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Lifetime Proteomic Profiling of an A30P α-Synuclein *Drosophila* Model of Parkinson's Disease

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A survey of the proteome changes in an A30P α-synuclein *Drosophila* model of Parkinson's disease (PD) in comparison to age-matched controls is presented for seven different ages across the adult lifespan. The data were acquired by a shotgun proteomic approach that involves multidimensional liquid chromatographies coupled to mass spectrometry and database searching techniques. Semi-quantitative analysis to assess relative changes in protein expression between the *Drosophila* PD model and age-matched controls provides evidence that 28, 19, 12, 5, 7, 23, and 17 proteins are significantly differentially expressed at days 1, 10, 20, 30, 40, 50, and 60, respectively. From the experimental approach employed, it appears that most dysregulated proteins are associated with narrow distributions of ages, such that disease-associated differences change substantially across the lifespan. Previous measurements [*J. Proteome Res.* **2007**, *6*, 348] at days 1, 10, and 30 showed dysregulation of actin cytoskeletal proteins at day 1 and mitochondrial proteins at day 10, suggesting that defects in the actin cytoskeleton and the mitochondria are associated with dopaminergic neuron degeneration in PD. Analysis of the day 20, 40, 50, and 60 animals supports the finding that these cytoskeletal and mitochondrial changes predominate in the youngest (pre-symtomatic and early disease stages) animals. Although studies across many time points appear to be important for characterizing disease state, an understanding of molecular changes at the youngest ages should be most important for addressing causation.

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

Introduction

Parkinson's disease (PD) is characterized by slow degeneration of dopaminergic neurons in the substantia nigra and formation of intracytoplasmic inclusions called Lewy bodies (LBs).[1,2] The molecular mechanisms associated with the deterioration of dopaminergic neurons remain unclear,[3] and whether or not LBs are neurotoxic or neuroprotective is controversial.[4] The symptoms in PD patients are clinically observable after the death of ~70% of dopaminergic neurons, at which time the disease has reached an advanced stage.[5] Although several medications can mitigate symptoms (e.g., tremor, poor balance, and walking difficulty), none prevents the degradation of dopaminergic neurons.[6,7] Direct studies of PD on humans are mostly limited to postmortem brain tissues, which prevents acquisition of important information from pre-symptomatic and early disease stages.[8−11] Studies encompassing different disease stages are important for understanding causations of PD, developing diagnostic tools, and ultimately, discovering cures to the disease.

In contrast to the challenge and intractability of direct studies of PD on humans, *Drosophila melanogaster* (hereafter referred to as *Drosophila*) expressing the human α-synuclein protein with an A30P mutation provides a good model system for studying human PD.[12] Its utility can be attributed to several factors. The most attractive feature is that the A30P α-synuclein transgenic animals exhibit physiological and phenotypic characteristics that mimic those found in humans, including: slow degeneration of dopaminergic neurons; formation of LB-like inclusions; and loss of locomotor functions.[12] Also important is that compared with mammalian models (e.g., rats and mice),[13−15] *Drosophila* models have shorter life spans (~60 days)[16] and it is relatively easy to manipulate large populations; this allows for greater throughput in studies over the course of organism lifespan. Additionally, the genome has been sequenced for some time[17] such that there are now a substantial number of relevant studies.[12,18−23] Of particular interest to the work conducted here are transcriptome[18] and proteome analyses[24] of the A30P α-synuclein transgenic *Drosophila* PD model.

In this study, we performed proteomic analyses of an A30P α-synuclein transgenic *Drosophila* PD model and age-matched controls across the organism lifespan at seven different ages (days 1, 10, 20, 30, 40, 50, and 60). We utilized multidimensional liquid chromatographies (LC) including offline strong cation exchange (SCX) chromatography and reversed-phase (RP) liquid chromatography coupled with tandem mass spectrometry (MS/MS) and database searching techniques for large-scale proteome analysis. A label-free peptide hits technique (PHT) was utilized for semiquantitative assessment of relative protein

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abundance at all time points.[24−31] To test biological and technical variation in some protein changes observed with the PHT, a global internal standard technology (GIST) isotopic labeling approach[32,33] was applied to 1-day-old flies. Herein, we present a survey of proteins that may change in expression levels in the *Drosophila* PD model when compared to age-matched controls as the disease gradually progresses. Although several types of analyses (e.g., time-dependent analyses of group, age, and combination of group and age) could be applied to these data to assess changes in protein expression, this study focuses only on examining proteome changes in A30P α-synuclein *Drosophila* PD-like flies in comparison to age-matched controls. In this nature, we are able to explore proteins that are substantially different in expression and that may be associated with PD. This work extends our previous study, which compared changes in gene expression profiles at the level of proteome with the patterns of change at the level of transcriptome at three disease stages (pre-symptomatic −day 1, early disease stage −day 10, and advanced-disease stage −day 30).[24]

Experimental Section

Fly Sample Preparation. In this study, we utilized the same control (i.e., *elav*::*Gal4*) and Parkinson's-like (i.e., *elav*::*Gal4*=>UAS::A30P a-synuclein) fly genotypes as described previously.[24] Protocols for fly sample preparation are described in detail elsewhere.[24] Briefly, progeny male flies of the appropriate genotype were transferred to new vials every 4 days. Control and PD-like flies were maintained at identical conditions and harvested at the same time. To avoid differences that arise from gender, only male flies were used. Fly heads were collected on dry ice and stored at −80 °C at days 1, 10, 20, 30, 40, 50, and 60 post-eclosion for each genotype. In the present study, no biological replicate experiments were performed; instead, a population of 250 adult fly heads was used at each individual age to account for potential biological variability in individual flies. Fly head proteins were extracted using a mortar and electric pestle in a 0.2 M phosphate buffer saline solution (pH 7.0) containing 8.0 M urea and 0.1 mM phenylmethylsulfonyl fluoride, and the supernatant was collected. A Bradford assay indicated that ∼2.5 mg of proteins was obtained from 250 fly heads. Reduction, alkylation, and trypsin digestion of the proteins were performed under identical conditions for each sample.[24] Finally, tryptic peptides were cleaned, dried, and stored at −80 °C until future use.

