Pathogenic chaperone-like RNA induces congophilic aggregates and facilitates neurodegeneration in *Drosophila*

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Abstract Protein aggregation is a hallmark of many neurodegenerative diseases. RNA chaperones have been suggested to play a role in protein misfolding and aggregation. Noncoding, highly structured RNA recently has been demonstrated to facilitate transformation of recombinant and cellular prion protein into proteinase K-resistant, congophilic, insoluble aggregates and to generate cytotoxic oligomers in vitro. Transgenic *Drosophila melanogaster* strains were developed to express highly structured RNA under control of a heat shock promoter. Expression of a specific construct strongly perturbed fly behavior, caused significant decline in learning and memory retention of adult males, and was coincident with the formation of intracellular congophilic aggregates in the brain and other tissues of adult and larval stages. Additionally, neuronal cell pathology of adult flies was similar to that observed in human Parkinson's and Alzheimer's disease. This novel model demonstrates that expression of a specific highly structured RNA alone is sufficient to trigger neurodegeneration, possibly through chaperone-like facilitation of protein misfolding and aggregation.

INTRODUCTION

Human neurodegenerative diseases (HNDs), such as Alzheimer's (AD), Parkinson's (PD), Huntington (HD), prion protein (PrP)-associated diseases and tauopathies, are devastating pathological, progressive neurodegenerative conditions accompanied by severe memory decline, as well as cognitive and motor impairments in afflicted individuals. Protein misfolding and aggregation into soluble oligomers and insoluble fibrils, tangles, and plaques are emerging as common hallmarks of HNDs (Prusiner et al 1997; Growdon and Rossor 1998; Ross and Poirier 2004). Despite differences in amino acid composition of

tau, A β , α -synuclein, HD, and PrP, misfolding of each of these proteins launches a cascade of events that culminates in phenotypically similar neuronal dysfunction and neurodegeneration (Caughey and Lansbury 2003).

Proper protein folding usually requires a complex set of interactions with cellular entities called molecular chaperones (Hartl and Hayer-Hartl 2002; Macario and Conway de Macario 2002; Walter and Buchner 2002). The term chaperonopathies recently was coined for diseases in which abnormal protein deposition results from either improper functioning of molecular chaperones (eg, due to aging) or involvement of pathogenic chaperones that facilitate protein misfolding and aggregation, rather than from alterations in the protein itself (Macario and Conway de Macario 2002). The observation that amyloid protein deposits seen in HNDs can result from an impaired

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Received 10 August 2006; Revised 5 September 2006; Accepted 25 September 2006.

chaperone system or the presence of pathogenic chaperones suggests that HNDs may be chaperonopathies as well (Leem et al 2002; Dou et al 2003). Nucleic acids (NAs) recently have been considered as possible chaperone mediators that facilitate structural modification of proteins through transformation (alpha-helix to beta-sheet conversion), which eventually might lead to protein aggregation (Biro 2005). Pathogenic NA chaperones were suggested as putative participants in various HNDs (Chenpenik et al 1998; Johnson 2003), including transmissible spongiform encephalopathies (Adler et al 2003; Deleault et al 2003; Vasan et al 2006), AD, and other tauopathies (Kampers et al 1996).

Traditional transgenic rodent and nonrodent models of HNDs have been used to study the pathology of HNDs even though these animals often are weakened physiologically by overexpression of foreign (usually human) protein(s) and do not recapitulate the features of neurodegenerative pathologies to their full extent and complexity (von Horsten et al 2003; Jinnah et al 2005). Transgenic models for HNDs can be divided into two major groups with (1) loss of function and (2) directed overexpression of exogenous transgenes. It is noteworthy that all animal models described so far, including *Drosophila*, recapitulate only some features of PD and AD, polyglutamine, and tau-associated pathologies (Bonini and Fortini 2003; Sang and Jackson 2005).

