Gene expression changes presage neurodegeneration in a Drosophila model of Parkinson’s disease

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Transgenic Drosophila expressing human α-synuclein faithfully replicate essential features of human Parkinson’s disease, including age-dependent loss of dopaminergic neurons, Lewy-body-like inclusions and locomotor impairment. To define the transcriptional program encoding molecular machinery involved in α-synuclein pathology, we characterized expression of the entire Drosophila genome at pre-symptomatic, early and advanced disease stages. Fifty-one signature transcripts, including lipid, energy and membrane transport mRNAs, were tightly associated with α-synuclein expression. Most importantly, at the pre-symptomatic stage, when the potential for neuroprotection is greatest, expression changes revealed specific pathology. In age-matched tau transgenic Drosophila, the transcription of α-synuclein associated genes was normal, suggesting highly distinct pathways of neurodegeneration. Temporal profiling of progressive gene expression changes in neurodegenerative disease models provides unbiased starting points for defining disease mechanisms and for identifying potential targets for neuroprotective drugs at pre-clinical stages.

INTRODUCTION

Parkinson’s disease (PD) in humans is a slowly progressive neurodegenerative disease that becomes clinically manifest only after an estimated 70% of vulnerable dopaminergic neurons in the substantia nigra have already died (1). Thus, modeling changes in pre-symptomatic patients is crucial for understanding pathogenesis and, perhaps even more importantly, for identifying therapeutic targets that might help to slow the disease process before it reaches the threshold for clinical symptoms.

Drosophila expressing human α-synuclein (αS) carrying the disease-linked A30P mutation in a panneural pattern faithfully replicate human Parkinson’s disease. αS transgenic Drosophila develop adult-onset, progressive degeneration of dopaminergic cells, have widespread Lewy-body-like inclusions and show impaired locomotor function as monitored by progressive loss of climbing ability (2). Loss of dopaminergic neurons and inclusion formation are first detected at 10 days of age, while at day 1 post-eclosion, the A30P-αS Drosophila are histologically and behaviorally normal. Pathological mechanisms in other disease paradigms, such as mediation of cell death or defense against toxic molecules, appear to be finely regulated on both the enzymatic and the transcript levels (3,4). Thus we hypothesized that a detailed genome-wide analysis of progressive gene expression changes in Drosophila brain would reveal the molecular machinery mediating αS toxicity in vivo.

To identify gene expression changes at pre-symptomatic, early and advanced disease stages, we hybridized RNA extracted from fly heads to high-density oligonucleotide arrays spotted with probes representing the entire Drosophila melanogaster genome. Rigorous statistical analysis was performed to minimize false positives due to biological or technical variation, and to correct for multiple testing. Permutation tests (significance analysis of microarrays; SAM) (5) were performed to estimate the number of false positives expected by chance alone by repeatedly permuting the samples’ class labels and computing t statistics for all genes in the scrambled data. This analysis determined that 36 of 13,500 transcripts (6) were significantly differentially expressed in brains of 1-day-old A30P-αS flies as compared with age-matched control fly brains. Thirty-seven genes were differentially expressed at day 10 post-eclosion, and 44 genes at day 30, with false discovery rates predicted by SAM (5) of 10.8, 5.0 and 1.4%, at day 1, 10 and 30, respectively.
RESULTS

Transcription in pre-symptomatic \(\alpha S\) transgenic Drosophila

Although 1-day-old \(\alpha S\) transgenic flies appear histologically and behaviorally normal (2), expression of 36 genes was abnormal (Fig. 1A; with less than four expected false positives by SAM). These genes encode enzymes involved in pathways previously associated with \(\alpha S\) pathology and physiology: regulation of catecholamine synthesis (Fig. 1B and C) (7), mitochondrial dysfunction (Fig. 1D) (8) or lipid binding (Fig. 1E) (9). Furthermore, expression of a number of genes not previously implicated in \(\alpha S\) toxicity was significantly altered (Fig. 1A).