LC−MS/MS Experiments. The LC−MS/MS experiments carried out are the same as described elsewhere.[24] Briefly, an equal amount of 1.33 mg of tryptic peptide solids were reconstituted into a 5.0 mM potassium phosphate buffer solution in 75:25 water/acetonitrile at pH 3.0 and injected onto a javelin guard column (10 × 2.1 mm^2) that preceded a polysulfoethyl aspartamide column (100 × 2.1 mm^2, 5 μm, 200 Å; PolyLC Inc., Southboro, MA) for SCX separation. 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, 100−0%; 125−141 min, 0%. One minute collections into 96 well plates (Corning Incorporated, Corning, NY) over the 125 min 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, dried, and stored at −80 °C until further analysis.

Individual SCX fractions were subjected to triplicate RP-LC−MS/MS measurements for a total of 252 analyses on an LCQ Deca XP mass spectrometer (Thermo Finnigan, San Jose, CA) coupled to a nanoflow LC system (LC Packings Inc., San Francisco, CA).[24] A sample volume of 4 μL was injected onto a trapping column (100 μm i.d., IntegraFrit capillary, New Objective Inc., Woburn, CA) packed to a length of 1.5 cm with Magic C18AQ (5 μm, 200 Å; Microm BioResources Inc., Auburn, CA) and separated on a pulled tip fused silica column (75 μm i.d., Polymicro Technologies, Phoenix, AZ) packed to a length of 15 cm with Magic C18AQ (5 μm, 100 Å; Microm BioResources Inc., Auburn, CA). Binary mobile phases consisted of 96.95:2.95:0.1 water/acetonitrile/formic acid (solvent A) and 99.9:0.1 acetonitrile/formic acid (solvent B). 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%. The mass spectrometer was operated in a data-dependent mode, where the top three ions in the mass-to-charge (*m/z*) range of 250−1500 were selected for fragmentation. An exclusion duration time of 60 s and a collision energy of 35% were employed.

Data Analysis and Semiquantitation Using the PHT. Raw MS/MS spectra were processed, submitted to MASCOT,[34] and searched against the National Center for Biotechnology Information nonredundant *Drosophila* database[35] and the FlyBase database[16] for peptide and protein identifications. Carbamidomethylation of cysteine residues was used as a fixed modification. Acetylation of proteins at the N-terminus and oxidation of methionine residues were used as variable modifications. Peptide assignments (hits) were 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 (which defines spectral match at a 95% confidence level, i.e., 35 for these data) were assigned to specific peptide sequences; only those peptide sequences that were unique to a single protein were considered here.

The relative abundances of proteins were estimated (as described previously)[24−31] by comparing the total raw number of peptide hits obtained from triplicate measurements, as used by Smith and co-workers,[25] rather than the normalized number of peptide hits, as utilized by Opiteck and co-workers[26,29,30] for a given protein between two samples. Normalization of peptide hits data between two samples (to the same total number of peptide hits) is normally applied to correct for variations in sample injections or instrumental fluctuations, as is normally applied to microarray data (termed "brightness adjustment").[29,30] In this study, the total number of peptide hits identified for PD-like flies and age-matched controls are highly reproducible (i.e., 0.4−15.1% across all ages studied); thus, normalization of our data is not necessary. We also established a set of conservative criteria for the determination of significant changes in protein expression.[24] Briefly, the first criterion is that the total change in the number of peptide hits for a particular protein between the *Drosophila* PD model and age-matched controls changes by more than 100% (i.e., ≥ a factor of 2 difference in the total number of hits). The second criterion is that the difference in total number of peptide hits between transgenic and control animals is ≥9. Last, a *P*-value from a Student's *t*-test

Table 1. Summary of the Number of Proteins and Unique Peptides Identified for PD-Like Flies and Age-Matched Controls

age (days)	PD-like[a]	control[a]	common[a]
1	1095 (3363)	1083 (3451)	802 (2046)
10	762 (2360)	835 (2704)	569 (1463)
20	782 (2707)	787 (2699)	581 (1719)
30	770 (2662)	725 (2447)	577 (1733)
40	786 (2442)	831 (2708)	592 (1580)
50	805 (2587)	901 (3164)	622 (1777)
60	834 (2862)	849 (3120)	631 (1803)

[a] First value before the parenthesis shows the total number of proteins assigned from the total number of unique peptides (indicated in parenthesis) from triplicate RP-LC−MS/MS experiments.

(two-tailed distribution and two-sample unequal variance) for peptide hits between the PD-like flies and age-matched controls is ≤0.05.

Protein Quantitation Using GIST. For protein quantitation by GIST, new batches of male control and PD-like flies were raised, harvested, and processed as described above. Myoglobin (~6 nmol) from horse heart was spiked into control and PD-like samples in a 1:1 ratio before trypsin digestion. Tryptic peptides from 1-day-old control and PD-like fly proteins were labeled with *N*-acetoxy-succinimide (light) and *N*-acetoxy-d_3-succinimide (heavy), respectively, as described previously.[32,33] Light and heavy labeled peptides were combined in a 1:1 ratio, treated with an excess amount of *N*-hydroxylamine, desalted, dried, and resuspended into a 5.0 mM potassium phosphate buffer solution in 75:25 water:acetonitrile at pH 3.0 for SCX fractionation as described above. Individual SCX fractions were subjected to triplicate nanoflow LC−MS/MS analysis using a MicroMass QTOF Ultimate Global mass spectrometer (Waters, Milford, MA) equipped with a CapLC system (Waters, Milford, MA). The gradient was the same as described above, and the capillary voltage was set at 3.5 kV. Data were processed and submitted to MASCOT for protein identification. Variable modifications include acetylation (light or heavy) of lysine residues and the N-termini of peptides. Abundance ratios of light and heavy labeled peptides were manually computed by integrating peak intensities from the extracted peptide ion chromatograms. For peptides identified from more than one charge state, peak intensities were summed to obtain the heavy: light peptide ratios. Relative quantitation of proteins was obtained by averaging the intensity ratios of multiple derived unique peptides.