Here, we describe a new type of HND animal model, transgenic *Drosophila* that expresses noncoding, highly structured RNA with chaperone-like activity. Such RNA represents a well-defined family of highly structured, mostly double-stranded species that are in the size range of 100–300 nucleotides long. In vitro, under near-physiological conditions, these RNAs bound PrP and tau protein, but differentially affected their solubility, resistance to proteinase K, and formation of insoluble, congophilic aggregates (Adler et al 2003; Vasan et al 2006). These RNAs generated different populations of soluble and membrane-disrupting PrP oligomers and facilitated oligomerization of endogenous PrP in normal sheep brain extracts (Vasan et al 2006). Thus, based on our in vitro experiments, these RNAs have characteristics of pathogenic chaperones that facilitate amyloid protein transformation. Here we show that expression of one of these RNAs in *D melanogaster* correlates with the accumulation of congophilic aggregates and a neurodegenerative phenotype but did not affect viability. The use of RNA chaperones as a tool for developing animal models for HNDs has advantages over traditional animal models in which overexpression of foreign proteins tends to reduce animal viability and disrupt normal physiology. RNA chaperone-based models offer an opportunity to study previously unknown molecular mechanisms of pathogenesis in HNDs, to identify both natural pathogenic NA and non-

NA chaperones, and to design new anti-HND therapeutic drugs and strategies.

MATERIALS AND METHODS

Construction of transgenic flies with noncoding RNAs

Noncoding RNAs with chaperone-like activity, RQT, and RQ were used for construction of transgenic *Drosophila*. Nucleotide composition and the thermodynamically most favorable secondary structures of RQ and RQT RNAs created with RNA draw (Matzura and Wennborg 1996) were presented previously, where RQ was referred to as RQ11+12 and RQT was referred to as RQT157 (Zeiler et al 2003; Vasan et al 2006). The sequences encoding these RNAs were amplified by polymerase chain reaction (PCR) and cloned into EcoRI and XbaI restriction sites of the pCaSpeR-hs vector described previously (Pirrota 1988). Both transcribed RNAs of approximately 800 nucleotides have the same 3' and 5' untranslated regions (UTRs) of hsp70 so that the only nucleotide differences are defined by the RQ and RQT sequences themselves.

Plasmid DNA used for transformation of *D melanogaster* was purified by *HiSpeed Plasmid Midi Kit* (Qiagen) and used for *D melanogaster* *y w^{67c23}* strain (referred in the paper as Df-1). Embryo injections were carried out as described previously (Rubin and Spradling 1982). Transposase activity was provided by the helper plasmid *Turbo D2-3* (Robertson et al 1988). Adults emerging from the injected embryos were crossed with Df-1 flies of the opposite sex, and the eye color of their progeny was examined. Transformed *Drosophila* strains that were homozygous for the transgene were established by full-sib mating. To remove the known suppressive effect on behavioral displays of the *yellow* mutation, it was outcrossed from the strains to be tested. The presence of homozygous transgene copies was confirmed by Southern blotting and in situ hybridization as described previously (Lim 1993). Flies of 3 strains were used in experiments: wild type Canton-S (CS) and 2 transgenic strains—1 strain with 2 transgene RQ RNA inserts (RQ2-flies) and 1 transgenic strain with 2 transgene RQT RNA inserts (RQT-flies). In various viability-related experiments, the parental Df-1 strain served as a control as well.

Heat shock treatment

A previously designed heat shock (hs) treatment protocol for *Drosophila* (Nikitina et al 2003) was used to modulate hs stress response and to induce expression of hs-driven transgenes. Behavioral patterns, brain morphology, and/or cytochemistry of adult 5-day-old flies were assessed after hs treatment was applied to either embryo-larvae I stage during formation of the mushroom bodies (HS1

group), to larvae III pupa during formation of the central complex of the brain (HS2 group), or to adult 5-day-old flies, 1 hour before a test (HS group). For studies of remote effects of hs treatment and RQ and RQT RNA expression during *Drosophila* development, adult flies were allowed to lay eggs for 5 hours; the parents were then discarded and first instar larvae or prepupae were subjected to heat shock as described by Nikitina et al (2003). After eclosion, newly hatched males were collected and staged until the experiment at the age of 5 days.

Preparation of RNA and Northern blot hybridization

RNA from control and transgenic larvae and flies was prepared by standard methods with isothiocyanate (Choczynski and Sacchi 1987), resolved by agarose gel electrophoresis, and transferred to a membrane for hybridization (Maniatis et al 1982) with a ^{32}P -labeled PCR fragment containing RQ-DNA. Hybridization was overnight at 42°C in 50% formamide, followed by two 20-minute washes in 2xSSC, 0.2 SDS at 42°C; two 20-minute washes in 1xSSC, 0.2% SDS at 42°C, and one 20-minute wash in 0.2xSSC, 0.2SDS at 68°C.

