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Protein Expression in a *Drosophila* Model of Parkinson's Disease

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Liquid chromatographies coupled to mass spectrometry and database analysis techniques are used to carry out a large-scale proteome characterization for a *Drosophila* model of Parkinson's disease. Semiquantitative analysis is performed on A30P α-synuclein expressing transgenic *Drosophila* and a control lacking the gene at presymptomatic, early, and advanced disease stages. Changes in gene expression at the level of the proteome are compared with changes reported from published transcriptome measurements. A summary of the comparison indicates that ~44% of transcripts that show changes can also be observed as proteins. However, the patterns of change in protein expression vary substantially compared with the patterns of change observed for corresponding transcripts. In addition, the expression changes of many genes are observed for only transcripts or proteins. Proteome measurements provide evidence for dysregulation of a group of proteins associated with the actin cytoskeleton and mitochondrion at presymptomatic and early disease stages that may presage the development of later symptoms. Overall, the proteome measurements provide a view of gene expression that is highly complementary to the insights obtained from the transcriptome.

Keywords: Parkinson's disease • *Drosophila* • α-synuclein • proteomics • liquid chromatography • mass spectrometry

Introduction

Parkinson's disease (PD), first described 189 years ago, is the most common age-related movement disorder and the second most common age-associated neurodegenerative disease.[1−3] The pathological features of PD are slow degeneration of the dopaminergic neurons in the substantia nigra and formation of intracytoplasmic Lewy body (LB) inclusion structures.[1,4] The disease is clinically manifested after the death of ~70% of these neurons.[4,5] Although several treatments that appear to alleviate PD symptoms are available, none are capable of halting the degeneration.[6,7] The molecular mechanisms that lead to the degeneration of dopaminergic neurons are unclear.[3] Arguably, studies of presymptomatic stages as well as the onset of stages where symptoms appear are crucial for developing a molecular understanding of disease etiology and new strategies for early diagnosis and intervention.

Direct studies of the molecular origins of PD in humans are intractable because of difficulties in obtaining tissues from pre- and early symptomatic disease stages. Therefore, there is great interest in developing model organisms that develop PD-like symptoms.[8] Two key factors in the development of these models arise from the discoveries that missense mutations (A30P, A53T, and E46K)[9−11] in the α-synuclein gene are associated with PD and that the α-synuclein protein is one of the major components of LBs found in the brains of PD patients.[12] The A30P and A53T α-synuclein gene mutations found in human PD have been used to develop several promising animal models that produce PD-like symptoms or related morphological changes.[13−16] Understanding the fundamental nature of neuronal degeneration in these models may provide insights into human PD.[8]

One model system that has been explored in some detail is *Drosophila melanogaster* (commonly known as the fruit fly, and hereafter referred to as *Drosophila*). The human wild-type and mutated (A30P or A53T) α-synuclein genes have been introduced into the *Drosophila* genome and expressed in the nervous system.[16] The resultant animals are viable but gradually display human PD-like symptoms, including slow degeneration of dopaminergic neurons with age; formation of LB-like inclusions in the brain; and malfunction in locomotor ability, which is most severe for the A30P mutation.[16] Fortunately, the lifespan of *Drosophila* is short (~60 days at 25 °C),[17] and it is straightforward to carry out experiments on populations of aging animals. Thus, it is relatively easy to gain insight into molecular changes that correlate with the above-noted temporal changes in cellular and behavioral activity.

Perhaps the most important aspect of the *Drosophila* system, in light of the work presented below, is the abundance of existing genomic work and recent characterization of the proteome.[16,18−22] Especially relevant to the work reported here are several studies of neurodegeneration,[20−22] including a recent publication by Feany and co-workers that describes a global analysis of gene expression at the level of the transcriptome for the PD-model transgenic animals at pre- (day 1), early- (day 10), and advanced- (day 30) symptomatic phases of Parkin-

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son's-like disease.[22] This approach is important because it provides an unbiased look at the influence of the introduced gene on the entire genome. In total, 102 transcripts appear to be influenced by expression of A30P α-synuclein from all three disease stages.[22] Included are signatures (transcripts that are significantly differentially expressed relative to a control) that are found at presymptomatic, early-, and advanced-disease stages. Those differences that are found at presymptomatic and early disease stages potentially provide important clues about pathways involved in neuroprotection.

Although transcriptome analysis may provide key insight into biological processes, these data alone are insufficient for a comprehensive understanding of gene expression.[23−26] Specifically, this type of analysis misses all downstream post-transcriptional events associated with protein expression, modification, and elimination.[27−29] In this paper, we report a proteomic analysis of the A30P α-synuclein *Drosophila* PD model. These studies were carried out with the same genotype flies and at the same timepoints as were used in the transcriptome work; thus, it is possible to make direct comparisons. In our approach, protein mixtures were digested with trypsin and the resulting peptides were analyzed by strong cation exchange (SCX) and reversed-phase (RP) liquid chromatographies (LC) coupled to tandem mass spectrometry (MS/MS) and sequence database searching techniques. Relative changes in protein expression were obtained using a peptide hits technique (PHT, described in more detail below), which provides a semiquantitative estimation of changes in protein abundance.[30−34] Similar to the published transcriptome work, this is an unbiased approach (in the sense that we have not targeted any specific class of proteins); the results provide a somewhat different view of the molecular changes that are involved in and thus are highly complementary to the transcriptome study.

Experimental Section

***Drosophila* Stocks and Harvesting.** This study utilized the following control and Parkinson's-like fly genotypes: *elav*::*Gal4* (P{$w^{+mW.hs}$ = GawB}$elav^{C155}$, Bloomington Stock Center, Indiana University); and *UAS-A30P α-synuclein* (the P{UAS-Hsap\SNCA.A30P}40.1 line was obtained from Mel Feany, Harvard Medical School), respectively. To obtain the elav::Gal4 ⇒ UAS::a-synuclein experimental flies, females from the elav::Gal4 line were crossed to males from the *UAS-A30P α-synuclein* stock. Progeny animals of the appropriate genotype were selected and used for aging and protein extraction. Flies were cultured on standard cornmeal medium and maintained at 25 ± 1 °C. Flies from each genotype were separated according to sex within 24 h post-eclosion. Male flies were used in the present study to avoid gender variance. Flies were transferred to new vials (∼40 flies per vial) every 4 days. A total of 250 adults were collected of each genotype at day 1, 10, and 30 post-eclosion. Flies were anesthetized with CO_2 gas, transferred to Corning centrifuge tubes (Corning Incorporated, Corning, NY), flash frozen with liquid N_2 and the tubes were shaken vigorously to separate heads from the bodies. Heads were collected on dry ice and stored at −80 °C until future use.