Dopamine and other catecholamines (Fig. 1A–C) have been strongly implicated in \(\alpha S\) toxicity. Dopamine was found to stabilize formation of potentially toxic \(\alpha S\) protofibrils in vitro (10) and in cultured neurons, catecholamines determined selective vulnerability to \(\alpha S\) (11). We found significant differential expression of three genes involved in regulation of catecholamine synthesis in Drosophila: henna, encoding phenylalanine hydroxylase, and punch and purple, encoding GTP cyclohydrolase and 6-pyruvoyl tetrahydrobipterin synthase. Changes in transcript abundance for henna (Fig. 1B; 0.58-fold change) and purple (Fig. 1C; 1.95-fold change) were confirmed by real-time PCR [Fig. 1D; fold change ± standard error (SE) 0.41 ± 0.059, \(P = 0.0037\) by unpaired \(t\)-test; fold change ± SE 1.57 ± 0.088, \(P = 0.0007\), respectively]. Phenylalanine hydroxylase catalyzes conversion of phenylalanine to tyrosine, the substrate for dopamine and other catecholamine synthesis. GTP cyclohydrolase and 6-pyruvoyl tetrahydrobipterin synthase catalyze subsequent reactions in the biosynthesis of tetrahydrobipterin (BH4), the rate-limiting co-factor of phenylalanine hydroxylase, tyrosine hydroxylase and tryptophan hydroxylase. The clinical importance of GTP cyclohydrolase for dopamine synthesis is demonstrated by the fact that autosomal dominant mutations in GTP cyclohydrolase cause childhood parkinsonism-dystonia (12) and can mimic Parkinson’s disease in adults (13). Homozygous mutations in 6-pyruvoyl tetrahydrobipterin synthase lead to hyperphenylalaninemia and loss of dopamine and serotonin (12). Interestingly, reduced levels of tetrahydrobipterin have also been found in CSF of patients with sporadic PD (14).

Mitochondrial dysfunction (Fig. 1A and D) is a biochemical hallmark of sporadic PD (15). We found that in 1-day-old transgenics, mutant \(\alpha S\) causes down-regulation of the mitochondrial genes walrus (Fig. 1D), an electron transfer flavoprotein (ETF; validated by real-time PCR, fold change ± SE 0.796 ± 0.043, \(P = 0.016\); Fig. 1F) and ATPase gamma-subunit, a component of complex V in the respiratory chain.

Lipid gene regulation (Fig. 1A and E) follows two different patterns. First, transcripts of two genes involved in lipid binding, retinoid- and fatty-acid-binding glycoprotein (RFABG), and an unnamed gene with homology to RFABG (CG15828; RFABG-like) were transiently reduced only in 1-day-old \(\alpha S\) flies. Down-regulation of RFABG was independently confirmed by real-time PCR (Fig. 1F; fold change ± SE 0.32 ± 0.04, \(P < 0.0001\)). Second, down-regulation of other lipid genes [Fig. 1F, real-time PCR validation, hom. long chain fatty acid CoA synthetase 3 (LCAS3) fold change ± SE 0.74 ± 0.02, \(P < 0.0001\); yip2, fold change ± SE 0.6 ± 0.073, \(P = 0.0022\)] involved in fatty acid turnover heralds changes seen in older animals (Fig. 2A and B).

Early and advanced disease stages show predominant up-regulation of energy genes

In 10-day-old \(\alpha S\) transgenic Drosophila, initial loss of dopaminergic cells is found (2). At 30 days post-eclosion, pathology is advanced with severe loss of dopamine neurons, formation of fibrillar Lewy-body-like inclusions, and impaired locomotion (2). In 10-day-old transgenics 37 genes were significantly differentially expressed (Fig. 1G) and in 30-day-old transgenics 44 genes (Fig. 1H) with less than two and one expected false positives by SAM, respectively. In contrast to pre-symptomatic animals most genes were up-regulated at these early and advanced disease stages. Although the combinatorial effects of multiple changes in energy gene transcription are unknown, it is intriguing that energy genes were down-regulated in behaviorally and pathologically normal 1-day-old transgenic animals, but were highly upregulated later in the disease when neurons have been damaged. This may reflect an adaptive cellular response to functional and structural impairment caused by \(\alpha S\) toxicity. Three closely related cytochrome P450 subunits were highly expressed in end-stage animals and might reflect an adaptive detoxification response (16). One transcript each in 10- and 30-day-old transgenics was chosen for technical validation by real-time PCR. For either transcript, fold changes measured by real-time PCR closely confirmed array results (Fig. 1I and J; n-synaptobrevin, fold change ± SE 1.81 ± 0.30, \(P = 0.014\); yip2, 0.52 ± 0.068, \(P = 0.0003\), respectively).

