding regime, normal (control) or AR or RS-supplemented food, is indicated above each column of the confocal projections (***a***–***c***, ***e***–***g***, ***i***–***k***) of eye discs immunostained for the protein noted on the left of a row. White arrows indicate position of the morphogenetic furrow in eye discs. The scale bar in (***a***) corresponds to 20 μm and applies to all the confocal projection image panels. The inset in (***a***) is a Western blot of total protein from eye discs of 127Q expressing larvae reared on normal (control) or RS or AR-supplemented food to show the relative levels of Hrp36 (upper row in inset in ***a***); *β*-tubulin (lower row) was used as loading control; the values below each column indicate the mean (± SE, $N = 3$) levels of Hrp36 relative to those in control sample, which was taken as 1.0. Histograms in (***d***), (***h***) and (***l***) represent the mean (±SE) fluorescence intensities (in arbitrary fluorescence units) of Hrp36 (***d***), Bancal (***h***) and CBP (***l***) respectively, in *127Q* expressing larval eye imaginal discs following different feeding regimes; numbers in parentheses after the bar legend indicate the number of eye discs examined.

reduced levels of 127Q IBs in eye discs from AR or RS fed larvae (Figure 3 *b*, *c*) were paralleled by a substantial reduction in immunostaining for Hsp70 (Figure 4 *b*, *c*) as well as Hsp60 (Figure 4 *f*, *g*). Compared to AR (Figure 4 *b*, *f*), RS feeding (Figure 4 *c*, *g*) resulted in a greater reduction in immunostaining for Hsp70 as well as Hsp60. This is further confirmed by a quantitation of fluorescence intensities in eye discs immunostained for Hsp70 (Figure 4 *d*) or for Hsp60 (Figure 4 *h*).

AR or RS feeding enhanced levels of Hrp36, Bancal and CBP

Earlier studies[10] and other unpublished data have shown that dietary AR or RS significantly enhances the levels of various hnRNPs and CBP in different wild-type larval tissues and since levels of these proteins are known to modulate polyQ toxicity[1,30], we examined the cellular levels of two hnRNPs, viz. Hrp36 (hnRNP-A homolog) and Bancal (hnRNP K homolog) and CBP in *GMR-GAL4 > UAS-127Q* expressing eye discs in larvae reared on normal food and those reared on AR or RS-supplemented food. Immunostaining with appropriate antibody and fluorescence intensity values showed that compared to normally fed larvae (Figure 5 *a*, *e* and *i*), dietary supplement of either of the formulations resulted in significant increase in cellular levels of Hrp36 (Figure 5 *b*–*d*) and Bancal (Figure 5 *f*–*h*). The increase was more apparent in RS-fed larval eye discs (Figure 5 *c*, *d*, *g*, *h*). Co-immunostaining with antibody against polyQ also revealed that the increase in the cellular level of these hnRNPs following formulation feeding is associated with a reduction in the accumulation of IBs (Figure 5 *a*–*h*). Increase in levels of Hrp36 was further confirmed by Western blotting and in this case too, RS-fed larval samples showed a greater increase (inset, Figure 5 *a*).

Immunostaining for CBP/p300 in *GMR-GAL4 > UAS-127Q* expressing discs (Figure 5 *i*–*l*) showed that AR or RS feeding significantly enhanced the levels of CBP, more so in RS-fed samples (Figure 5 *k*, *l*).