Results and Discussion

Summary of SCX-RP-LC−MS/MS Analysis. Tryptic peptides of control and PD-like fly head proteins at each of the seven time points were subjected to off-line SCX prefractionation. A total of six SCX fractions were collected at each time point for each sample. Each SCX fraction was subjected to triplicate RP-LC−MS/MS analysis to give a total of 252 LC−MS/MS measurements. At each time point, one replicate of each of the six SCX fractions were combined and treated as a single analysis. Table 1 lists the number of unique peptides and corresponding proteins obtained from triplicate measurements for the *Drosophila* PD model and age-matched controls. A similar number of proteins and/or unique peptides were identified for the PD-like flies and age-matched controls at a specific age. For example, at day 1, a total of 1095 proteins were identified from 3366 unique peptides in PD-like flies; similarly, a total of 1083 proteins were identified from 3451 unique peptides in controls.

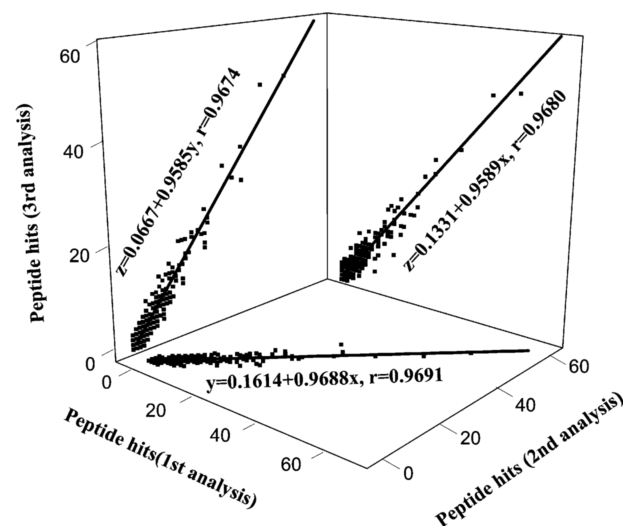

Figure 1. Plot of the number of peptide hits from triplicate analyses of tryptic peptides from 50-day-old control flies. The dots on the *xy* plane represent proteins with peptide hits from the first and the second analyses; the dots on the *yz* plane represent proteins with peptide hits from the second and the third analyses; and the dots on the *xz* plane represent proteins with peptide hits from the first and the third analyses. Linear regression was performed and the best-fit equation as well as the Pearson product-moment correlation coefficient are shown for any two replicates from the triplicate measurements.

Table 2. Pearson Product-Moment Correlation Coefficients of Peptide Hits Identified for Proteins among Triplicate Analyses for PD-Like Flies and Age-Matched Controls

age	control			PD-like		
	analysis[a]					
(days)	1 and 2	2 and 3	1 and 3	1 and 2	2 and 3	1 and 3
1	0.9539	0.9502	0.9548	0.9442	0.9444	0.9486
10	0.9388	0.9396	0.9366	0.9629	0.9681	0.9652
20	0.9614	0.9559	0.9601	0.9582	0.9587	0.9606
30	0.9476	0.9454	0.9455	0.9435	0.9453	0.9486
40	0.9532	0.9548	0.9605	0.9533	0.9498	0.9542
50	0.9691	0.9674	0.9680	0.9610	0.9503	0.9517
60	0.9651	0.9676	0.9626	0.9530	0.9520	0.9570

[a] This refers to the comparison of triplicate analyses.

At each individual age, ~70% of either unique peptides or proteins are in common between PD-like flies and age-matched controls. Cumulatively, 1271 and 1266 proteins were identified with a minimum of two peptide hits for control and PD-like flies, respectively. A total of 313 (control) and 337 (PD-like) proteins identified from a single peptide sequence were detected multiple times. A complete list of protein and peptide identifications is provided in Supporting Information Table S1.

Evaluation of Reproducibility in Peptide Hits for RP-LC−MS/MS Analyses. As mentioned above (also provided in Table S1), hundreds of proteins were identified from the *Drosophila* PD model and age-matched controls at each individual age. To obtain information about molecular changes that occur at different ages in the *Drosophila* PD model, the semiquantitative PHT was employed.[24−31] The reproducibility of the RP-LC−MS/MS analyses was evaluated by determining the variation in the number of peptide hits identified for proteins from replicate measurements. Figure 1 shows a plot of the correlations of peptide hits from triplicate measurements of the 50-day-old

Table 3. Proteins That May Be Significantly Differentially Expressed between PD-Like Flies and Age-Matched Controls