Disease-stage-independent \(\alpha S\)-associated signature genes

We next hypothesized that transcription of genes most directly linked to A30P-\(\alpha S\) toxicity would be consistently modulated independent of age or disease stage. To address this question, we identified which genes were significantly up- or down-regulated over the entire dataset of 20 Drosophila genome arrays independent of disease stage. We compared genomic expression from eight microarray experiments using RNA extracted from brains of A30P-\(\alpha S\) transgenic flies (four independent experiments with 1-day-old, two each with 10- and 30-day-old transgenics) with 12 independent matched control experiments (Fig. 2A; four microarrays each for 1-, 10- and 30-day-old controls). Permutation analysis (5) demonstrated 51 A30P-\(\alpha S\)-associated genes (with less than one expected false positive) that were significantly differentially expressed over the entire dataset of 1-, 10- and 30-day-old \(\alpha S\) transgenics when compared with matched control Drosophila (Fig. 2A; false discovery rate 0.83%).

Four lipid genes were down-regulated in all Drosophila carrying the \(\alpha S\) transgene, but not in matched normal or disease controls (Fig. 2B): CG11124, a phospholipase A2 (−3.5-fold); CG8732, a novel Drosophila gene highly similar to human and rodent long chain fatty acid CoA synthetase 3 (−1.5-fold); yippee interacting protein 2, a thiolase (yip2;
Changes in energy genes (pyruvate dehydrogenase kinase, isocitrate dehydrogenase, mitochondrial aspartate aminotransferase, and transaldolase) discussed previously for 40- and 30-day-old animals were also significant over the entire dataset, although results are driven by the overwhelming changes at later disease stages (Fig. 2F).

Novel pathologic or protective mechanisms are suggested by genes not previously associated with PD. Transcripts for membrane transport proteins were differentially expressed in all zS Drosophila. Expression levels of a voltage-gated calcium channel and an amino-acid permease (Fig. 2C) were increased, while expression of a novel lysosomal H\(^+\) ATPase was repressed (Fig. 2A). In addition, two peptidoglycan recognition proteins highly conserved in humans (17) [PGRP-SC1A (CG14746) and SC1B (CG8777)], were strikingly up-regulated in zS transgenics (up to 8- and 27-fold, respectively; Fig. 2D) relative to controls.

A disease control: transcription in the Drosophila model of tauopathy

To test whether expression changes in zS transgenics are specific to the Parkinson’s disease model, we examined genome-wide expression in another neurodegenerative disease model (Fig. 3). Transgenic Drosophila expressing a mutant form of human tau (R406W), linked to familial frontotemporal dementia with parkinsonism (FTDP), show features of the human tauopathy: adult-onset, progressive neurodegeneration, early death and accumulation of normally phosphorylated and folded tau (18). In patients, deposition of abnormally phosphorylated tau is the unifying histological hallmark of a number of neurodegenerative diseases (19), the most prevalent being Alzheimer’s disease (AD).

Twenty-three of 13 500 genes were significantly differentially expressed in brains of 1-day-old R406W flies as compared to closely matched non-transgenic control fly brains (Fig. 3B). Sixteen genes were differentially expressed at day 10 post-eclosion (Fig. 3D), and 25 genes at day 30 (Fig. 3E), with false discovery rates at day 1, 10 and 30 of 9.39, 5.12 and 3.72\%, respectively.

In pre-symptomatic histologically normal 1-day-old R406W-tau transgenics repression of a serine/threonine specific phosphatase (CG8889) was validated by real-time PCR (Fig. 3C; fold change $\pm$ SE 0.64 $\pm$ 0.031, $P = 0.025$ by unpaired t-test). Up-regulation of genes involved in tetrahydrofolate biosynthesis was also confirmed by real-time PCR (Fig. 3C; pugilist, fold change $\pm$ SE 3.06 $\pm$ 0.15; CG3011 fold change $\pm$ SE 2.48 $\pm$ 0.24, $P < 0.0001$ by unpaired t-test, respectively).

Beginning at 10 days of age tau transgenic flies show progressive neurodegeneration (18). Microarray analysis revealed that expression of cytoskeletal genes was significantly repressed at this early disease stage (Fig. 3D). The expression profile at an advanced disease stage (Fig. 3E; 30-day-old transgenics) showed up-regulation of cellular stress response and lysosomal genes (Fig. 3E and F; DNAJ-1, fold change $\pm$ SE 2.22 $\pm$ 0.26, $P = 0.0061$; cathepsin D, fold change $\pm$ SE 1.98 $\pm$ 0.27, $P = 0.005$ by unpaired t-test on real-time PCR validation, respectively). Three genes predicted to increase levels of the antioxidant glutathione, were also up-regulated (Gclm, and two thioredoxin-like genes, CG6852, CG10157 (20).