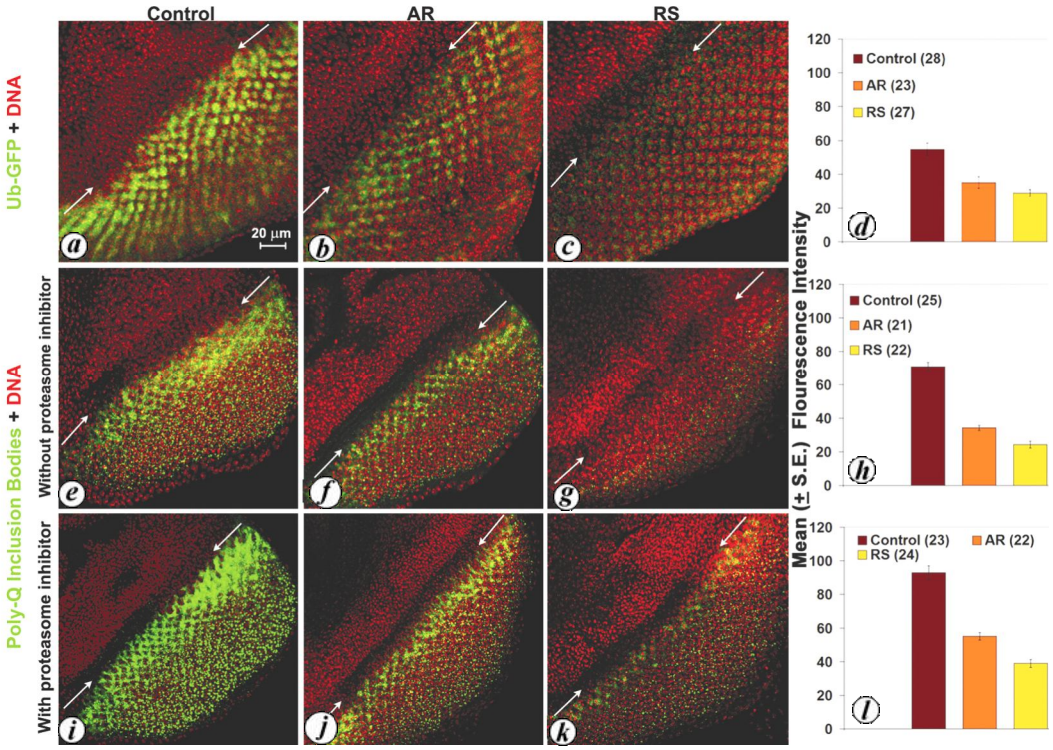

Figure 6. AR or RS feeding improved UPS activity in *–127Q* expressing eye discs. The feeding regime, normal (control) or AR or RS-supplemented food, is indicated above each column of the confocal projections of four mid *z*-axis optical sections of eye discs showing Ub-GFP (green, ***a***–***c***) or polyQ IBs (green, ***e***–***g*** and ***i***–***k***). ***e***–***g***, Confocal projection images of polyQ IBs (green) in *127Q* expressing larval eye discs without the 2 h *in vitro* exposure to proteasome inhibitors. ***i***–***k***, Confocal projection images of polyQ IBs in *127Q* expressing larval eye discs exposed to the proteasome inhibitors. DAPI-stained nuclei are shown in red (***a***–***k***). White arrows indicate position of the morphogenetic furrow in eye discs. The scale bar in (***a***) corresponds to 20 µm and applies to all the confocal projection image panels. Histograms in (***d***), (***h***) and (***l***) represent the mean (± SE) fluorescence intensities (in arbitrary fluorescence units) of Ub-GFP, polyQ IBs without and after treatment with proteasome inhibitor respectively, in *127Q* expressing larval eye imaginal discs following different feeding regimes; numbers in parentheses after the bar legends indicate the number of eye discs examined for each data point.

AR or RS feeding improved proteasome activity

The UPS activity, involved in degradation and clearance of unwanted proteins in cells[28], is compromised in the affected neuronal cells in polyQ/HD and AD, leading to enhanced accumulation of pathogenic protein[1,6]. Therefore, we examined the UPS activity in *GMR-GAL4 > UAS-127Q* expressing eye discs using the *UAS-Ub^{G76V}-GFP* transgenic line[26] in which the GFP is tagged with ubiquitin so that under conditions of compromised UPS activity, GFP fluorescence persists. As expected because of the compromised UPS activity, eye discs of normally fed *GMR-GAL4 > UAS-127Q* expressing larvae showed high levels of GFP fluorescence (Figure 6 *a*). However, in AR or RS-fed larval eye discs, the GFP fluorescence was significantly reduced, especially in RS-fed samples (Figure 6 *b–d*).

In order to further assess whether the improved UPS is indeed playing a role in reducing the accumulation of IBs and disappearance of *UAS-Ub^{G76V}-GFP* fluorescence, *GMR-GAL4 > UAS-127Q* expressing eye discs from differently fed late third instar larvae were incubated *in vitro* for 2 h in a medium containing proteasome inhibitor prior to immunostaining for polyQ IBs. As expected, the accumulation of IBs was much higher in discs from normally fed larval eye discs in which the proteasomal activity was inhibited for 2 h (Figure 6 *i*, compare it with Figure 6 *e*). Interestingly, however, the accumulation of IBs even in the presence of proteasome inhibitor was much less in discs from AR (Figure 6 *j*, *l*) or RS (Figure 6 *k*, *l*)-fed larvae, although they were slightly more abundant than in discs which were not exposed to the proteasomal inhibitor (Figure 6 *f*, *g*). Taken together, these results confirm that AR or RS feeding indeed improves the proteasomal activity.