Congo red and Giemsa staining

In histopathology studies, adult flies were placed in mass-histology collars as described previously (Heisenberg and Bohl 1979), fixed for 3 hours in 4% paraformaldehyde, embedded in paraffin, and sliced into 7- μm serial frontal sections. Each collar contained heads of hs-treated (HS1, HS2, and HS groups) and nontreated adult flies. The presence of control and experimental flies on the same collar provided simultaneous and uniform fixation, histological treatment, and slide preparation. The histological preparations from adult flies' heads and larval tissues, including salivary glands and brains, were made according to the standard protocols described previously (White 1998).

The sections of frontal protocerebral cellular mass of the brains of hs-treated and untreated RQ2 adults were stained with modified Giemsa method for nervous tissue (Iniguez et al 1985) and Congo red (CR) stain for amyloid. CR staining was performed using Putter's modification with Mayer's Hematoxylin counterstaining to reveal amyloid deposits in pink-red and nuclei in blue (Elghetany and Saleem 1988).

Statistical comparisons were performed with computer program Statistica 6 using nonparametric Wilcoxon test and parametric *t*-test for independent samples.

Test for learning and memory of transgenic flies in conditioned courtship suppression paradigm

Tests were performed either immediately (to test for learning acquisition), or 3 hours after training (to test for

memory retention). Particularly, the time spent in courtship (orientation, following, wing vibration, licking, and attempted copulation) was recorded for 300 seconds by pressing an appropriately coded key on a computer, using a specially designed program (Kamyshev et al 1999). The resulting courtship index ([CI], percentage of time spent in courtship) was calculated for each male. The learning index (LI) was computed according to the formula: $\text{LI} = ([\text{CI}_{\text{na}} - \text{CI}_{\text{tr}}] / \text{CI}_{\text{na}}) \times 100 = (1 - \text{CI}_{\text{tr}} / \text{CI}_{\text{na}}) \times 100$ (Gallagher and Rapp 1997), where CI_{na} and CI_{tr} are the mean courtship indices for independent samples of naive and trained males, respectively. Statistical comparisons of behavioral data were made by a one-sided randomization test, by directly computing the probability of rejection of the null-hypothesis α_R . The sampled randomization test with 10 000 permutations was used. The null-hypothesis was rejected at $\alpha_R < 0.05$.

Viability measurements

Flies from parental control (Df-1) and 2 transgenic strains RQ2 and RQT were used for viability measurements. The flies were either kept at 25°C or heat shocked (37°C for 30 minutes plus 1 hour of recovery at 25°C) either at the adult stage (5 days old) or at the first or third instar larva stage. In the experiments where larvae were used, the percent of pupation and eclosion was determined, in which the percent of successful pupation and new-hatched flies were determined. In the longevity experiments, 10 males and 10 females of each genotype tested were maintained in the vial at 25°C. The adults were transferred to new vials with fresh medium every 3 days and dead flies were counted every day. The survival of males and females were measured separately; however, because in most cases there were no differences between males and females in longevity, we represented pooled data in corresponding Figures S1, S2 (see *Supporting Information*). In the longevity experiments with imago and third instar larvae, about 600 flies were tested for each genotype in the control and experimental (hs) series. In experiments where the first instar larvae were treated with hs, more than 1200 individuals were tested in each case.

RESULTS

Characterization of transgenic *Drosophila* strains

The RQ2 strain had 2 inserts of the RQ construct that were mapped to 57A and 87B regions of *D melanogaster* polytene chromosomes. The RQT strain also had a double insertion for the RQT construct that was mapped to 86F and 3D regions by in situ hybridization (not shown). It is noteworthy that in the preliminary screen we have used

Fig 1. Heat shock-inducible expression of RQ and RQT RNA in flies. Upper panel: Northern blot hybridization of ^{32}P -labeled RQ construct DNA probe with total RNA isolated from adult flies of transgenic strains. RNA from uninduced flies is compared with RNA from hs-induced flies at 0, 5, and 15 hours after treatment for 30 minutes at 37°C . Lane 1: RQ2 flies, no hs; lane 2: RQT flies, no hs; lane 3: RQ2 flies immediately after hs; lane 4: RQT flies immediately after hs; lane 5: RQ2 flies after 5 hours of recovery from hs; lane 6: RQT flies after 5 hours of recovery from hs; lane 7: RQ2 flies after 15 hours of recovery from hs; lane 8: RQT flies after 15 hours of recovery from hs. Lower panel: ethidium bromide-stained gel before transfer to indicate even loading of RNA samples.

a few other transgenic strains with single insertions of RQ and RQT, and these strains also differed in terms of aggregate formation and behavioral disturbances (ie, only RQ strains exhibit the abnormalities). However, because the differences were more pronounced in the strains with double insertions, we have chosen these for detailed analysis.