Protein Extraction and Proteolysis. Proteins were extracted using a mortar and electric pestle (KONTES glass company, Vineland, NJ) in a 0.2 M phosphate buffer saline solution (pH 7.0) containing 8.0 M urea and 0.1 mM phenylmethylsulfonyl fluoride. The protein solutions were centrifuged at 15 700 g at 4 °C for 10 min and the supernatant was collected. Protein concentrations were determined with a Bradford assay (Pierce, Rockford, IL) and indicated that ∼2.5 mg of proteins was obtained from 250 heads. Extracted proteins were treated with a 1:40 molar ratio of protein:dithiothreitol for 2 h at 37 °C for the cleavage of disulfide bonds. A 1:80 molar ratio of protein: iodoacetamide was added and allowed to react for 2 h in an ice bath in complete darkness. A 1:40 molar ratio of protein: L-cysteine was then added to quench the reaction at 25 °C for 30 min. The concentration of urea in solution was diluted to a final concentration of 2.0 M with 0.2 M Tris buffer in 10 mM $CaCl_2$ (pH 8.0). Tryptic digestion was performed by adding 2% (by weight) TPCK-treated trypsin to the protein mixture and incubating at 37 °C for 24 h. Tryptic peptides were cleaned using Oasis HLB extraction cartridges (Waters, Milford, MA), dried in a centrifugal concentrator (Eppendorf North America, Inc., Westbury, NY), and stored at −80 °C.

SCX Chromatography. Tryptic peptide solids were resuspended in a 5.0 mM potassium phosphate buffer solution in 75:25 water/acetonitrile at pH 3.0. For each sample, 1.33 mg of tryptic peptides was injected onto a javelin guard column (10 × 2.1 mm) that preceded a polysulfoethyl aspartamide column (100 × 2.1 mm, 5 μm, 200 Å; PolyLC Inc., Southboro, MA). The gradient was delivered at a flow rate of 0.2 mL·min^{-1} by a Waters 600 multisolvent delivery system (Waters, Milford, MA) and peptides were detected at 214 nm by a Waters 2487 dual λ absorbance detector. Mobile phases consisted of 5 mM potassium phosphate in 75:25 water/acetonitrile at pH 3.0 (solvent A) and solvent A with the addition of 350 mM potassium chloride (designated as solvent B). Binary gradients with respect to the percentage of solvent B were as follows: 0−5 min, 0%; 5−45 min, 0−40%; 45−90 min, 40−80%; 90−100 min, 80−100%; 100−110 min, 100%; 110−125 min, 100 to 0%; 125−141 min, 0%. One minute collections into 96 well plates (Corning Incorporated, Corning, NY) over the first 125 min of the gradient were combined into six fractions as follows: (1) 0−38 min, (2) 38−41 min, (3) 41−45 min, (4) 45−49 min, (5) 49−55 min, and (6) 55−125 min. Pooled fractions were desalted and dried as described above and stored at −80 °C until further analysis.

RP-LC-MS/MS Analysis. Fused silica columns (75 μm i.d., Polymicro Technologies, Phoenix, AZ) with a pulled tip were packed with Magic C18AQ (5 μm, 100 Å; Microm BioResources Inc., Auburn, CA) in a methanol slurry to a length of 15 cm. The nanospray tip was pulled by a Flaming/brown micropipette puller (Sutter Instrument Company, Novato, CA). The trapping column (100 μm i.d., IntegraFrit capillary, New Objective Inc., Woburn, CA) was packed with 5 μm, 200 Å Magic C18AQ (Microm BioResources Inc., Auburn, CA) to a length of 1.5 cm. A sample volume of 4 μL was injected by a FAMOS microautosampler (LC Packings Inc., San Francisco, CA). A Switchos pump (LC Packings Inc., San Francisco, CA) was used to load samples onto the trapping column at a flow rate of 4 μL·min^{-1}. Mobile phases of 96.95:2.95:0.1 water/acetonitrile/formic acid (solvent A) and 99.9:0.1 acetonitrile/formic acid (solvent B) were delivered by an Ultimate pump (LC Packings Inc., San Francisco CA) at a flow rate of 0.25 μL·min^{-1}. The gradient with respect to the percentage of solvent B was as follows: 0−10 min, 3%; 10−90 min, 3−20%; 90−145 min, 20−40%; 145−150 min, 40−80%; 150−160 min, 80%; 160−161 min, 80−3%; and, 161−181 min, 3%.

Peptides eluting from the nanocolumn were electrosprayed into an LCQ Deca XP mass spectrometer (Thermo Finnigan, San Jose, CA). The electrospray voltage was set to 2.1 kV and the capillary temperature was 150 °C. The mass spectrometer

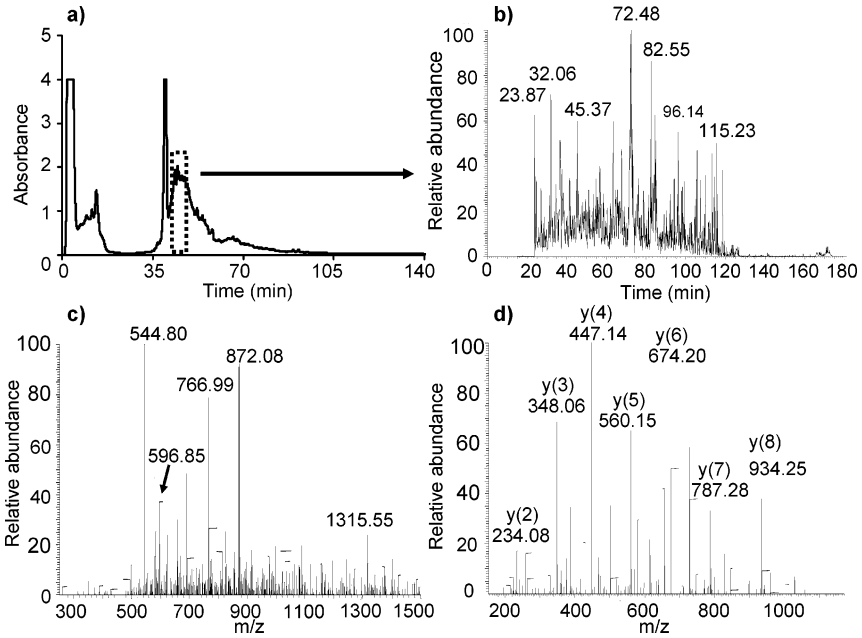

Figure 1. Example data of SCX−RP-LC-MS/MS analyses. (a) SCX chromatogram (monitored at 214 nm) of 1-day-old A30P α-synuclein transgenic *Drosophila* tryptic peptides. (b) Base peak chromatogram (BPC) of the RP separation of SCX fraction 3 (indicated by dashed rectangle in a). (c) Full-scan mass spectrum acquired at 54.46 min from the BPC shown in (b). (d) Fragmentation mass spectrum of the precursor ion at *m/z* 596.85 (shown with arrow in c), which was identified as EEFLNLVNSK belonging to the GTPCH protein.

was operated in a data-dependent mode, where the top three ions in the mass-to-charge (*m/z*) range 250−1500 were selected for fragmentation. Additional parameters included a 60 s exclusion duration time and a collision energy of 35%. Individual SCX fractions were subjected to triplicate RP-LC-MS/MS for a total of 108 analyses.