AR or RS suppresses eye damage and accumulation of amyloid plaques in AD

In order to see if the protective effects of AR or RS feeding extend to AD associated with formation of amyloid plaques[5,6], we examined adult eye phenotypes (Figure 7 *a–c*) and accumulation of amyloid plaques in third

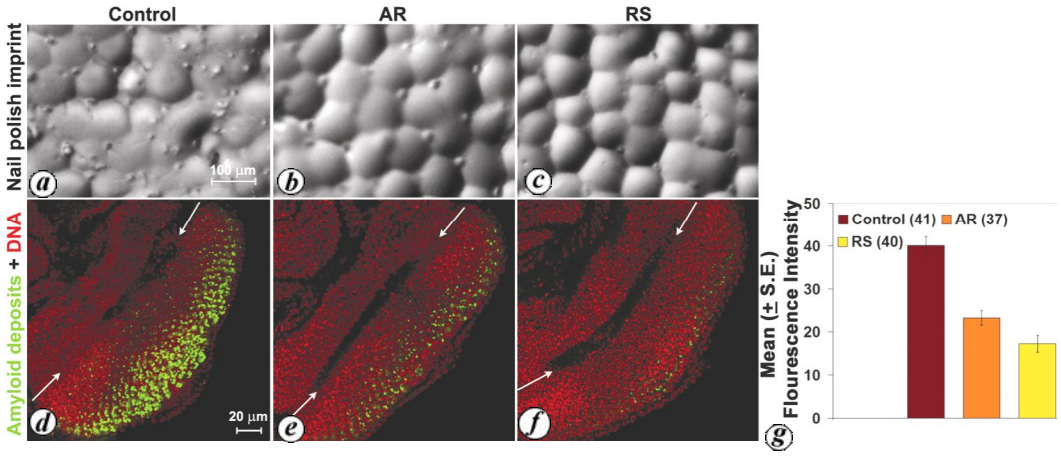

Figure 7. Dietary AR or RS reduced damage to eyes in *GMR-Aβ42* adult flies together with reduction in amyloid plaques. ***a–c***. Nail-polish imprints of eyes of 1-day-old *GMR-Aβ42* flies reared on normal (Control, ***a***) or AR (***b***) or RS (***c***) supplemented food. ***d–f***, Projections of four mid *z*-axis confocal optical sections showing amyloid deposits (green) in eye discs from larvae reared on normal (***d***) or AR (***e***) or RS (***f***) supplemented food. DAPI-stained nuclei are shown in red. White arrows indicate position of the morphogenetic furrow in eye discs. Scale bars in (***a***) (100 µm) and (***d***) (20 µm) apply to ***a–c*** and ***d–f*** respectively. Histogram in (***g***) shows the mean (±SE) fluorescence intensities (in arbitrary fluorescence units) of amyloid deposits in eye discs from differently fed larvae; numbers in parentheses after the legend bars indicate the number of eye discs examined in each case.

instar larval eye discs (Figure 7 *d–f*) of *GMR-Aβ42* (ref. 22) expressing flies/larvae reared on different feeding regimes. We found that damage to ommatidial arrays in flies expressing four copies of *GMR-Aβ42* transgene was substantially reduced when larvae were reared on food supplemented with AR or RS, so that a majority of *GMR-Aβ42* expressing flies reared on the formulation-supplemented food showed (67.22%, $N = 49$ in AR-fed and 71.23%, $N = 41$ in RS-fed) near normal or only mildly damaged eyes (Figure 7 *b* and *c*) when compared with those of control ($N = 40$). Immunostaining for the amyloid plaques in third instar eye imaginal discs and comparison of their respective fluorescence intensities revealed that unlike the very high levels of amyloid plaques in eye discs from *GMR-Aβ42* larvae reared on normal food (Figure 7 *d*), accumulation of plaques was greatly reduced in those from the formulation-fed larvae, especially those reared on RS-supplemented food (Figure 7 *e–g*). Thus, as in the case of the polyQ toxicity, dietary AR or RS suppresses AD pathology, with RS being more effective.