Northern blot analysis of total RNA was used to study expression of the RQ and RQT constructs (Fig 1). DNA from the RQ construct was labeled for the detection of both RQ and RQT transcripts because there is only a 40-nucleotide difference between these constructs. The size of each transcript appears to be about 800 ribonucleotides, which is consistent with the sums of the RQ and RQT constructs, 5' and 3' UTRs, and poly-A tail.

Therefore, the fusion sequences in the reported transgenes resulted in the production of RNA transcripts that triple the sizes of shs RNAs (Fig 1). This raises a question of whether these fusion RNAs could be used as effective shs RNAs, ie, a problem for the potential negative interference from the adjacent unrelated sequences in the

transgenic genes. Would the core RQ and RQT sequences be able to maintain their secondary pairing structures of shs RNAs? Would they have chaperone-like function in the context of RNAs with greatly increased sizes? Would they have chaperone-like function in the context of RNAs with greatly increased sizes?

In order to answer these questions we have performed predictions of the secondary structure of the resulted transcripts using Zuker secondary structure algorithm (Mathews et al 1999; Zuker 2003). The analysis showed that the shsRNA sequences remain as separate domains maintaining their secondary pairing structures regardless of 5' and 3' UTR sequences and polyA tail (data not shown). Because the secondary structures of RQ and RQT are maintained within the transcripts, it is anticipated that they may have similar function. However, this has not been tested *in vitro*. It is evident that advantages of having RQ and RQT within the context of 5' and 3' UTR sequences in polyadenylated transcripts include the export of the transcripts to the cytosol and increased stability.

Northern analysis showed that uninduced adult RQ2 (lane 1) and RQT (lane 2) flies did not have detectable expression in this exposure. A very weak signal was detected occasionally in the uninduced RQ2 and RQT flies after a long exposure, suggesting slight leakage of the hs promoter in these strains (not shown). Immediately after a 30-minute hs treatment at 37°C , RQ (lane 3) and RQT (lane 4) RNA expression was most abundant. The smear in lane 3 above the band may be due to the presence of transcripts with variable-length poly-A tails. Five hours after hs, signal intensity was almost as high as signal immediately after hs (lanes 5 and 6), but the smear was reduced markedly for RQ RNA (compare lanes 3 and 5) at 5 hours probably due to more complete processing of the transcripts. Signal for RQ and RQT RNA was visible but markedly reduced 15 hours after hs (lanes 7 and 8). To test for even loading of RNA, the gel was stained with ethidium bromide before transfer (lower panel). Similar results were found for hs-induced expression of RQ and RQT RNA in larvae (not shown). As expected, RQ and RQT RNAs were not detected in hs-induced and uninduced CS and Df-1 parental strains (not shown).

Cytoplasmic aggregates accumulate in RQ2 strain following hs

Amyloidogenic misfolded proteins in HNDs are enriched with β -sheet structures that bind thioflavines and CR, which are traditional histological stains for amyloid formations (Elghetany and Saleem 1988). CR staining was used to determine whether expression of RQ and RQT RNA facilitated formation of amyloid-like aggregates. A dramatic increase was found in congophilic aggregates in

Fig 2. Congophilic aggregate formation in salivary glands of larvae. Congo red staining accompanied by Mayer's Hematoxylin counterstaining to see the nuclei in blue, of salivary glands was performed on Canton-S (A) uninduced and (B) induced larvae and RQ2 (C) uninduced and (D) induced. Salivary glands from induced larvae were collected at 1 hour after hs at 37°C for 30 minutes.

the brain and other tissues of RQ2 larvae, but not in the brains of nontransgenic *Drosophila* after heat shock treatment. The most illustrative result was observed in salivary glands of RQ2 larvae (Fig 2). No aggregates were found in salivary glands of nontransgenic CS flies without hs (panel A) or after hs (panel B). Uninduced RQ2 larval salivary glands had no aggregates visible at the nuclear periphery (panel C), whereas numerous aggregates accumulated at the periphery of nuclei 30 minutes after hs induction (panel D). These aggregates migrated to the cytoplasm where they continued to be observed 24 hours after hs treatment, although they were less numerous (not shown).