Peptide and Protein Identification. Raw MS/MS spectra were processed, submitted to MASCOT[35] and searched against the National Center for Biotechnology Information nonredundant *Drosophila* database[36] and the FlyBase database.[17] Carbamidomethylation of cysteine residues was used as a fixed modification and both acetylation of proteins at the N-terminus and oxidation of methionine residues were used as variable modifications. Peptide assignments (hits) are made by searching MS/MS data against all possible assignments from the database. Spectra that lead to scores at or above the MASCOT assigned homology score (the homology score defines spectral match at a 95% confidence level, i.e., 35 for these data) are assigned to specific peptide sequences; only those peptides having sequences that are unique to a single protein are considered.

Semiquantitation from the Peptide Hits Technique. The relative abundances of different proteins were estimated (as described previously)[30−34] by comparing the total number of peptide hits (obtained across triplicate measurements) for a given protein between two samples. Typically, peptide hits data for each protein between two samples are normalized to the same total number of peptide hits to correct for variations in sample injections or instrumental fluctuations, as is normally applied to microarray data (termed "brightness adjustment").[31−33] In our study, we utilized the raw number of peptide hits identified for each protein to assess changes in relative protein abundance, as was performed by Smith and co-workers in the comparative proteome analyses of human plasma before and 9 h after lipopolysaccharide administration.[34] The total number of peptide hits identified for age-matched control and PD-like fly samples are highly reproducible (i.e., 3.7% difference at day 1, 5.2% difference at day 10 and 9.4% difference at day 30); thus normalization of our data is not necessary. MS/MS spectra were confirmed by manual inspection for proteins identified with a single peptide hit across all experiments.

An issue that arises in this type of analysis has to do with the criteria that are used to assess change in protein abundance. For example, in the comparative proteomic study of human plasma samples before and 9 h after lipopolysaccharide treatment, Smith and co-workers considered a protein to be up-regulated when it was observed only in the lipopolysaccharide treated sample and the number of peptide hits was greater than three.[34] In another study of protein expression changes in yeast as a function of carbon source, Opiteck and co-workers proposed a protein to be differentially expressed when the fold change in peptide hits was equal to or greater than 1.1 and the *P* value from a Student's *t*-test was equal to or less than 0.05.[33] In the present work, we have utilized a strict set of criteria that are designed to focus our attention to those proteins that have been identified by a large number of peptide hits and also appear to vary substantially in abundance (between the PD-model and control animals). The criteria used for selection are as follows. First, we require that the total change in the number of peptide hits for a particular protein between transgenic and control animals changes by more than 100% (i.e., ≥a factor of 2 difference in the total number of hits for PD-model and control animals). This criterion is employed in order to pull out the most substantial abundance differences. Second, we require that the difference in total peptide hits between transgenic and control animals is ≥ 9. This ensures that when a protein is observed in both control and transgenic animals it must have been identified a minimum of ten times. In fact, the number of hits for most of the proteins discussed below is substantially greater than this threshold value. Third, we require a *P* value ≤ 0.05 from a standard Student's *t*-test for peptide hits between PD-model and control flies. For those

Table 1. Comparison of Gene Expression Changes at the mRNA and Protein Levels in 1-Day-Old Transgenic *Drosophila* vs Age-Matched Controls

gene name or ID[a,b]	flybase ID[b]	PH[c] (control)	PH[c] (transgenic)	PH ratio[d]	PHP-value[e]	mRNA[f]
CG8732[a]	FBgn0010609	2	0	0	0.1835	↓
CG15515[a]	FBgn0039719	2	0	0	0.1835	↑
punch[a]	FBgn0003162	23	10	0.43*	0.0198*	↓
henna[a]	FBgn0001208	12	8	0.67	0.2065	↓
purple[a]	FBgn0003141	6	4	0.67	0.5666	↑
adenosine5[a]	FBgn0020513	14	11	0.79	0.3785	↓
CG13044[a]	FBgn0036599	5	4	0.8	0.5185	↑
glutactin[a]	FBgn0001114	6	5	0.83	0.6495	↓
walrus[a]	FBgn0010516	7	7	1	1	↓
CG2604[a]	FBgn0037298	1	1	1	1	↓
RFABG[a]	FBgn0016724	114	115	1.01	0.9141	↓
yip2[a]	FBgn0040064	10	16	1.6	0.0765	↓
CG15828[a]	FBgn0032136	0	1	NA	0.4226	↓
CG14688[a]	FBgn0037819	0	1	NA	0.4226	↓
fat body protein 1[b]	FBgn0000639	25	0	0.00 *	0.0202*	NC
larval serum protein 2[b]	FBgn0002565	37	2	0.05*	0.0024*	NC
troponin C at 73F[b]	FBgn0010424	18	1	0.06*	0.0034*	NC
PHGPx[b]	FBgn0035438	12	1	0.08*	0.0082*	NC
diphenol oxidase A2[b]	FBgn0000486	12	2	0.17*	0.0132*	NC
Rpt1[b]	FBgn0028687	13	3	0.23*	0.0419*	NC
muscle-specific protein 300[b]	FBgn0053715	23	6	0.26*	0.0273*	NC
ribosomal protein S17[b]	FBgn0005533	14	4	0.29*	0.0217*	NC
tropomyosin 2[b]	FBgn0004117	23	7	0.30*	0.0499*	NC
CG11089[b]	FBgn0039241	15	5	0.31*	0.0099*	NC
cheerio[b]	FBgn0014141	28	10	0.36*	0.0043*	NC
sta[b]	FBgn0003517	17	7	0.41*	0.0133*	NC
sallimus[b]	FBgn0003432	64	26	0.41*	0.0102*	NC
bentb[b]	FBgn0005666	156	66	0.42*	0.0022*	NC
regucalcin[b]	FBgn0030362	30	14	0.47*	0.0241*	NC
CG14961[b]	FBgn0035439	17	8	0.47*	0.0031*	NC
tropomyosin 1[b]	FBgn0003721	51	25	0.49*	0.0188*	NC
ribosomal protein S3A[b]	FBgn0017545	18	9	0.5*	0.0351*	NC
CG3523[b]	FBgn0027571	26	56	2.15*	0.0013*	NC
TER94[b]	FBgn0024923	18	40	2.22*	0.0012*	NC
CG3731[b]	FBgn0038271	9	26	2.89*	0.0027*	NC
elongation factor 1 β[b]	FBgn0028737	4	13	3.25*	0.0286*	NC
ferritin 1 heavy chain homologue[b]	FBgn0015222	3	12	4.00*	0.0350*	NC
ras opposite[b]	FBgn0004574	3	15	5.00*	0.0080*	NC
comatose[b]	FBgn0000346	3	22	7.33*	0.0019*	NC
stoned A[b]	FBgn0016976	2	15	7.50*	0.0059*	NC
Trehalose−6-phosphate synthase 1[b]	FBgn0027560	1	13	13.00*	0.0011*	NC