Discussion

A few earlier studies have examined efficacy of Ayurvedic and other herbal/traditional formulations in ameliorating neurodegenerative disease phenotypes[35–40]. However, most of these studies used individual constituents ('active principle') rather than the complete traditional formulation. As noted earlier[9,10,41], search for the so-called 'active principle' in an Ayurvedic formulation defies the holistic concept of Ayurveda which prescribes specific combinations of the herbal/organo-metallic components and *Anupana* for different ailments since different combinations are believed to have specifically varying effects. Therefore, we used the complete formulations as prepared for human consumption. As noted in our earlier studies with wild-type flies[10], the mercury-sulphide based RS had no toxic effects in the expanded polyQ or Aβ42 expressing genotypes as well. On the other hand, it has been seen (V. Dwivedi and S. C. Lakhotia, unpublished) that feeding on fly food supplemented (0.5% w/v) with dried *Kajjali*, an intermediary product which is subsequently sublimed at 600°C to produce the final RS[10], caused substantial developmental delay in wild type and more so in the polyQ expressing larvae. This confirms that the complex preparatory processes are necessary for activity of the Ayurvedic formulations and converting compounds like mercury sulphide into non-toxic but active formulation[42].

The substantial improvements in the different neurodegeneration phenotypes, viz. eye morphology, formation and organization of rhabdomeres, phototaxis, inclusion bodies/amyloid deposits, enhanced levels of Hsp70 and Hsp60, etc. following rearing on the AR or RS-supplemented diet clearly show that these two traditional Ayurvedic formulations effectively suppress neurodegeneration in fly models of polyQ toxicity and AD. The absence of a complete recovery in the eye phenotype of adult flies may be because while there was no intake of formulation during the 4–5 days of non-feeding pupal period between the larval and adult stages, the expanded polyQ or the amyloid protein continued to be synthesized in developing pupal eyes and cause neuronal damage. Our observation that feeding on formulation-supplemented diet during the adult stage only does not have any suppressive effect (Figure 2) and also indicates that these Rasayanas suppress neurodegeneration when it is being

inflicted, but do not restore the lost neurons in the fly model. This may partly be related to the fact that cell division in somatic cells is completely absent in adult flies, except in certain specific stem cells and, therefore, the neural cells that are lost during development because of the polyQ or mutant Aβ toxicity cannot be regenerated.

Abundance of the polyQ or amyloid aggregates is generally indicative of the degree of neurodegenerative manifestation. The significantly reduced accumulation of 127Q or the amyloid aggregates following the AR or RS feeding suggests that either these formulations inhibited the synthesis of the toxic proteins or facilitated clearance of the toxic proteins. Since we found the levels of polyQ transcripts in formulation-fed larvae to be similar to those in normally fed larvae, we believe that these formulations reduce the accumulation of IBs through clearance of toxic proteins rather than inhibiting transcription of the *polyQ* transgenes. The improved proteasomal activity in formulation-fed larval tissues is likely to be responsible for the significant reduction in polyQ IBS or the amyloid plaques through more efficient proteolysis.

Each of the two Ayurvedic formulations also enhanced cellular levels of hnRNPs and CBP, which are well known to suppress polyQ or amyloid plaque toxicity[1,14,17–20,30]. In another study (V. Dwivedi *et al.*, unpublished), it has been found that AR or RS supplement significantly suppressed induced apoptosis in eye discs caused by expression of the pro-apoptotic Reaper, Grim or Hid proteins. In the present study also we found a substantial suppression of apoptosis in *GMR-GAL4 > UAS-127Q* expressing discs from formulation-fed flies. Thus, besides the protection offered by elevated levels/activity of hnRNPs, CBP, 26S proteasomal components, inhibition of induced apoptosis by the dietary AR or RS also appears to contribute to suppression of neurodegeneration caused by polyQ or Aβ42 toxicity. It is significant that the generally greater suppressive effect of RS feeding in polyQ and AD models is paralleled by a greater enhancement in the levels of hnRNPs, CBP and UPS activity and a greater inhibition of apoptosis. An important role of hnRNPs in mediating the suppression of neurodegeneration by AR or RS feeding is supported by our other observations (V. Dwivedi and S. C. Lakhotia, unpublished) that if the Hrp36 is reduced or completely absent because of genetic mutation[43], neither of the Rasayanas brings about any suppression of the neurodegeneration caused by 127Q or Htt-ex1P Q93 toxic proteins. Further studies are needed to understand the pathways through which the dietary AR or RS elevate levels of hnRNPs, CBP and UPS activity. It also remains to be seen if any or both of these formulations affect the lysosomal activity as well, since autophagy too is involved in clearance of the toxic polyQ or amyloid aggregates[6,44–46].