Histological analysis of frontal brain sections of uninduced 5-day-old control adult males, nontransgenic CS, and RQ2 flies was performed (Fig 3). No congophilic aggregates were found in brains of uninduced RQ2 flies in representative sections shown (panels A and C), whereas congophilic aggregates were observed in brains of RQ2 males tested 1 hour after 30 minute hs treatment (panels B and D). Aggregates were not found in brains of RQ2 adults when larval stages of RQ2 strain were treated with hs (results not shown). Congophilic aggregates generally were not observed in brains of nontransgenic CS males regardless of hs treatment, whereas RQ2 males showed both the increase in number of affected animals and a 20-fold increase in aggregates in each brain after heat shock compared to untreated RQ2 flies. Histology was not performed on brains of RQT flies because no congophilic aggregates were found in RQT larval tissues including salivary glands. A quantitative comparison of congophilic aggregate formation in brains of CS and RQ2 flies is summarized in Table 1. It is evident that, although congo-

Fig 3. Congophilic aggregates in the brains of RQ2 flies and neural pathology. (A) Frontal fly brain sections at the level of central complex from RQ2 transgenic flies stained with Congo red (CR): (A) uninduced; (B) hs-treated, arrows indicate congophilic aggregates. Brain sections at the level of the optic lobes from RQ2 transgenic adult flies stained with CR: (C) uninduced; (D) hs-treated, arrows indicate congophilic aggregates. Frontal protocerebral cellular mass of RQ2 transgenic adult flies: (E) uninduced; (F) hs-treated, arrows indicate Giemsa-positive pycnomorphic cells with high level of basophilia; and (G) hs-treated individual, arrows indicate dying nerve cells. The Giemsa-positive, needle-like structures are seen near the dying cells. Magnification: 10 × 100, immersion.

philic aggregates are lacking in CS flies, their quantity varies significantly in hs-treated individual RQ2 flies.

Cytopathological consequences of RQ RNA expression

To determine cytopathological consequences of RQ RNA expression on neuronal cell morphology, the frontal protocerebral cellular mass from the brains of hs-treated and untreated RQ2 adults were subjected to a more detailed comparison with control CS specimens. Two main stages of neurodegeneration can be recognized in Giemsa-

Table 1 Number of Congo Red-positive aggregates scattered over the brain sections at the level of the central complex

Number of analyzed brains	CS	CS hs	RQ2	RQ2 hs
1	0	0	0	3
2	0	0	0	310
3	0	0	0	206
4	0	0	0	0
5	0	0	0	33
6	0	0	0	15
7		0	8	185
8		0	0	120
9			0	0
10			0	0
11			0	5
12			8	0
13			6	
14			0	
15			30	
Total number	6	8	15	12
Mean number of aggregates	0	0	3.4	73.1*

* $p < 0,05$ RQ2 vs. RQ2HS, Wilcoxon Matched Pairs Test.

stained sections. Pycnomorphic cells with a higher level of basophilic staining represent early stage of degeneration. On later stage of degeneration, the cells lose their integrity and were classified as dying cells. Numerous pycnomorphic cells were found in Giemsa-stained RQ2 adult brains within 1 hour of hs treatment (Fig 3, panel F). No such neural pathology was seen in RQ2 brains without hs treatment (Fig 3, panel E).

A large number of dying nerve cells, as well as Giemsa-positive needle-like and filamentous structures, were adjacent to the pycnomorphic cells (Fig 3G) that also were observed frequently in the brains of RQ2 flies after hs treatment. Neural pathology was not observed in the CS strain with or without heat shock. Neural pathology was not observed in the RQT transgenic flies with or without heat shock, with the exception of rare pycnomorphic cells observed after hs treatment. Quantitative analysis of the cytopathology observed in brains of RQ2 and control CS strains are summarized in Table 2.

Expression of noncoding RNA reduced learning acquisition and memory retention

The conditioned courtship suppression paradigm (CCSP) is used widely for learning ability and memory retention in *Drosophila* (Siegel and Hall 1979; Kamyshev et al 1999). Although the dynamics of memory retention were evident from the analysis of CI for a given strain, the LI also were computed to enable comparisons to be made irrespective of genotype- or hs-introduced fluctuations in the courtship levels of naive males. Figure 4 presents the results of such analyses. CS flies showed a tendency of improved memory formation after experiencing hs; there was no time-dependent decline of these flies in LI as well (0 vs 3 hours after training). A profound decline, however, in LI 3 hours after training is evident in RQ2; the most dramatic was a 3.5-fold decrease in memory formation manifested by males that experienced hs at the prepupal stage. These males also were defective in learning acquisition as compared to RQT males. At the same time, RQT males demonstrated perfect learning and memory formation both at normal temperature and after hs, and did not differ from CS flies in this respect.