gene name or ID[a]	FBgn No.[a]	PH[b] (control)	PH[b] (PD-like)	PH ratio (PD-like/control)	*P*-value[c]
			Day 1		
Fat body protein 1	FBgn0000639	25	0	0.00	0.0202
Larval serum protein 2	FBgn0002565	37	2	0.05	0.0024
Troponin C at 73F	FBgn0010424	18	1	0.06	0.0034
PHGPx	FBgn0035438	12	1	0.08	0.0082
Diphenol oxidase A2	FBgn0000486	12	2	0.17	0.0132
Rpt1	FBgn0028687	13	3	0.23	0.0419
Muscle-specific protein 300	FBgn0053715	23	6	0.26	0.0273
Ribosomal protein S17	FBgn0005533	14	4	0.29	0.0217
Tropomyosin 2	FBgn0004117	23	7	0.30	0.0499
CG11089	FBgn0039241	15	5	0.33	0.0099
cheerio	FBgn0014141	28	10	0.36	0.0043
sallimus	FBgn0003432	64	26	0.41	0.0102
stubarista	FBgn0003517	17	7	0.41	0.0133
bent	FBgn0005666	156	66	0.42	0.0022
Punch	FBgn0003162	23	10	0.43	0.0198
regucalcin	FBgn0030362	30	14	0.47	0.0241
CG14961	FBgn0035439	17	8	0.47	0.0031
Tropomyosin 1	FBgn0003721	51	25	0.49	0.0188
Ribosomal protein S3A	FBgn0017545	18	9	0.50	0.0351
CG3523*	FBgn0027571	26	56	2.15	0.0013
TER94*	FBgn0024923	18	40	2.22	0.0012
CG3731	FBgn0038271	9	26	2.89	0.0027
Elongation factor 1 β	FBgn0028737	4	13	3.25	0.0286
Ferritin 1 heavy chain homologue	FBgn0015222	3	12	4.00	0.0350
Ras opposite	FBgn0004574	3	15	5.00	0.0080
comatose	FBgn0000346	3	22	7.33	0.0019
stoned A	FBgn0016976	2	15	7.50	0.0059
Trehalose-6-phosphate synthase 1*	FBgn0027560	1	13	13.00	0.0011
			Day 10		
Calbindin 53E	FBgn0004580	13	2	0.15	0.0399
Ubiquitin activating enzyme 1	FBgn0023143	11	2	0.18	0.0031
chickadee	FBgn0000308	12	3	0.25	0.0351
CG3011	FBgn0029823	20	5	0.25	0.0193
CG4685	FBgn0039349	16	5	0.31	0.0177
CG6439	FBgn0038922	15	5	0.33	0.0132
Elongation factor 1a48D*	FBgn0000556	19	7	0.37	0.0011
CG3731	FBgn0038271	31	15	0.48	0.0303
G11876	FBgn0039635	12	24	2.00	0.0366
Myosin alkali light chain 1	FBgn0002772	8	17	2.13	0.0031
CG6287	FBgn0032350	12	26	2.17	0.0159
CG30045	FBgn0050045	9	20	2.22	0.0148
ATP synthase-β*	FBgn0010217	39	93	2.38	<0.0001
chaoptic	FBgn0000313	21	57	2.71	0.0119
Retinin*	FBgn0040074	11	30	2.73	0.0019
obp44a*	FBgn0033268	12	33	2.75	0.0010
CG6543*	FBgn0033879	11	35	3.18	0.0019
CG11015*	FBgn0031830	3	12	4.00	<0.0001
ATP synthase-γ chain	FBgn0020235	3	24	8.00	0.0067
			Day 20		
CG12120	FBgn0030106	12	2	0.17	0.0132
Thiolester containing protein IV	FBgn0041180	14	3	0.21	0.0315
Chd64	FBgn0035499	26	9	0.35	0.0273
arginase	FBgn0023535	18	8	0.44	0.0132
porin	FBgn0004363	54	27	0.50	0.0121
no receptor potential A	FBgn0004625	13	26	2.00	0.0271
Glutamate oxaloacetate transaminase 2	FBgn0001125	13	27	2.08	0.0159
Succinyl coenzyme A synthetase a subunit	FBgn0004888	6	21	3.50	0.0199
nervana 2	FBgn0015777	4	15	3.75	0.0322
Trehalose-6-phosphate synthase 1	FBgn0027560	4	18	4.50	0.0230
CG3699*	FBgn0040349	2	11	5.50	0.0031
inactivation no afterpotential C	FBgn0004784	1	10	10.00	0.0286
			Day 30		
CG6543*	FBgn0033879	11	22	2.00	0.0015
Calcium ATPase at 60A	FBgn0004551	9	19	2.11	0.0132
Elongation factor 1a48D	FBgn0000556	13	29	2.23	0.0102
Glutamate oxaloacetate transaminase 2	FBgn0001125	12	28	2.33	0.0303
G protein &bgr;-subunit 13F	FBgn0001105	4	17	4.25	0.0271

Table 3 (Continued)

gene name or ID[a]	FBgn No.[a]	PH[b] (control)	PH[b] (PD-like)	PH ratio (PD-like/control)	*P*-value[c]
Day 40					
Eukaryotic initiation factor 4a	FBgn0001942	9	0	0.00	0.0351
Photoreceptor dehydrogenase	FBgn0011693	32	16	0.50	0.0491
ade5	FBgn0020513	17	7	0.41	0.0494
CG16936	FBgn0027590	14	5	0.36	0.0286
CG4169*	FBgn0036642	49	24	0.49	0.0008
thioredoxin peroxidase 1	FBgn0040309	21	9	0.43	0.0202
CG7217*	FBgn0038570	22	9	0.41	0.0061
Day 50					
Acetyl Coenzyme A synthase	FBgn0012034	11	1	0.09	0.0021
sallimus	FBgn0003432	15	2	0.13	0.0059
Annexin IX	FBgn0000083	14	3	0.21	0.0148
CG9914	FBgn0030737	14	3	0.21	0.0322
Tropomyosin 2	FBgn0004117	13	3	0.23	0.0419
TER94	FBgn0024923	13	3	0.23	0.0419
PHGPx	FBgn0035438	13	3	0.23	0.0377
Dak1	FBgn0028833	20	5	0.25	0.0335
Odorant-binding protein 56d	FBgn0034470	14	4	0.29	0.0021
CG11089	FBgn0039241	23	7	0.30	0.0166
CG30337	FBgn0050337	23	7	0.30	0.0129
Neuroglian	FBgn0002968	13	4	0.31	0.0286
Pyruvate kinase	FBgn0003178	36	12	0.33	0.0052
lethal (1) G0255	FBgn0028336	15	5	0.33	0.0202
Elongation factor 1a48D	FBgn0000556	22	8	0.36	0.0157
Zeelin1	FBgn0038294	27	11	0.41	0.0284
Phosphoglucose isomerase	FBgn0003074	17	8	0.47	0.0031
Triose phosphate isomerase	FBgn0003738	21	10	0.48	0.0100
lethal (1) G0030	FBgn0026708	64	32	0.50	0.0042
ATP citrate lyase	FBgn0020236	9	21	2.33	0.0080
Calcium ATPase at 60A*	FBgn0004551	19	51	2.68	0.0011
Ejaculatory bulb protein III	FBgn0011695	2	12	6.00	0.0132
Black cells	FBgn0000165	1	10	10.00	0.0031
Day 60					
Glycerol 3 phosphate dehydrogenase	FBgn0001128	19	1	0.05	0.0124
Glutamine synthetase 2	FBgn0001145	15	2	0.13	0.0061
β-Tubulin at 56D*	FBgn0003887	17	5	0.29	0.0011
Succinyl coenzyme A synthetase a subunit	FBgn0004888	16	5	0.31	0.0424
GDP dissociation inhibitor	FBgn0004868	20	7	0.35	0.0198
Phosphofructokinase	FBgn0003071	16	6	0.38	0.0132
Heat shock protein 83	FBgn0001233	22	9	0.41	0.0433
ATP synthase, subunit b	FBgn0019644	20	9	0.45	0.0148
Mitochondrial phosphate carrier protein	FBgn0026409	19	9	0.47	0.0419
α actinin	FBgn0000667	33	16	0.48	0.0136
Elongation factor 1a48D*	FBgn0000556	8	23	2.88	0.0004
Turandot C	FBgn0044812	4	13	3.25	0.0031
Triose phosphate isomerase	FBgn0003738	5	17	3.40	0.0133
lethal (2) 37Cc	FBgn0002031	3	12	4.00	0.0213
CG9629	FBgn0036857	3	13	4.33	0.0099
capulet	FBgn0028388	2	11	5.50	0.0031
CG6415	FBgn0032287	1	10	10.00	0.0031