Although we did not examine the levels of different proteins in the HD model, we believe that the AR or RS-induced changes seen in the 127Q model apply to this model as well since many previous studies have shown that the conditions that aggravate or ameliorate the polyQ toxicity have similar actions in the HD model[1,5].

The reactive oxygen species (ROS) are known to be significant causative factors in the neurodegenerative disorders[47,48]. Amalaki extracts are known to have very high antioxidant activity[49–52]. As expected, AR-fed larvae have also been found to display improved oxidative stress tolerance (V. Dwivedi and S. C. Lakhotia, unpublished). Thus boosting of oxidative stress tolerance may be an additional path through which dietary AR may ameliorate neurodegeneration. Although 'Makardhwaja', which like RS is a mercury-containing Ayurvedic preparation, is reported to significantly improve the oxidative stress scavenging system[53], we did not find any improvement in oxidative stress tolerance in RS-fed wild type larvae/flies (V. Dwivedi and S. C. Lakhotia, unpublished). This suggests that scavenging of ROS by RS may not be a contributing factor, but the greater enhancement in levels of hnRNPs, CBP and UPS activity following RS feeding may explain its greater suppressive effect on neurodegeneration in polyQ as well as AD than that of AR.

The traditional Ayurvedic literature does not appear to specifically indicate use of either of the two formulations for the polyQ or amyloid toxicity. It is quite likely that such ailments were not specifically identified in ancient times. However, these Rasayanas are indicated to generally improve health and brain functions, especially during ageing. Mercury-based *Bhasma* like the RS has been considered in traditional literature[54,55] as *Maharasa*, which promotes good physique, stable mind and good vision, improves memory and cures all diseases. RS has also been shown to significantly improve behaviour of geriatric dogs[53]. It has been reported[56] that WSHFD, a traditional Chinese drug containing 10% Cinnabar (HgS) and 10% Realgar (As_2S_4) together with certain plant products, exerts protection against LPS-induced neurotoxicity via the inhibition of microglial activation and the production of pro-inflammatory factors. This study[56] further showed that Cinnabar and Realgar in WSHFD are critical for the neuro-protection since removal or reduction of either of them rendered the treatment ineffective. Apparently, the mercury present in these traditional formulations is rendered non-toxic by the specific steps required for their preparation.

The multiple and varying phenotypes in patients of inherited neurodegenerative disorders due to mutation in a specific single gene, and the existence of a large number of genetic inter actors and possible therapeutic agents for such single gene defect disorders reflect the complexity of the underlying networks[1,5,31]. Unlike the target-specific chemicals/drugs which often have desirable therapeutic as well as undesirable side-effects, the Ayurvedic formulations containing a complex mix of mostly molecules of biological origin are likely to have more balanced effects on the systems biology of the body and thus help achieve

homeostasis[9,42]. Further, most of the oral Ayurvedic formulations are administered with one or more vehicle material/s or *Anupana*, which improve the main drug's acceptability and absorption of the main drug, besides acting as antidote. Our earlier studies[10] indeed showed that in agreement with the principles of Rasayana therapy[8,54], AR or RS supplements affect multiple pathways and thereby, each formulation improves homeostasis and general health. We believe that such multi-pronged actions of these Rasayanas provide a balanced defence to neuronal cells against the toxic protein aggregates.

The present study extends the beneficial effects of traditional Ayurvedic formulations in suppressing inherited neurodegenerative disorders. Since studies on fly models for diverse human neurodegenerative diseases have contributed significantly to our understanding of the genetic and cellular bases of these inherited disorders[1,2,5,57], it is expected that further studies on the Ayurvedic formulations in other model systems will be useful in developing them as convenient therapeutic formulations for combating the increasing burden of neurodegenerative disorders[9].

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