To determine the genes most directly linked to tau toxicity independent of disease stage (‘tau-associated genes’), we identified which genes were significantly dysregulated over the entire dataset of eighteen tau and control arrays. In striking contrast to zS transgenics, we found only five genes consistently differentially expressed over all time points in mutant tau flies (with one expected false positive by SAM; Fig. 3A). Three of these five tau-associated genes were also significantly differentially expressed in the analysis of individual timepoints.

Overall, these results are in accordance with previous data on tau pathology. For example, protein phosphatase activity is reduced in Alzheimer’s disease brains, and genetic inhibition of protein phosphatase 2A leads to chronic tau hyperphosphorylation in mice (21). Our data in pre-symptomatic 1-day-old tau transgenics suggest that reduction in another protein phosphatase (CG8889) could be contributing to tau hyperphosphorylation in vivo. Furthermore, lysosomal gene expression in aged tau transgenics (cathepsin D and CG9953) parallels lysosomal activation in brains of Alzheimer’s disease patients and some tau transgenic mice (22). In particular, in Alzheimer’s disease autopsies material cathepsin D mRNA is increased (23) and there is abnormal cathepsin D activity in early endosomes (24).

Distinct and common pathways of neurodegeneration

A striking result of this comprehensive temporal and genome-wide expression analysis in two Drosophila models of human neurodegenerative diseases was that the overwhelming majority of genes associated with zS or tau pathology were highly distinct (Fig. 4). Specifically, catecholamine biosynthesis, energy, and lipid binding genes are not perturbed in 1-day-old tau transgenics (Fig. 1B–E; and compare Fig. 1A and 3B). Also, zS signature genes were not significantly changed in tau transgenics (Fig. 2B–D and F).

To test independently whether the zS-associated genes (extracted from the ‘training set’ of zS and control samples) constitute a specific zS signature, we performed unsupervised cluster analysis of all samples, including tau samples (‘test set’; Fig. 2E). Using the zS-associated genes as classifier, blinded cluster analysis correctly distinguished the eight zS samples from tau transgenics and, as expected, from controls. Similarly, using the five tau-associated genes as classifier, blinded cluster analysis correctly distinguishes tau samples from zS samples (data not shown).

Addition of the genes significant in the four analyses performed for zS and tau transgenics, respectively (at day 1, 10, 30, and over the entire data set) yields 122 selective mutant zS and 64 selective mutant tau genes. Only three genes are common (either significant up- or down-regulation in each model) to both neurodegenerative Drosophila models (pugilist, CG6870, and CG15065; Fig. 4; the number of selective genes is less than the number of genes significant in all individual comparisons, because some genes were significant in multiple comparisons).

Pugilist was upregulated in 1-day-old tau transgenics and 30-day-old zS transgenics. It catalyzes interconversion of three...
derivatives of tetrahydrofolate to provide cofactors for de novo purine nucleotide biosynthesis. Several additional genes in this common pathway are dysregulated in one of the models, e.g. adenosine2 in zS, and adenosine3 in tau transgenics.

The second gene common to both neurodegenerative models, CG6870, is down-regulated in both 1-day-old zS and tau transgenics and encodes a cytochrome b5, an ubiquitous microsomal electron transporter. No information is available on the third common gene, CG15065, that is up-regulated in 1-day-old flies carrying either transgene.

**DISCUSSION**

The present study provides an unbiased genome-wide blueprint of the transcriptional programs associated with neurodegeneration in *Drosophila* models of Parkinson's disease and tauopathy. PD is a slowly progressive neurodegenerative disease that becomes clinically manifest only after an estimated 70% of vulnerable dopaminergic nigral neurons have already died (1). The power of high-density oligonucleotide microarrays in investigating this chronic progressive neurodegenerative disease is best revealed in pre-symptomatic, normal appearing *Drosophila*. In pre-symptomatic zS transgenics, microarray analysis was more sensitive than conventional neuropathological techniques in elucidating disease-associated changes (2). First, in 1-day-old zS transgenics, transcription of 36 genes was significantly and reproducibly dysregulated presaging neuronal loss, Lewy-body-like inclusion formation and locomotor impairment at later stages (Fig. 1). Second, zS signature genes are dysregulated independent of disease-stage in both pre-symptomatic and symptomatic animals (Fig. 2). Therefore, parts of the same molecular machinery dysregulated during the disease are altered in pre-symptomatic transgenics prior to the onset of neurodegeneration (Fig. 2).