Effect of RQ and RQT RNA expression on viability

Efficiency of pupation, eclosion of adult flies from pupae, and longevity were compared for nontransgenic Df-1 and transgenic RQ2 and RQT strains to estimate the effects of RQ and RQT RNA expression on viability.

Pupation

Pupation levels of hs-treated and untreated series were similar for all variants studied when third instar larvae were hs treated. However, differences were more pronounced in the series where first instar larvae were used. First instar RQ2 larvae were most affected by hs treatment, which reduced the number of pupated larvae by about 25%, whereas nontransgenic and RQT larvae had

Table 2 Cytopathology observed after hs treatment in RQ and Canton-S (CS) flies

Genotype	hs	n flies	Hyperchromatophilia			Strong pycnosis			Dying cells		
			% of cells	Standard error	P hs (-) vs hs (+)	% of cells	Standard error	P hs (-) vs hs (+)	% of cells	Standard error	P hs (-) vs hs (+)
CS	-	6	—			1.8	0.49		—		
CS	+	8	—			4.6	0.50	0.002	—		
RQ2	-	2	11.5	3.50		0.5	0.50		—		
RQ2	+	4	54.0	10.62	0.057	8.3	0.63	0.001	3.5	1.26	0.137

Quantitative analysis has been performed in the brain sections (frontal protocerebral cellular mass) of hs-treated and control flies; 67–118 cells were counted for each animal.

Cells scored as: hyperchromatophilic cells manifested drastic increase in Giemsa staining; strong pycnosis showed nuclear condensation and shrinkage; dying cells strongly deformed in shape with distorted inner organization.

Statistical comparisons were performed with computer program Statistica 6 using *t*-test for independent samples.

Variables were treated as independent samples.

Significant differences are underlined.

Fig 4. Learning indices (LI) in wild type and transgenic strains. Abscissa: time after training (min); ordinate: LI. The sample size for each time point was 20 males. * = LI after heat shock significantly differ from LI at 25°C (one-sided randomization test, $\alpha_R < 0.05$); & = LI significantly lower than that of Canton-S strain under similar conditions (one-sided randomization test, $\alpha_R < 0.05$); @ = LI significantly lower than that of RQT157 strain under similar conditions (one-sided randomization test, $\alpha_R < 0.05$); # = LI 3 hours after training significantly differ from LI 0 hours after training (one-sided randomization test, $\alpha_R < 0.05$); ^ = LI after HS1 or HS2 significantly differ from LI after HS (one-sided randomization test, $\alpha_R < 0.05$).

about 15% reduction in pupation (*Supporting Information, Fig S1A*).

Ecdysis

No significant differences were found between hs-treated and untreated first and third instar larvae for nontransgenic, RQ, and RQT strains (*Supporting information, Fig S1B*).

Longevity

No significant differences were found between transgenic and nontransgenic strains with or without hs when male and female data were pooled. The maximal longevity average among all strains was estimated as 12 weeks. Generally, when males and females were counted separately in all strains tested, both in the control and hs series, it was demonstrated that the majority of males die faster than females. In most cases no significant differences were found in the survival of RQ2 and RQT transgenic

strains compared with nontransgenic Df1 flies (*Supporting information, Fig S2I-L*). However, there was a subtle hs-dependent difference on longevity of RQ2 strain males when third instar larvae were treated. In this case, significantly lower survival of males after hs treatment is evident (*Fig 5*), whereas no statistically significant hs-dependent differences were found in RQ2 females (not shown).