[a] Gene name or ID was obtained from ref 22. [b] Gene name or ID was obtained from ref 17. [c] The number of peptide hits (PH) is the total number of PH from the triplicate analyses. [d] The PH ratio is relative to the control. The * indicates that the protein meets our criteria (described in the experimental section) and is considered to be significantly changed. [e] *P*-value was obtained from Student's *t*-test (two-tailed distribution and two sample unequal variance) of PH from triplicate analyses in Microsoft Excel. [f] mRNA information was obtained from ref 22. The symbols ↓, ↑, and NC indicate that the mRNA abundance was down regulated, up regulated, and not changed in A30P α-synuclein transgenic flies compared to age-matched controls, respectively.

proteins with many hits (in each of the three analyses, for both the PD-model and control animals) this criterion provides an assessment of the significance of the variation that is observed. It is important to note that fold change in peptide hits does not imply exact change in protein abundance. Rather, this approach indicates only the direction of change in relative protein abundance.[30−34]

One final note regarding this analysis is that we have spent considerable time modeling the profiles of random distributions of hits for these and other types of data based on Monte Carlo algorithms (written in house). These models take into account the probability of randomly hitting the same protein multiple times over the course of many measurements. The criteria that are described above for assessing abundance changes result in selection of only proteins that are well above the expected noise level for these experiments.

Results

SCX-LC-MS/MS Data. It is useful to show a small fraction of the raw data. Figure 1 shows an example of typical data that are obtained from SCX-LC-MS/MS analysis of tryptic digest mixtures. Briefly, Figure 1a shows that SCX chromatography leads to a separation in which only a few broad features are observed. This is a typical result for large mixtures of *Drosophila* tryptic peptides separated using the gradient described above (see Experimental). We use this approach to obtain smaller fractions of peptides as indicated in the figure. The individual SCX fractions collected were subjected to triplicate RP-LC-MS/MS experiments. Figure 1b shows an example base peak chromatogram that is obtained for a single fraction. These data show that many components are pulled apart; however, most peptides are ionized and injected into the mass spectrometer as a complex mixture of components. Once inside the mass spectrometer we obtain a precursor ion mass spectrum, such as the one shown in Figure 1c. Different ions are then selected and exposed to energizing collisions in order to obtain MS/MS spectra, such as that shown in Figure 1d. Shown in Figure 1d are typical fragment ion patterns (here primarily y-type fragments) that are used to assign a peptide sequence. The example that is shown gives a MASCOT score of 66 and the

Table 2. Comparison of Gene Expression Changes at the mRNA and Protein Levels in 10-Day-Old Transgenic *Drosophila* vs Age-Matched Controls

gene name or ID[a,b]	flybase ID[b]	PH[c] (control)	PH[c] (transgenic)	PH ratio[d]	PH P-value[e]	mRNA[f]
CG4233[a]	FBgn0001125	13	12	0.92	0.8305	↑
Khc[a]	FBgn0001308	3	2	0.67	0.7972	↑
ATPalpha[a]	FBgn0002921	86	134	1.56	0.0027	↑
Pfk[a]	FBgn0003071	13	8	0.62	0.1908	↑
PyK[a]	FBgn0003178	37	35	0.95	0.7580	↑
n-syb[a]	FBgn0013342	9	17	1.89	0.0153	↑
His4r[a]	FBgn0013981	0	1	NA	0.4226	↑
CG11899[a]	FBgn0014427	3	1	0.67	0.3868	↑
ATPsyn-b[a]	FBgn0019644	10	7	0.7	0.2739	↑
rack1[a]	FBgn0020618	7	2	0.29	0.0241	↑
glob1[a]	FBgn0027657	8	13	1.63	0.0241	↑
CG11901[a]	FBgn0029176	5	6	1.20	0.6495	↑
CG5325[a]	FBgn0032407	0	1	NA	0.4226	↑
CG9394[a]	FBgn0034588	10	2	0.2	0.0782	↑
CG4784[a]	FBgn0036619	31	40	1.29	0.1395	↑
CG7592[a]	FBgn0039685	1	0	0.00	0.4226	↑
calbindin 53E[b]	FBgn0004580	13	2	0.15*	0.0399*	NC
ubiquitin activating enzyme 1[b]	FBgn0023143	11	2	0.18*	0.0031*	NC
chickadee[b]	FBgn0000308	12	3	0.25*	0.0351*	NC
CG3011[b]	FBgn0029823	20	5	0.25*	0.0193*	NC
CG4685[b]	FBgn0039349	16	5	0.31*	0.0177*	NC
CG6439[b]	FBgn0038922	15	5	0.33*	0.0132*	NC
elongation factor 1α48D[b]	FBgn0000556	19	7	0.37*	0.0011*	NC
CG3731[b]	FBgn0038271	31	15	0.48*	0.0303*	NC
CG11876[b]	FBgn0039635	12	24	2.00*	0.0366*	NC
myosin alkali light chain 1[b]	FBgn0002772	8	17	2.13*	0.0031*	NC
CG6287[b]	FBgn0032350	12	26	2.17*	0.0159*	NC
CG30045[b]	FBgn0050045	9	20	2.22*	0.0148*	NC
ATP synthase-β[b]	FBgn0010217	39	93	2.38*	<0.001*	NC
chaoptic[b]	FBgn0000313	21	57	2.71*	0.0119*	NC
retinin[b]	FBgn0040074	11	30	2.73*	0.0019*	NC
obp44a[b]	FBgn0033268	12	33	2.75*	0.0010*	NC
CG6543[b]	FBgn0033879	11	35	3.18*	0.0019*	NC
CG11015[b]	FBgn0031830	3	12	4.00*	<0.001*	NC
ATP synthase-γ chain[b]	FBgn0020235	3	24	8.00*	0.0067*	NC

[a] Gene name or ID was obtained from ref 22. [b] Gene name or ID was obtained from ref 17. [c] The number of peptide hits (PH) is the total number of PH from the triplicate analyses. [d] The PH ratio is relative to the control. The * indicates that the protein meets our criteria (described in the experimental section) and is considered to be significantly changed. [e] *P*-value was obtained from Student's *t*-test (two-tailed distribution and two sample unequal variance) of PH from triplicate analyses in Microsoft Excel. [f] mRNA information was obtained from ref 22. The symbols ↓, ↑, and NC indicate that the mRNA abundance was down regulated, up regulated, and not changed in A30P α-synuclein transgenic flies compared to age-matched controls, respectively.

peptide EEFLNLVNSK is assigned to the guanosine triphosphate cyclohydrolase (GTPCH) protein.