[a] Gene name or ID was obtained from the FlyBase database www.flybase.org. Gene name or IDs were labeled with "*" to indicate that they pass the conservative Bonferroni correction. [b] Peptide hits (PH) is the total number of PH from triplicate analyses. [c] *P*-value was obtained from Student's *t*-test (two-tailed distribution and two sample unequal variance) of PH from triplicate analyses uisng Microsoft Excel.

control fly sample (collective data from six SCX fractions were treated as one measurement of the sample). As shown in Figure 1, variations in the number of peptide hits for some proteins are found between the three measurements. However, the number of peptide hits for most proteins is fairly reproducible. Linear regression analysis indicates Pearson product-moment correlation coefficients of 0.9691 between the first and the second analyses, 0.9680 between the second and the third analyses, and 0.9674 between the first and the third analyses (Pearson product-moment correlation coefficient is a measure of the correlation between two sets of measurement on the same object; a perfect fit has a correlation coefficient of 1).[36] Table 2 lists Pearson product-moment correlation coefficients of any two replicates among triplicate measurements of all datasets for the PD-like flies and age-matched controls. As shown in Table 2, all the Pearson product-moment correlation coefficients are greater than 0.93, which indicates high reproducibility of peptide hits from run to run across all samples. This ensures the applicability of peptide hits for semiquantitative estimation of relative abundance of proteins identified from PD-like flies and age-matched controls.

Semiquantitation of Relative Protein Expression Using the PHT. It has previously been demonstrated that a change in peptide hits does not imply the exact fold change in protein abundance, rather it indicates the direction of change for a protein that is up or down regulated.[26−31] Reliability of PHT

Table 4. Comparison of Differentially Expressed Proteins between PHT and GIST Measurements

gene name or ID[a]	peptide sequences identified[b]	mean ± SD[c]	mean ± SD[d]	PH[e] (control)	PH[e] (PD-like)	PH ratio[e] (PD-like/ control)	*P*-value[e]
Larval serum protein 2	(Ac)YDEHGHEIPLEHNYQNFFELEHFK(Ac)	0.34 ± 0.01	0.45 ± 0.12	37	2	0.05	0.0024
	(Ac)HDYYFDVHNFK(Ac)	0.58 ± 0.09					
	(Ac)LDQSEAHCGVPNR	0.56 ± 0.20					
	(Ac)TYYGVPQWHR	0.47 ± 0.01					
	(Ac)VHLEAGVNHIK(Ac)	0.31 ± 0.11					
Ribosomal protein S17	(Ac)LLDFHNIR	0.85 ± 0.03	0.52 ± 0.20	14	4	0.29	0.0217
	(Ac)IAGYVTHLMGR	0.38 ± 0.04					
Punch	(Ac)LLLGGLGENPDR	0.55 ± 0.10	0.55	23	10	0.43	0.0198
CG8036[#]	(Ac)VVVVEDHYQQGGLGEAVLSALAGER	0.59 ± 0.04	0.59 ± 0.13	14	9	0.64	0.03775
	(2Ac)KIDSDLEGHPTPR	0.46 ± 0.05					
	(Ac)IDSDLEGHPTPR	0.72 ± 0.08					
Chaoptic[#]	(Ac)TFFDGNPIHTLR	0.64 ± 0.12	0.65 ± 0.12	39	25	0.64	0.0065
	(Ac)ISGNHLTEIPDDAFTGLER	0.83 ± 0.05					
	(Ac)LAVLDLSHNR	0.56 ± 0.02					
	(Ac)LLELHDNR	0.74 ± 0.05					
	(Ac)LLLTDNILSEIPYDALGPLK(Ac)	0.53 ± 0.10					
	(Ac)LNLDNLHLEYNHIEVLPPNSFK(Ac)	0.57 ± 0.06					
α-Tubulin at 84B[#]	(Ac)QLFHPEQLITGK(Ac)	0.66 ± 0.22	0.66 ± 0.15	46	44	0.96	0.5384
	(Ac)AVFVDLEPTVVDEVR	0.86 ± 0.11					
	(Ac)IHFPLVTYAPVISAEK(Ac)	0.52 ± 0.10					
	(Ac)NLDIERPTYTNLNR	0.58 ± 0.12					
Rtnl1[#]	(Ac)VQNIAGVAVAHINGFISELR	0.61 ± 0.04	0.67 ± 0.08	6	4	0.67	0.3868
	(Ac)QSIDTHLDLVR	0.72 ± 0.16					
Odorant-binding protein 99b[#]	(Ac)THEDLTNYR	0.78 ± 0.23	0.68 ± 0.11	3	0	0	NA
	(Ac)IAHCAETHSK(Ac)	0.70 ± 0.29					
	(Ac)IHIQLAGPGVEVHESDEVHQK(Ac)	0.57 ± 0.02					
sallimus	(Ac)LVEGQSAHFEAR	0.74 ± 0.08	0.68 ± 0.08	64	26	0.41	0.0102
	(Ac)EGENAHFEAR	0.62					
β-Tubulin at 56D[#]	(Ac)SGPFGQIFRPDNFVFGQSGAGNNWAK(Ac)	0.57 ± 0.03	0.69 ± 0.16	8	5	0.63	0.2739
	(Ac)LTTPTYGDLNHLVSLTMSGVTTCLR	0.80 ± 0.05					
retinal degeneration A[#]	(Ac)VTPNDDVTDDHVDR	0.53 ± 0.03	0.69 ± 0.22	11	9	0.82	0.4926
	(Ac)ELGQTALHIAAEQNR	0.84 ± 0.16					
bent	(Ac)FLKPHIDR	0.83 ± 0.07	0.72 ± 0.08	156	65	0.42	0.0022
	(Ac)YDILEEIGTGAFGVVHR	0.72 ± 0.05					
	(Ac)DGSHLDSGPYR	0.66					
	(Ac)NPFDVSAPPGLPELEDWDEHHVK(Ac)	0.65					
cheerio	(Ac)FNGVHIPDSPFR	0.78 ± 0.07	0.74 ± 0.11	28	10	0.36	0.0043
	(Ac)ELGVHTVSVR	0.83 ± 0.02					
	(Ac)ENGIHAIHVK(Ac)	0.62 ± 0.10					
Tropomyosin 1[*]	(Ac)IQLLEEDLER	1.02 ± 0.04	0.94 ± 0.12	51	25	0.49	0.0188
	(Ac)IVELEEELR	0.85 ± 0.25					
Tropomyosin 2[*]	(Ac)LLEATQSADENNR	1.01 ± 0.02	1.01	23	7	0.3	0.0499
Fatty acid synthase	(Ac)RPGTQAPPTLER	1.91 ± 0.28	2.01 ± 0.13	26	56	2.15	0.0013
	(Ac)AAPQLDLGGGHYVPR	2.11 ± 0.03					