DISCUSSION

Necessity of adequate HND animal models

The development of animal models for HNDs is of great importance for studying these devastating human diseases and for the development of therapeutics or neuroprotective strategies in humans. As for any other human diseases, the optimal HND animal model should reproduce the full spectrum of disease manifestations. Particularly, HND models at least should illuminate the most prominent among them: (1) amyloid formation, (2) cytopathol-

Fig 5. Lifespan of RQ2 males hs-induced at stage of third instar. Heat shock treatment of RQ2 males at the stage of third instar larvae decreased the lifespan of adult flies.

ogy, and (3) behavioral abnormalities (Gallagher and Rapp 1997; Zoghbi and Botas 2002). Unfortunately, the existing HND models do not represent all aspects of the neurodegenerative phenotype or even significant features of these disorders (Gotz 2001; Janus and Westaway 2001). Furthermore, existing transgenic animal models for HNDs are based on misexpression or overexpression of exogenous human protein, which often disrupts normal physiology, development, and viability of the transgenic organism and obscures the neuropathological profile of the model so that it cannot be used to study mechanisms of environmentally induced sporadic disorders with unknown triggers. Therefore, a transgenic animal with a strong HND phenotype (and normal viability) that is based on misfolding and aggregation of endogenous proteins would represent an attractive alternative model to study HNDs.

Noncoding hsRNA with chaperone-like activity in vitro facilitated a HND phenotype in vivo

In vitro RQ RNA facilitated transformation of PrP (Adler et al 2003), and RQ and RQT RNAs facilitated the formation of toxic PrP oligomers (Vasan et al 2006). Work in progress suggests that interaction of RQ and RQT RNAs with tau differentially facilitate tau oligomerization and filament formation. It is fascinating that one of these RNAs, RQ, also had a profound effect in vivo, generating 3 prominent HNDs manifestations, ie, congophilic aggregates, neural pathology, and cognitive impairment.

The congophilic nature of the aggregates is indicative

of beta-sheet structure characteristic for amyloid. Interestingly, numerous aggregates have been observed in the optic lobes of *Drosophila* brain, which contains high levels of tau protein (Hiedary and Fortini 2001). Preliminary results with antibody against human tau seem to suggest that *Drosophila* tau is a component of aggregates that form at the nuclear periphery of cells from salivary glands of hs-induced RQ2 larvae (data not shown). A thorough biochemical analysis of the composition of the aggregates would help to formulate a mechanism for their formation and the resulting neurodegenerative phenotype.

Importantly, together with the congophilic aggregates, we found neuronal death in the RQ2 strain as well as in the Giemsa-positive, needle-like filamentous structures seen near dying cells in the fronto-lateral protocerebral cellular mass in the brains of transgenic flies 1 hour after hs treatment. Similar structures found in close proximity to nerve cells often are observed in post mortem brains of Alzheimer's and Parkinson's patients (Wenning et al 1997; Stark et al 2005).

The hs promoter was used to drive expression of the RNA constructs. One consequence of hs is the upregulation of heat shock proteins to ameliorate protein misfolding and aggregation caused by elevated temperature. The facilitation of aggregate formation in the post-hs environment demonstrates the pathogenic strength of RQ RNA. The applied scheme of hs administration at crucial preadult developmental stages and in adults enables the assessment of the remote and immediate effects of hs treatment per se with the hs-induced proteins or hs-driven induction of RNA and hs proteins combinations. The

hs treatment applied at specific developmental stages, coinciding with the time of formation of the brain structures known to be involved in learning, memory storage, and control of locomotion, provides the ability to monitor the probable contribution of certain structures (ie, mushroom body vs central complex) to behavioral phenotypes observed in wild type flies. However, in the RQ2 strain, the abnormal behavioral phenotypes are the consequence of aggregate formation and neuronal cell pathology facilitated by the chaperone-like activity of the hs-induced RQ RNA. Thus a comparison can be made of the consequences of hs modulation vs hs-induced RNA chaperone lesioning.

Although we observed aggregate formation in a number of tissues of RQ2 transgenic larvae, the remote effects of RQ chaperone expressed at preadult stages (HS1 and HS2 series) is absent at the histological level in the adult brains that was registered as a lack of aggregates. The absence of brain pathology in adult flies may result from the fact that lesioned neurons and aggregates per se do not pass through metamorphosis. However, the remote effects of hs treatment on the preadult stages clearly are manifested at the behavioral level of the adult flies, leading to impairment of their learning ability and memory formation (Fig 4).

One can ascribe these phenomena to the deleterious effects of formation of cytoplasmic amyloid aggregates, formed immediately after heat shock and scattered throughout the *Drosophila* central complex, the optic lobes, and the fronto-lateral protocerebral cellular mass. The central complex has polysynaptic connections both with ascending interneurons of different motor and sensory systems and with descending interneurons, controlling activity of motor centers during realization of different behavioral acts (Wolf et al 1998; Popov et al 2004).