A summary of the number of unique peptides (and corresponding proteins) for the PD-model and control animals that are obtained from the triplicate analyses is as follows. At day 1, there is evidence for 3366 peptides (corresponding to 1095 proteins) for the PD-model animals and 3451 peptides (corresponding to 1083 proteins) for the control. At days 10 and 30, the number of assigned peptides (and proteins) varies somewhat. PD-model flies show evidence for 2360 peptides (corresponding to 762 proteins) at day 10 and 2662 peptides (corresponding to 770 proteins) at day 30. The control shows evidence for 2704 peptides (corresponding to 835 proteins) at day 10 and 2447 peptides (corresponding to 725 proteins) at day 30. When the number of hits (obtained from triplicate analyses) for the age-matched PD-model and control animals are considered, we find that a total of 49 unique proteins meet our criteria for varying in abundance, specifically 28 proteins at day 1, 19 proteins at day 10, and 5 proteins at day 30. These proteins (along with relevant transcript data) are summarized in Tables 1, 2, and 3 and are discussed in more detail below.

Gene Expression at the mRNA and Protein Levels at the Presymptomatic Stage. Table 1 provides a complete list of proteins we identified that are significantly differentially expressed in 1-day-old transgenic animals at the mRNA level[22] as well as those we identified as changing at the protein level but for which there is no corresponding evidence for a change in transcription. At day 1 post-eclosion, 36 of 13 500 transcripts were significantly dysregulated in the brains of PD-model flies.[22] We identified 14 proteins of these 36 transcripts which are encoded by genes CG8732, CG15515, *Punch*, *Henna*, *purple*, *adenosine 5*, CG13044, *Glutactin*, *Walrus*, CG2604, *RFABG*, *yip2*, CG15828 and CG14688. GTPCH, encoded by the *Drosophila* gene *Punch*, is the only protein that we detected to be significantly differentially expressed ($P = 0.0198$) in PD-model flies that also changed significantly in mRNA abundance. GTPCH was identified with 23 peptide hits in control flies whereas it was identified with 10 peptide hits in experimental animals, giving an ~0.43 fold change. The remaining 13 proteins did not pass our criteria (described in the experimental section) to be considered as significantly differentially expressed.

Table 1 also shows 27 additional genes that were significantly differentially expressed at the protein level but not at the mRNA level. Specifically, there are 18 proteins that are down regulated and 9 proteins that are up regulated in PD-model *Drosophila* compared to controls. A total of 7 of the 18 down-regulated proteins are actin cytoskeleton-associated, including those encoded by *sallimus* (fold change 0.41, $P = 0.0102$),[37] *bent* (fold

Table 3. Comparison of Gene Expression Changes at the mRNA and Protein Levels in 30-Day-Old Transgenic *Drosophila* vs Age-Matched Controls

gene name or ID[a,b]	flybase ID[b]	PH[c](control)	PH[c](transgenic)	PH ratio[d]	PHP-value[e]	mRNA[f]
adenosine 2[a]	FBgn0000052	1	1	1.00	1.0000	↑
CG8732[a]	FBgn0010609	6	6	1.00	1.0000	↓
CG11899[a]	FBgn0014427	1	5	5.00	0.0474	↑
Acp1[a]	FBgn0014454	1	3	3.00	0.1835	↑
Ahcy13[a]	FBgn0014455	2	0	0.00	0.1835	↑
pugilist[a]	FBgn0020385	7	4	0.57	0.4970	↑
CG2827[a]	FBgn0023477	4	4	1.00	1.0000	↑
globin 1[a]	FBgn0027657	7	6	0.86	0.6495	↑
CG7203[a]	FBgn0031942	3	4	1.33	0.4226	↑
CG7224[a]	FBgn0031971	1	1	1.00	1.0000	↑
CG8736[a]	FBgn0033308	0	3	NA	NA	↑
CG3088[a]	FBgn0036015	0	4	NA	0.0572	↑
CG8329[a]	FBgn0036022	1	1	1.00	1.0000	↑
CG4784[a]	FBgn0036619	23	28	1.22	0.2113	↑
yip2[a]	FBgn0040064	26	32	1.23	0.1394	↓
CG6543[b]	FBgn0033879	11	22	2.00*	0.0015*	NC
calcium ATPase at 60A[b]	FBgn0004551	9	19	2.11*	0.0132*	NC
elongation factor 1α48D[b]	FBgn0000556	13	29	2.23*	0.0102*	NC
Got2[b]	FBgn0001125	12	28	2.33*	0.0303*	NC
G protein β-subunit 13F[b]	FBgn0001105	4	17	4.25*	0.0271*	NC

[a] Gene name or ID was obtained from ref 22. [b] Gene name or ID was obtained from ref 17. [c] The number of peptide hits (PH) is the total number of PH from the triplicate analyses. [d] The PH ratio is relative to the control. The * indicates that the protein meets our criteria (described in the experimental section) and is considered to be significantly changed. [e] *P*-value was obtained from Student's *t*-test (two-tailed distribution and two sample unequal variance) of PH from triplicate analyses in Microsoft Excel. [f] mRNA information was obtained from ref 22. The symbols ↓, ↑, and NC indicate that the mRNA abundance was down regulated, up regulated, and not changed in A30P α-synuclein transgenic flies compared to age-matched controls, respectively.

change 0.42, $P = 0.0022$),[38] *cheerio* (fold change 0.36, $P = 0.0043$),[39] *tropomyosin 1* (fold change 0.49, $P = 0.0188$),[40] *tropomyosin 2* (fold change 0. 30, $P = 0.0499$),[41] *troponin C at 73F* (fold change 0.06, $P = 0.0034$),[42] and *muscle-specific protein 300* (fold change 0.26, $P = 0.0273$).[43] The 9 proteins up regulated are encoded by *trehalose-6-phosphate synthase 1* (fold change 13.00, $P = 0.0011$), *comatose* (fold change 7.33, $P = 0.0019$), *Ras opposite* (fold change 5.00, $P = 0.0080$), *stoned A* (fold change 7.50, $P = 0.0059$), *TER94* (fold change 2.22, $P = 0.0012$), *elongation factor 1β* (fold change 3.25, $P = 0.0286$), *Ferritin 1 heavy chain homologue* (fold change 4.00, $P = 0.0350$), and two unnamed genes, CG3731 (fold change 2.89, $P = 0027$) and CG3523 (fold change 2.15, $P = 0.0013$). Some of these up-regulated proteins are known to be involved in metabolic pathways, such as trehalose-6-phosphate synthase 1, which participates in carbohydrate biosynthesis, and elongation factor 1β, which functions in polypeptide synthesis.[44,45] Two proteins, which are encoded by *comatose* and *Ras opposite*, have been shown to be involved in neurotransmitter secretion and synaptic transmission.[46,47]