Fifty-one disease-stage-independent genes were tightly associated with zS expression in *Drosophila*, including lipid, energy, membrane transport and defense response genes. zS is structurally related to apolipoproteins, binds to phospholipid vesicles and inhibits PLD2, an important regulator of vesicle membrane budding (9,25). zS is enriched at the presynaptic terminal and might play a modulatory role in release of dopamine vesicles (7 and references therein). How might lipid genes identified in this study interact with A30P-zS in *Drosophila* neurons? PLA2, the lipid gene most highly downregulated in zS transgenics (−3.5-fold), is enriched in synaptic vesicles of rats (26). PLA2 regulates exocytosis of catecholamines and other neurotransmitters in cultured cells, perhaps by generating 'fusogenic' lipid products that directly regulate fusion between secretory vesicles and plasma membrane (26–32). For the predicted product of gene CG8732, a specialized function as neuronal long-chain fatty-acid CoA synthase is suggested by sequence homology to a brain-type isoform of vertebrate long-chain fatty acid CoA synthase (LCAS3) (33). In addition, the retinoid- and fatty-acid-binding glycoprotein gene (RFABG) is transiently but specifically down-regulated in 1-day-old zS transgenics. RFABG is an insect lipoprotein that transports fatty acids and retinoids, and has an apolipoprotein B homology domain (34). Thus, the persistent A30P-zS-associated stage-independent repression of lipid enzymes together with the transient dysregulation of RFABGs in pre-symptomatic zS transgenic *Drosophila* strongly suggests that a major effect of zS in the *Drosophila* model of PD is perturbation of normal processing of lipids. There is evidence supporting the relevance of this interaction for the human disease. First, lipids (35) and vesicle membranes (36) are found in Lewy bodies on autopsy. Second, lipid-bound soluble cytosolic zS oligomers, the potentially pathogenic zS species, are increased in extracts of brains from patients with PD or dementia with Lewy bodies (37).

In summary, these alterations might lead to perturbed synaptic vesicle exocytosis in catecholaminergic neurons and increase levels of potentially toxic zS oligomers in lipid-associated complexes.

Mitochondrial dysfunction in nigral neurons is a biochemical hallmark of human Parkinson's disease (15) and might be one cause of sporadic Parkinson's disease. More recently, cell culture experiments have shown that mitochondrial inhibition can accelerate zS aggregation (38). In addition, overexpression of zS leads to enlarged and abnormal mitochondria in culture (8), suggesting that initial mitochondrial dysfunction caused by zS could further accelerate zS aggregation. Our data link zS toxicity and oxidative phosphorylation dysfunction of Parkinson's disease. In fruit flies, zS expression leads to early down-regulation of specific mitochondrial respiratory chain components in pre-symptomatic 1-day-old animals with progressive, and perhaps compensatory, up-regulation of other energy gene transcripts paralleling disease progression (Figs 1D and 2F). Thus, in the *Drosophila* model of Parkinson's disease two primary suspects of familial and sporadic disease are causally and progressively linked.

Novel pathologic and protective mechanisms are suggested by genes previously not associated with PD. Transcripts for