[a] Gene name or ID were obtained from the FlyBase database, www.flybase.org. Proteins labeled with # only change in the GIST measurements. Proteins labeled with * only change with the PHT. [b] The site of light or heavy acetylation is indicated in parenthesis by Ac. [c] The mean ratio and standard deviation (SD) were obtained from triplicate measurements of intensities of light and heavy labeled peptides. The ratios with no SD denote that they were quantitated from one replicate. [d] The mean ratio and SD were obtained from multiple peptides of corresponding proteins. [e] Refer to Table 3 for annotation.

for semiquantitation of relative protein abundance has been partially validated using the Western blotting assay by Opiteck and co-workers[29] and the $^{16}O/^{18}O$ isotopic labeling strategy by Smith and co-workers.[25,37] However, like other quantitative or semiquantitative approaches employed in proteomics analysis, the PHT has its own limitations in detecting quantitative changes. One of the major limitations is that this approach is only valid over ~2 orders of magnitude.[26,38] Low abundant proteins may go undetected or detected with only a few peptides in both control and experimental samples and thus will fall below the limits at which change can be confidently assessed. In the case of some highly abundant proteins, the PHT will not be sufficient to detect changes in concentration between control and experimental samples. This is because the analytes have already reached the saturation point in the analysis, and no more hits will be detected with the same protocol (e.g., certain dynamic exclusion) even if there are concentration changes. Additionally, the detectability of a peptide in LC–MS/MS analysis is based on its intrinsic properties (e.g., hydrophobicity and charge) as well as the complexity of the sample matrix. Despite the limitations of the PHT, it offers a promising approach for assessing relative protein abundance and screening changes in large-scale proteome analysis.[24–31,39]

Currently, no standard criteria exist for the determination of whether or not there is a change in relative protein abundance using the PHT. 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 the ratio of peptide hits was four standard deviations greater than the average ratio or when the protein was only observed in the lipopolysaccharide treated sample with more than three peptide hits.[25] In another proteomic study of yeast protein expression changes 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.[27] Because our goal is to identify protein candidates for further investigation of their roles in PD, we need suitable criteria to identify a targeted set of proteins. Thus, the criteria that we established for the determination of variation in protein

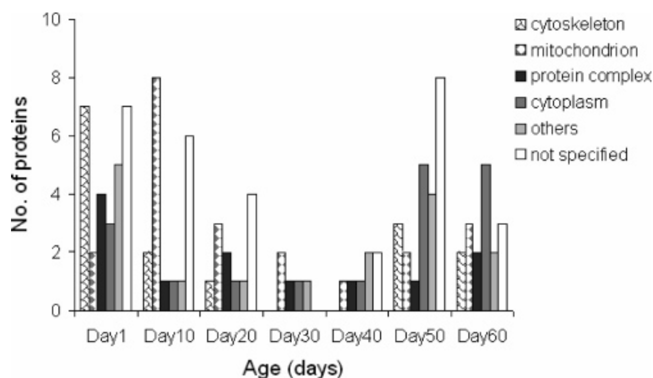

Figure 2. Bar graph representation of Gene Ontology categorization of cellular components for the dysregulated proteins at each individual age. The cellular component categorizations are cytoskeleton (outlined triangles), mitochondrion (shaded triangles), protein complex (shaded black boxes), cytoplasm (shaded dark gray boxes), others (shaded light gray boxes), and not specified (white boxes).

expression are conservative (as briefly discussed in the experimental section).[24]

Table 3 lists detailed information of the proteins that are significantly differentially expressed at each of the various ages, including encoding gene names, FlyBase IDs, corresponding peptide hits from control and PD-like flies, and P-values. Overall, 28, 19, 12, 5, 7, 23, and 17 proteins meet our established criteria to be considered as significantly changed between PD-like flies and age-matched controls at days 1, 10, 20, 30, 40, 50, and 60, respectively. We note that when assuming independence of multiple comparisons of peptide hits data only 16 of the total 111 proteins from all seven time points (Table 3) pass the conservative Bonferroni method. The Bonferroni method states that if an investigator is making k independent significance test, the statistical significance level used for each individual test should be $1/k$ times of what it would be if only one significance test were made.[40] This is carried out to lower the overall experimentwise error rate.[40] A significance level of 0.0015, 0.0022, 0.0036, 0.0071, 0.0071, 0.0019, and 0.0021 is required to achieve an experimentwise error rate of $a = 0.05$ at days 1, 10, 20, 30, 40, 50, and 60, respectively. However, proteins that are not deemed significant based on the Bonferroni method appear to change with other analytical approaches (Table 4).