Figure 2 shows Gene Ontology (GO) categorization of biological processes (obtained using the web-based FatioGo data mining approach)[48] for those significantly differentially expressed genes identified at the mRNA and protein levels. It should be noted that individual proteins may be associated with multiple categories; thus, the total percentage is not equal to 100%. A total of 17 of the 36 (47.2%) transcripts that were significantly differentially expressed have GO biological process annotations whereas the remaining transcripts have unknown biological functions. In contrast, 24 of 28 genes with significant differential expression at the protein level have GO biological process annotations. As shown in Figure 2, these 24 proteins are involved in versatile biological processes, such as locomotion, cell communication, response to stress, and cell differentiation. Similarly for both mRNAs and proteins, the top three biological processes are cellular physiological process, metabolism, and localization. More than 90% of these proteins participate in cellular physiological processes, and more than 50% are associated with metabolism.

Gene Expression at the mRNA and Protein Levels at the Early Disease Stage. Table 2 provides a complete list of proteins we identified that are significantly differentially expressed at the mRNA level[22] as well as proteins that we identified changing only at the protein level in 10-day-old PD-model animals. At day 10 post-eclosion, 36 transcripts were up regulated and one transcript was down regulated in PD-model *Drosophila*.[22] Table 2 lists 16 proteins that were detected from these 37 transcripts. These proteins are encoded by the following genes: CG4233, *Khc*, *ATPalpha*, *Pfk*, *Pyk*, *n-syb*, *His4r*, CG11899, *ATPsyn-b*, *Rack1*, *glob1*, CG11901, CG5325, CG9394, CG4784, and CG7592. None of these proteins changed in relative abundance in our studies.

Table 2 also lists the 19 genes that were significantly differentially expressed only at the protein level. Particularly, 8 proteins are down regulated and 11 proteins are up regulated. The 8 down-regulated proteins are encoded by genes *Calbindin 53E* (fold change 0.15, $P = 0.0399$), *Ubiquitin activating enzyme 1* (fold change 0.18, $P = 0.0031$), *chickadee* (fold change 0.25, $P = 0.0351$), CG3011 (fold change 0.25, $P = 0.0193$), CG4685 (fold change 0.31, $P = 0.0177$), CG6439 (fold change 0.33, $P = 0.0132$), *Elongation factor 1α48D* (fold change 0.37, $P = 0.0011$) and CG3731 (fold change 0.48, $P = 0.0303$). In contrast to the presymptomatic stage, only one of these down-regulated proteins (encoded by *chickadee*) in experimental animals is actin cytoskeleton-associated.[49] Rather, a total of eight of the 19 dysregulated proteins are mitochondrial-associated. These are encoded by CG3011, CG4685, CG6439, CG3731, CG6543, *ATP synthase-γ chain*, CG11015 and *ATP synthase-β*. Finally, proteins encoded by *ATP synthase-γ chain*, CG11015 and *ATP synthase-β* are up regulated and are related to oxidative phosphorylation.[50]

Gene Expression at the mRNA and Protein Levels at the Advanced Disease Stage. At day 30 post-eclosion, 44 mRNAs were significantly differentially expressed between PD-model

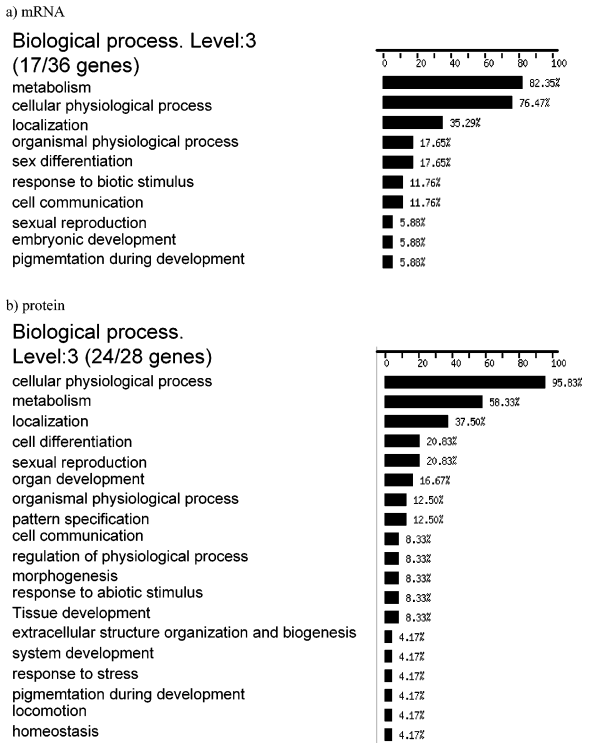

Figure 2. Histogram of gene ontology (GO) categorization of biological processes (at level 3) for (a) mRNAs and (b) proteins that were significantly differentially expressed in transgenic *Drosophila* at the presymptomatic PD stage. Only 17 of 36 genes that show significant differential expression at the mRNA level have biological process annotations. In (b), 24 of 28 genes that show significant differential expression at the protein level have GO annotations. The total percentage is not equal to 100% because a protein may be associated with multiple categories. The histogram was plotted by web-based FatiGO data mining (see ref 48).

flies and controls.[22] Table 3 shows the 15 proteins from these 44 mRNAs that were identified in this study. These proteins are encoded by genes *adenosine 2*, CG8732, CG11899, *Acp1*, *Ahcy13*, *Pugilist*, CG2827, *globin 1*, CG7203, CG7224, CG8736, CG3088, CG8329, CG4784, and *yip2*. None of these proteins were significantly differentially expressed. Table 3 also lists five genes that only changed in abundance at the protein level, including *G-protein β-subunit 13F* (fold change 4.25, $P = 0.0271$), *elongation factor 1α48D* (fold change 2.23, $P = 0.0102$), *Got2* (fold change 2.33, $P = 0.0303$), *calcium ATPase at 60A* (fold change 2.11, $P = 0.0132$), and CG6543 (fold change 2.00, $P = 0.0015$). The protein encoded by *G-protein β-subunit 13F* is a G-protein coupled receptor protein, which plays a role in cell communication by transducing extracellular signals into intracellular signals.[51] The protein encoded by *Got2* plays a pivotal role in the synthesis of the neurotransmitter glutamate in *Drosophila*.[52]