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**Figure 1.** Transcription profile of pre-symptomatic, early and advanced symptomatic stages of Parkinson’s disease in *Drosophila*. (A) In histologically and behaviorally normal 1-day-old zS transgenics, transcription of 36 of 13 500 genes was significantly up- or down-regulated presaging neurodegeneration. In the colorgrams columns represent samples of human A30P-zS transgenic *Drosophila* and age-matched controls; rows represent genes significantly differentially expressed by permutation analysis. Expression higher than the mean is displayed as shades of red, lower than the mean as shades of blue. (B–F) Expression changes in pre-symptomatic zS transgenics are specific and reproducible. Mean transcript abundance (average differences) detected by microarray analysis in 1-day-old zS transgenics (n = 4), age-matched normal controls (n = 4), and R406W-tau transgenics (n = 2) for selected genes involved in (B and C), regulation of catecholamine synthesis (blue font in A); (D) energy production (red font in A); (E) retinoid- and fatty-acid binding (green font in A). For all genes displayed, transcript abundance in zS transgenics is significantly different from normal controls by permutation analysis. The transcript abundance in the disease controls, *Drosophila* expressing mutated tau (R406W-tau), is not significantly different from controls by SAM. See text for details. Error bars show standard error of the mean. (F) Results for select genes in each functional class were validated by real-time PCR (***P < 0.0001; **P < 0.0007; *P = 0.0037; **P = 0.0022; *P = 0.016, by unpaired t-test, respectively). (G) Early disease-stage: at day 10 post-eclosion compared to matched controls, 37 genes were differentially expressed. (H) Advanced disease stage: 44 genes were significantly changed at day 30. (I and J) Real-time PCR validation for select genes in early and advanced disease-stages, respectively (**P = 0.014; ***P = 0.0003, by unpaired t-test, respectively).

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membrane transport proteins are differentially expressed in all αS Drosophila but not in controls. Expression of a voltage-gated calcium channel and an amino-acid permease are increased (Fig. 2C), while expression of a lysosomal H⁺ ATPase is repressed. In vitro data indicate that toxic αS protofibrils form pores in vesicular membranes (39). An analogous mechanism is conceivable in vivo and could lead to perturbed ion and amino acid exchange.

In summary, dysregulation of lipid processing, membrane transport and energy genes might indicate perturbed vesicle membrane fusion and permeability with early mitochondrial damage as result of A30P-αS toxicity.

In matched tau transgenics, expression of the overwhelming majority of αS-associated genes was unchanged, suggesting highly distinct pathways of neurodegeneration (Fig. 4). The relevance of select gene expression changes in early and advanced disease-stages of tau transgenic fruit flies (Fig. 3) for the human disease is corroborated by previous large-scale profiling studies of autopsy material from patients with Alzheimer’s disease [e.g. up-regulation of cathepsin D (40) and DNJ (41), and down-regulation of cytoskeletal genes (41)].

Ninety-eight novel genes were significantly differentially expressed in the Drosophila models of neurodegeneration. Forty-four percent of these novel genes (27 αS and 16 tau genes) are predicted to have human homologs based on sequence similarities and might serve as novel drug target candidates in human neurodegenerative disease. Temporal profiling of gene expression in neurodegenerative disease models opens avenues for defining mechanisms of disease and, most importantly, for identifying targets for drugs designed to slow disease progression at pre-clinical stages.

MATERIALS AND METHODS

Fly stocks
Control genotypes: elav-GAL4 and elav-GAL4/+.
Transgenic strains, including neuropathologic analyses, have been previously described (2,18).

Fly collection and replicate experiments
Two to four independent collections, head separations, RNA extractions and array hybridizations, were performed for each genotype at each time point. See the Results section for a detailed description of experimental design. Flies were maintained on standard cornmeal medium. A total of 200–300 adult flies were collected for each genotype at 1, 10 or 30 days post-eclosion. Flies were frozen, stored at −80°C, and rapidly decapitated by vortexing on dry ice for 8 s. Heads and bodies were mechanically separated using a sieve of appropriate pore size. Frozen heads were collected, and homogenized in Trizol (Gibco BRL Life Technologies, Rockville, MD, USA). Special care was taken to ensure that all steps were performed at temperatures <−20°C in a standardized manner to obtain high-quality RNA and to minimize technical variation.

RNA preparation for hybridization
RNA was isolated from fly heads using Trizol solution. We essentially followed the manufacturer’s protocol except for adding a centrifugation step (11 000 g for 10 min) to remove cuticle membranes prior to the addition of chloroform. RNA quality was confirmed on a 1% agarose gel. 7.9 μg of total RNA was used for amplification and the amplified product was labeled with biotin following a procedure described previously (42). The hybridization was then carried out at 45°C for 16–18 h using Affymetrix Drosophila Genome Arrays (Affymetrix Inc., Santa Clara, CA, USA) spotting 13 500 genes. After being washed, the array was stained and amplified as described (42) and scanned on an HP Gene Array scanner (Affymetrix Inc., Santa Clara, CA, USA). Visual inspection was performed to identify arrays with production defects.

Data analysis
The intensity for each feature of the array was captured with Affymetrix Microarray Suite 4.0 software, according to standard Affymetrix procedures by performing global scaling with an average ‘target intensity’ of 100 for all probe sets. Stringent significance thresholds were set in order to control for false positive results due to biological and technical noise, and to correct for multiple testing.