Figure 2 shows a bar graph representation of Gene Ontology[41] categorization of cellular components for the dysregulated proteins at each individual age. Dysregulated proteins at days 1, 10, and 30 have previously been discussed.[24] Additionally these data were compared to a corresponding transcriptome from Feany's laboratory.[18] At day 1, seven of the dysregulated proteins are actin cytoskeleton associated (i.e., the proteins encoded by *sallimus, bent, cheerio, tropomyosin 1, tropomyosin 2, troponin C at 73F,* and *muscle-specific protein 300*) and most interestingly, they are all down-regulated. In contrast, within the detection limits of the present approach employed there are fewer cytoskeletal proteins that are dysregulated at other ages, i.e., two (encoded by *chickadee* and *myosin alkali light chain 1*) at day 10, one (encoded by *Chd64*) at day 20, none at days 30 and 40, three (encoded by *sallimus*, *Tropomyosin 2* and *TER94*) at day 50, and two (encoded by β−Tubulin at 56D and *capulet*) at day 60. In eukaryotic cells, the actin cytoskeleton plays a pivotal role in cell morphology, cell motility, cell polarity, cell division, cell communication, and endocytosis, especially in maintaining specific shapes of cells and supporting synaptic transmission and plasticity.[42] Dysregulation of actin cytoskeletal proteins suggests that the actin cytoskeleton network may be disrupted, which can cause the collapse of specific structures of cells in the CNS and consequently their dysfunction. Moreover, early disturbance of the actin cytoskeletal proteins may play a role in late degeneration of dopaminergic neurons, formation of LB-like inclusions, and declining locomotor ability observed in PD-like flies.[12] 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.

Mitochondrial proteins were perturbed across different ages. Specifically, there are two (encoded by *PHGPx* and an unnamed gene CG3731) at day 1, eight (encoded by *ATP synthase-γ chain*, CG11015, *ATP synthase-β*, CG3011, CG4685, CG6439, CG3731, and CG6543) at day 10, three (encoded by *Glutamate oxaloacetate transaminase 2*, *arginase*, and *porin*) at day 20, two (encoded by *Glutamate oxaloacetate transaminase 2* and CG6543) at day 30, one (encoded by CG4169) at day 40, two (encoded by *PHGPx* and *lethal (1) G0030*) at day 50, three (encoded by *Mitochondrial phosphate carrier protein*, *lethal (2) 37Cc*, and *ATP synthase subunit b*) at day 60. An important point is that at day 10, ~42% (8/19) of the dysregualted proteins are mitochondrion-associated. It is interesting to mention that in the proteome analysis of human substantia nigra in PD patients, ATP synthase D chain was observed at higher abundance relative to controls;[8] at the early disease stage in PD-like flies, both ATP synthase-γ chain (fold change 8.00, $P = 0.0067$) and ATP synthase-β subunit (fold change 2.38, $P < 0.0001$) are up-regulated compared to age-matched controls. Because ATP synthase plays pivotal roles in mitochondrial function, the consistent finding of the dysregulation of ATP synthase in both postmortem human substantia nigra and the *Drosophila* PD model supports the notion that mitochondrial dysfunction may be associated with neurodegeneration in PD.[3]

Proteins associated with other cellular components (e.g., cytoplasma and protein complex) also changed at various ages. For instance, at day 50 and 60, many of the dysregulated proteins are from the cytoplasm and/or some unknown cellular components. Some of these proteins also play important roles in cell communication and cell viability, such as proteins encoded by *calcium ATPase at 60A* and *glutamine synthetase 2*.[43,44]

GIST Analysis of 1-Day-Old Flies. To validate some protein changes observed with the PHT by another approach, we have applied a GIST strategy to one of the seven time points (i.e., 1-day-old control and PD-like flies). The internal standard myoglobin spiked at an equal amount into control and PD-like samples shows an average ratio of 1.04 ± 0.13 ($N = 11$); thus, a protein is considered as significantly differentially expressed if the abundance ratio differs from 1.04 with more than 3 standard deviation (either >1.42 or <0.75). Figure 3 shows examples of mass spectra obtained for peptides belonging to larval serum protein 2. From the relative intensities of the peak pairs it is apparent that the peptides are down regulated in the PD-like samples. The average ratio of five peptides detected for larval serum protein 2 is 0.45 ± 0.12 (Table 4). Down regulation of larval serum protein 2 agrees with the results from the PHT (Table 3), which shows 37 hits in control and two hits in PD-like flies. In addition, if the GIST