Summary of Gene Expression at the mRNA and Protein Levels. Overall, there are a total of 102 unique mRNAs (calculated from reference 22) and 49 unique proteins that were significantly differentially expressed across the three disease stages for the *Drosophila* PD model. Figure 3 shows a Venn diagram of gene expression changes at the mRNA level (thin black circles) and the protein level (thick gray circles) across the three disease stages. At the transcriptome level, the majority of dysregulated mRNAs are symptom-dependent; specifically,

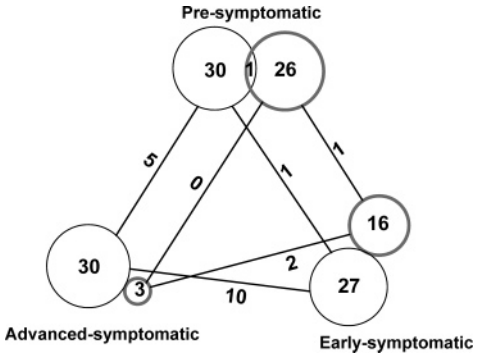

Figure 3. Venn diagrams showing mRNAs (thin black circles) and proteins (thick gray circles) that significantly changed across all disease stages. The values in the circles refer to the number of proteins (or mRNAs) that changed between transgenic *Drosophila* and age-matched controls exclusively at one specific disease stage. The values next to the lines indicate the additional number of proteins (or mRNAs) that changed at both two disease stages.

31 mRNAs are unique to the presymptomatic stage, 27 mRNAs are unique to the early disease stage and 30 mRNAs are unique to the advanced disease stage. There is one additional transcript that changed at both the presymptomatic and early disease stages; 10 additional transcripts changed at both the early and advanced-symptomatic disease stages; and, five additional transcripts changed at both the presymptomatic and advanced disease stages. Similarly, dysregulated proteins also demonstrate symptom-dependent characteristics; specifically, 27 proteins are unique to the presymptomatic stage, 16 proteins are unique to the early disease stage, and 3 proteins are unique to the advanced disease stage. There is one additional protein that changed at both the presymptomatic and early disease stages; two additional proteins changed at both the early and advanced disease stages; and, no proteins changed at both the presymptomatic and advanced disease stages. As indicated in the overlapping circles in Figure 3, there is only one gene (encoded by *Punch*, described above) whose expression is dysregulated at both the mRNA and protein levels.

Figure 4 shows pie chart representations of the cellular component categorization of significantly differentially expressed genes combined from all three PD stages. As shown in Figure 4a (at the mRNA level), 57 of 102 (~55.9%) total genes have unknown cellular locations while nine are associated with the mitochondrion, 16 are associated with the membrane, and one is a cytoskeleton associated transcript. In contrast, at the protein level, Figure 4b shows that only 5 of the 49 proteins are associated with the membrane. However, 20.4% are cytoskeleton-associated proteins and another 20.4% are mitochondrion proteins.

Discussion

Variability of Gene Expression at the mRNA and Protein Levels. A comparison of gene expression at the mRNA and protein levels in a *Drosophila* PD model vs the control reveals substantial differences. These differences demonstrate the importance of posttranscriptional mechanisms in controlling gene expression. A poor correlation between mRNA and protein expression levels has also been observed in other systems.[23−26] For instance, in the study of induced gene expression changes at the transcriptome and proteome levels in yeast grown on either galactose or ethanol, Aebersold and co-workers found

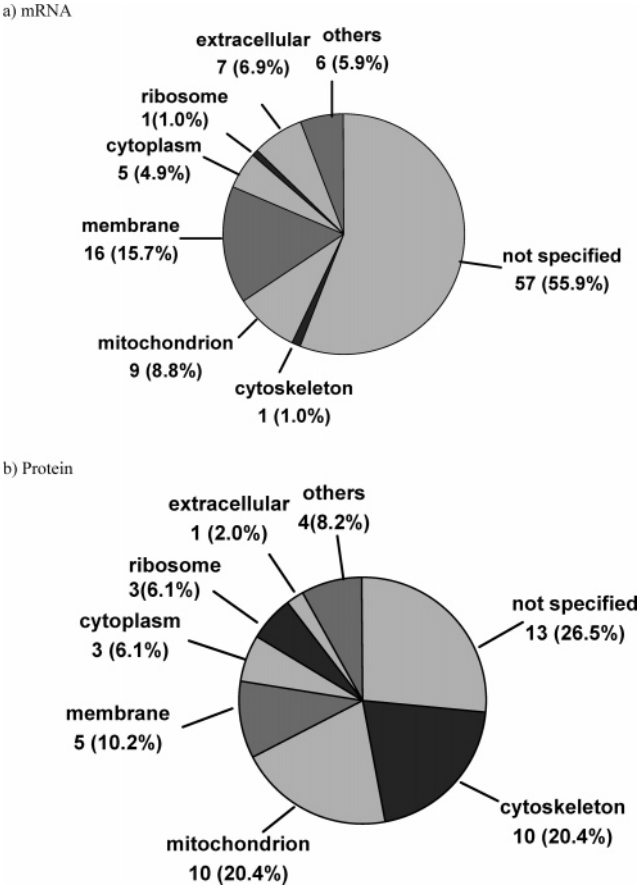

Figure 4. Pie chart representation of cellular localization of significantly differentially expressed gene products combined across all disease stages at (a) the mRNA level and (b) the protein level. In total, 102 mRNAs are represented in (a) and 49 proteins are represented in (b). A protein (or mRNA) was grouped into a single well-known category if it was associated with several cellular components.

that a remarkable number of genes show striking discrepancy between mRNA abundance ratios and protein abundance ratios.[25] Similarly, in the study of correlation between protein and mRNA abundance in yeast, Gygi et al. found that protein levels could vary more than 20-fold for some invariant levels of mRNAs and vice versa.[26] These findings in yeast as well as our observations in *Drosophila* demonstrate the necessity for integration of trancriptome and proteome studies for a comprehensive understanding of biological systems. Variations in gene expression can be accounted for in several ways. First, some mRNAs might not be translated into protein. Second, mRNAs might not have been considered significantly changed in the microarray experiments used for comparison with our study. Third, the half-lives of some proteins might be too short and thus are not detected within the timescales of our experiment. Fourth, some proteins may be in low abundance and therefore go undetected because they are outside of the instrumental dynamic range or are not selected for fragmentation in the ion trap. Discordant proteome and transcriptome expression profiles do however, offer complementary information to the understanding of molecular machinery associated with the development of PD-like symptoms in the α-synuclein transgenic *Drosophila* model.