Because technical variation is high for genes with low average expression intensities, only genes with intensities of ≥300 in at least one sample were considered for further analysis. In addition, small changes in expression were eliminated by requiring fold changes of at least 1.5. To correct for multiple testing stringent permutation analysis, SAM (5), was applied. This conservative statistical analysis keeps the number of false positives at a minimum, although the number of false negatives is likely to remain high. Unsupervised hierarchical clustering using an algorithm in the DNA-Chip

Figure 2. Disease-stage independent αS-associated signature genes. (A) Fifty-one signature genes tightly associated with A30P-α-synuclein expression independent of disease stage are clustered by hierarchical average-linkage analysis and visualized in a colorgram. The branches of the dendrogram comprising the cluster of four independent samples of pre-symptomatic 1-day-old transgenics is highlighted in pink. Expression levels higher than the mean are displayed in red, lower than the mean in blue. (B–D) While histology and behavior are normal in pre-symptomatic 1-day-old αS-transgenics, microarray profiles reveal a Parkinson’s disease-specific expression signature. Graphs show the average fold change of select genes in different functional classes at day 1, 10 and 30 for αS transgenics (left panels) and tau transgenics (right panels). In R406W-tau transgenics, expression of the αS-signature genes is generally unchanged (changes not significant by SAM). Time points representing symptomatic stages of Parkinson’s disease pathology are shaded gray. Signature expression of down-regulated lipid genes (B and green font in A), up-regulated membrane transporters (C, orange font), and defense response genes (D, blue font) is detectable at the pre-symptomatic stage. (F) Progressive upregulation of a set of energy genes also begins in pre-symptomatics. This increase may be a compensatory response different from the energy genes uniquely down-regulated at day 1 (Fig. 1A). (E) Using the αS-associated signature genes as classifier, blinded hierarchical average-linkage analysis correctly distinguishes the eight αS samples from tau transgenics and, as expected, from normal controls.
Analyzer (dChip) package (www.dchip.org) was used to group genes and samples according to relative variation in gene expression patterns. To generate graphical representations of relative gene expression levels, all expression levels were first normalized for each gene by setting the average (mean) to 0 and the standard deviation to 1 across all samples. Scaled levels were color-coded as a spectrum representing relative changes from the mean (red for higher than the mean, blue for lower than the mean). Low-quality probe sets spotted on the array, designated by the manufacturer as 'incomplete' or 'rules dropped', were excluded from final analysis.

Real-time quantitative RT–PCR

Primers identified using Primer Express 1.0 (Applied Biosystems, Foster City, CA, USA) following standard protocol, were synthesized by Invitrogen Life Technologies (Carlsbad, CA, USA) and used at a final concentration of 600–900 nM in the reaction mixture. Total RNA (3 μg) was reverse-transcribed into cDNA using Taq-Man Reverse Transcription reagents and random hexamers as the primer (Applied Biosystems, Foster City, CA, USA). PCR reactions were set up in a 50 μl reaction volume using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). PCR amplification was performed using an Applied Biosystems

Figure 3. A disease control: transcription in the Drosophila model of tauopathy. Colorgrams visualizing genes (rows) with significant differential expression by permutation analysis between R406W-tau Drosophila and matched controls. Expression higher than the mean is displayed in red, lower than the mean in blue. (A) Only five genes are significantly differentially expressed over the entire dataset of eighteen tau and control samples. Dendrograms illustrate genes and samples with similar expression patterns by cluster analysis. (B) Although 1-day-old R406W-tau transgenics appear histologically normal, expression of 23 genes was significantly repressed by permutation analysis (the purine nucleotide synthesis genes are in purple font). (C) For three genes, changes in transcript abundance are confirmed by real-time PCR (CG8889, unnamed serine/threonine phosphatase; ****P < 0.0001; **P = 0.026 by unpaired t-test respectively). (D) In brains of 10-day-old tau transgenics, expression of cytoskeletal genes (cyan font) is repressed. (E) Expression profile at advanced disease stage shows up-regulation of lysosomal and cellular stress response genes (pink and red fonts, respectively). (F) Validation in 30-day-old tau transgenics by real-time PCR, ***P = 0.005; **P = 0.006 by unpaired t-test, respectively).
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SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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