RQ2 and RQT strains displayed very different phenotypes, although they both had 2 copies of each construct and similar levels of construct expression. Differences in RQ and RQT RNA function in vivo may be explained partially by a 40-nucleotide region in RQ absent in RQT. This region contains a protein-binding site for the HIV rev protein and the Ricin/Sarcin cleavage domain from rRNA. These motifs may have the potential to interact with other proteins in vivo and facilitate a change in their conformations, leading to misfolding and aggregation.

Pathogenic RNA chaperones—a novel tool for developing HND animal models

Expression of RQ RNA appears to facilitate some common aspects of human neurodegenerative conditions, such as amyloid formation, cytopathology, and behavioral abnormalities characteristic for PD and AD. In contrast to the majority of existing murine, fish, or *Caenorhabditis ele-*

gans models that employ chemical lesions or overexpression of selected (usually a single) human protein, overexpression of an RNA chaperone does not produce foreign protein. Importantly, expression of pathogenic RQ chaperone barely impaired viability of the transgenic flies. With 1 exception, hs-treated RQ2 *Drosophila*'s viability is statistically comparable with viability components of control Df-1 flies and uninduced RQ2 flies (see *Supporting Information*). It is noteworthy that the lifespan of *D melanogaster* with hs-driven expression of *Syrian hamster* prion was the same as controls. However, in these transgenic flies neither aggregates nor other disease phenotypes were detected (Raeber et al 1995). Expression of RQ RNA in *Drosophila* confirmed the previously postulated (Adler et al 2003; Grossman et al 2003; Biro 2005) and recently demonstrated role of RNA chaperones as facilitators of protein transformation and neurodegenerative progression (Adler et al 2003; Vasan et al 2006). Furthermore, RQ transgenic flies by themselves may represent a model for HNDs because they manifest learning and memory impairment, cell neuropathology, and amyloid-like aggregate formation.

Disadvantages of the present model are that expression of the RNA transgene is not brain-specific, and that the expressed RNAs facilitate aggregation of proteins in other tissues. The ability of RNA chaperones to interact with such proteins outside the brain might explain the formation of the congophilic aggregates in various larval tissues. Therefore, this transgenic *Drosophila* represents a model for systemic amyloidosis with a strong neurodegenerative phenotype rather than a narrowly targeted particular HND model. The expression pattern of the RNA chaperones is dependent on promoter choice. Here we used the hs-promoter that lacks tissue specificity. Use of a neuron-specific promoter in combination with antibiotic- (Stebbins and Yin 2001) or sugar- (Osterwalder et al 2001) based induction systems may facilitate the use of RNA chaperones for HND models. RQ RNA and other RNA constructs also may be expressed in a variety of animals, including mice, to further assess their role as pathogenic chaperones and to develop novel HND models.

This work evokes numerous questions regarding the mechanisms that may be involved in the role of RNA pathogenic chaperones in HND pathology. Is RQ unique or are there natural RNAs with similar potential? Which proteins are interacting with RQ RNA? Does RQ RNA only function as an initiator of protein misfolding or does it become incorporated in the aggregates? How does the pathogenic RNA chaperone mediate neuronal dysfunction? What is the role of molecular chaperones in this effect? Ultimately, molecular genetic approaches will be required to enable us to answer these questions. This model can be useful in approaching these questions.

CONCLUSION

In conclusion, we have demonstrated that expression of a noncoding RNA with pathogenic chaperone-like properties in *Drosophila* facilitated formation of congophilic aggregates, neurocytopathology, and cognitive decline. Transgenic *Drosophila* males exhibited pronounced cognitive abnormalities in learning acquisition and memory retention. However, the viability of the transgenic flies generally was unaffected. This model supports the notion that pathogenic chaperones may be involved in the origin and progression of HNDs and that pathogenic RNA chaperones may be used to develop HND models without exogenous protein overexpression. Pathogenic RNA chaperone-based *Drosophila* strains would offer a means to search for and identify natural NA and non-NA pathogenic chaperones and validate them as new targets for designing novel therapeutic strategies.

ACKNOWLEDGMENTS

This study was supported by the Russian Foundation for Basic Research, project nos. 06-06-48993 and 06-04-48854, and by a grant of the Program of Cellular and Molecular Biology RAN and "GENOFOND" grants.

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