Presymptomatic Disease Stage in PD-like *Drosophila* Model. Although transgenic adult flies appear normal at day 1 post-eclosion, 28 genes were significantly dysregulated at the protein level. Specifically, the number of down-regulated proteins was more than twice that of up-regulated proteins (refer to Table 1). *Punch* is the only gene that was down regulated at both the mRNA and protein levels. GTPCH (encoded by *Punch*) is the first enzyme involved in the biosynthesis of biopterin, such as tetrahydrobiopterin, in *Drosophila*[53] as well as in a variety of mammalian cells and tissues.[54] Tetrahydrobiopterin is a co-factor of tyrosine hydroxylase in the synthesis of catecholamine neurotransmitters, including dopamine, norepinephrine and epinephrine.[55] In the brains of PD patients, tetrahydrobiopterin has been found at ~50% the level of that found in age-matched controls.[56] It is possible that a reduction in GTPCH might result in the deficiency of biopterin, tetrahydrobiopterin, and/or dopamine. The down-regulation of *Punch* at both the mRNA and protein levels in PD-model flies suggests that further studies are warranted in order to understand more completely the role(s) of GTPCH in the pathology and etiology of human PD.

More interestingly, a group of actin cytoskeletal proteins were found to be down regulated in PD-like flies. In eukaryotic cells, actin cytoskeleton plays a pivotal role in cell morphology, cell motility, cell polarity, cell division, cell communication and endocytosis.[57] However, the diverse functions of the actin cytoskeleton are regulated by a large number of actin binding proteins,[57] such as those identified in our study (e.g., filamin encoded by *cherrio* and muscle specific protein 300). Considering the importance of the actin cytoskeleton to the viability of cells-especially in maintaining specific shapes of cells and supporting synaptic transmission and plasticity-we hypothesize that disruption of the actin cytoskeleton network might cause the collapse of specific structures of cells in the CNS and consequently their dysfunction. More specifically, the early disruption of the actin cytoskeleton may play a role in the degeneration of dopaminergic neurons, formation of LB-like inclusions and thus declining locomotor ability. Moreover, our findings of down regulated actin cytoskeletal proteins suggest that A30P α-synuclein might interact directly or indirectly with this cytoskeletal component. Although it is currently unclear if defects in the cytoskeleton play a definitive role in human PD, our results indicate that actin cytoskeletal defects may provide new insights into the etiology of PD.

Early Disease Stage in PD-like *Drosophila* Model. At day 10 post-eclosion, when the loss of dopaminergic neurons was initially observed,[16] 19 genes were found to be significantly differentially expressed exclusively at the protein level. In contrast to the presymptomatic stage, the dysregulated proteins were predominantly associated with the mitochondrion. Mitochondria play key roles in many cellular processes, such as energy production, fatty acid metabolism, oxidative stress and cell signaling.[3,58] Particularly, energy metabolism is crucial to a cell's health and viability, and disorders in the energy metabolic pathways have been linked to some neurodegenerative diseases.[1−3] For example, the study of postmortem tissues taken from the brains of PD patients has revealed defects in mitochondrial proteins that constitute the respiratory chain or oxidative phosphorylation enzymes.[59,60] Abnormalities in mitochondrial associated metabolic proteins have also been demonstrated in the study of transgenic mice overexpressing A30P α-synuclein.[13] As shown above, three oxidative phosphorylation associated mitochondrial proteins were up regulated in 10-day-old A30P α-synuclein transgenic flies; thus, this seems to support the idea that α-synuclein may influence mitochon-

drial activity and mitochondrial dysfunction may lead to neurodegeneration in PD. The mitochondrial dysfunction parallels seen in the fly and vertebrate suggest that PD-model *Drosophila* could provide a rapid and inexpensive system to study this aspect of PD and provide insights into the etiology of the human condition.

Advanced Disease Stage in PD-like *Drosophila* Model. At day 30 post-eclosion, transgenic flies clinically mimic human PD-like symptoms.[16] At this advanced disease stage, however, we only detected five proteins that significantly changed in abundance. Interestingly, all five proteins were up regulated. The protein encoded by *Got2* is a glutamate oxaloacetic transaminase, an enzyme involved in the synthesis of the neurotransmitter glutamate.[52] In invertebrates, the activity of glutamate is associated with neuron excitation and muscle contraction.[52] Interestingly, poor motility has also been observed in *Got2* deficient flies.[52] Thus, our finding of the dysregulation of the *Got 2* gene at the protein level is consistent with the poor climbing ability of A30P α-synuclein expressing transgenic flies.[16] *Calcium ATPase at 60A* encodes the sarcoplasmic/endoplasmic reticulum-type Ca^{2+}-ATPase in *Drosophila*.[61] Ca^{2+}-ATPases are critical for cell survival and tissue morphogenesis by maintaining Ca^{2+} homeostasis.[61] Ca^{2+}-ATPases also interact with *Notch* and play a role in protein trafficking.[61] Thus, dysregulation of *Calcium ATPase at 60A* at the protein level might be associated with the symptoms observed in the advanced disease stage of A30P α-synuclein transgenic flies.

Conclusions

The present study carried out a large-scale proteomic analysis of a transgenic *Drosophila* model of PD and an age-matched control at three disease stages. Overall, 1727 proteins were identified with SCX-LC-MS/MS analyses, and 49 proteins changed in relative abundance across the three stages. Gene expression profiles at the protein level were compared with gene expression patterns at the mRNA level for each disease stage. Differences in mRNA and protein expression patterns for particular genes reveal that transcript levels provide little predictive value to protein levels. Our observation of the perturbation in two main groups of proteins including seven actin cytoskeletal proteins at day 1 and eight mitochondrial proteins at day 10 in PD-like flies vs controls suggest that dysregulation of actin cytoskeletal proteins and mitochondrial proteins at presymptomatic and early PD stages might be associated with the onset of PD-like symptoms in transgenic flies. Mitochondrial dysfunction is well accepted as a pathological characteristic of human PD.[3] Our findings of a group of dysregulated mitochondrial proteins in conjuction with Feany and co-workers' findings of many down-regulated mitochondrial transcripts (and up-regulated energy transcripts) propose that the *Drosophila* model of PD may share similar molecular pathways in the progression of neurodegeneration as in humans. Thus, comprehensive understanding of the pathogenesis and etiology of PD in this *Drosophila* model may provide clues for new therapeutic targets for human PD.

One final emphasis is that like many other proteome analyses (e.g., the study of changes in protein expression of yeast as a function of carbon source by Opiteck and co-workers),[33] the proteomic platform utilized is rather time-consuming, thus no biological replicate experiments were performed. Rather, we carried out triplicate LC-MS/MS measurements on a population of 250 flies at each disease stage to account for potential biological variability in individual flies. Additionally, the criteria that we utilized for the determination of variation in protein expression are more stringent in comparison to those applied by other authors using similar approaches (see Experimental Section for detail). This has allowed us to confidently assess credible changes in protein abundance. Like other large-scale analyses, the present study aims to provide a meaningful starting point and to provide useful directions for further investigations of PD using this *Drosophila* model. Thus, a possible future direction would be to incorporate biological assays, such as Western blotting to validate the changes that we see in expression of the cytoskeletal proteins.

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