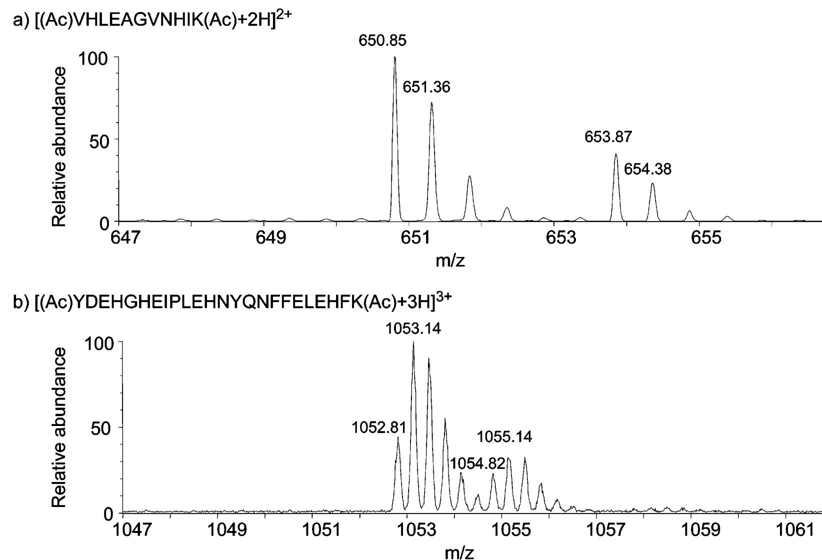

Figure 3. Example mass spectra for light and heavy labeled peptide pairs of (a) VHLEAGVNHIK and (b)YDEHGHEIPLEHNYQNFFELEHFK that are assigned to larval serum protein 2. The site of light or heavy acetylation is indicated in parentheses by Ac.

data are analyzed using the PHT, 11 peptides are detected for control samples whereas only one hit is detected for PD-like samples.

For other proteins that exhibit up- or down-regulation in the peptide hits data that were also detected in GIST experiments, overall qualitative agreement was obtained for most proteins (Table 4). Although these proteins show the same direction of change between the two techniques, the overall magnitude of change differs. Differences in the exact fold-change detected for common proteins identified with various techniques have also been reported by others.[25,29,30,45] Tropomyosin 1 and 2 do not appear to change based on the GIST data, whereas they exhibit a 2- and 3-fold change in peptide hits (Table 3), respectively. Table 4 also shows seven additional proteins that changed based on the GIST measurement, however did not meet the criteria established for consideration as a change with the PHT. Other proteomic studies have also reported differences in relative expression levels of proteins identified with different approaches.[25,37,45,46]

We note that there may be several reasons for differences in the changes of proteins associated with PHT and GIST approaches. For example, the second batch of flies utilized for the GIST measurement were generated a year after the peptide hits analysis. Thus, there could be some biological variability in the expression of some proteins; these may not be directly associated with the development of PD-like symptoms. In addition, many proteins may also undergo posttranslational modifications and thus are not identified in our analysis scheme. In this case, the changes that we observed with either the PHT or the GIST approach only correspond to the unmodified states of the peptides. Finally, the GIST values for some of these proteins are only based on one or two peptides that were measured in replicate analyses; it is possible that additional peptides belonging to these proteins may display differences in the heavy:light ratios. Thus, PHT and GIST approaches may provide complementary information, but further studies are also warranted to unambiguously determine the change.

Summary of Lifetime Protein Expression Profiles. Because PD is an age-associated neurodegenerative disease, it is informative to consider how many proteins and which proteins consistently change between the *Drosophila* PD model and age-

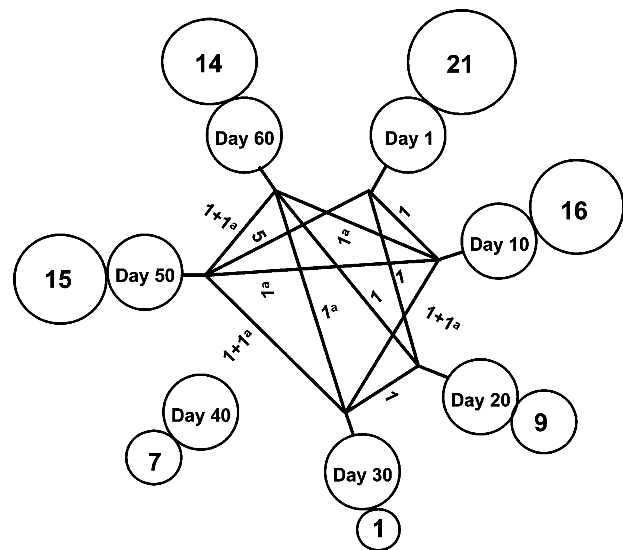

Figure 4. Diagram comparing the number of proteins that are significantly differentially expressed in PD-like flies vs age-matched controls at different ages. The values in the circles refer to the number of dysregulated proteins in PD-like flies vs age-matched controls that are unique to a specified age. The values next to the lines indicate the additional number of proteins that are significantly differentially expressed between any two disease stages.

matched controls as the PD-like flies age. Figure 4 shows a diagram comparing the number of proteins that are significantly differentially expressed in PD-like flies vs age-matched controls at each specified age from the PHT. In total, 21, 16, 9, 1, 7, 15, and 14 proteins are unique to day 1, 10, 20, 30, 40, 50, and 60, respectively. There are only a few proteins that consistently change between PD-like flies and age-matched controls across different ages. For example, when comparing the proteins that changed between the *Drosophila* PD model and controls at day 1 with other ages, there is one protein (encoded by CG3731) in common between day 1 and day 10; one protein (encoded by *trehalose-6-phosphate synthase 1*) is in common between day 1 and day 20; and five proteins

(encoded by *sallimus*, *TER94*, *PHGPx*, *Tropomyosin 2* and CG11089) are in common between day 1 and day 50; no proteins are in common between day 1 and day 30, day 40, and day 60. The protein (indicated by 1[a]) encoded by *elongation factor 1α48D* shows significant change at four different ages (i.e., day 10, 30, 50, and 60). The seven differentially expressed proteins at day 40 are all unique to that specific age. These results obtained from the experimental approach employed in this study appear to support that up or down regulation of proteins in PD-like flies in comparison with age-matched controls is age and subsequently disease stage dependent. Proteomic studies of the influence of fungal polysaccharide treatments on rat plasma protein changes as a result of diabetes induction also revealed that disturbed proteins exhibit a time-dependent manner.[47] Thus, these studies appear to support the notion that proteome changes resulting from stimuli as a function of time or disease stage are highly dynamic.

Because the A30P α-synuclein transgenic *Drosophila* slowly develop human PD-like symptoms and the degeneration of dopaminergic neurons emerges after day 10,[12] the protein expression changes that occur at day 1, before the onset of the neurodegeneration, may provide key insights to the understanding of the molecular mechanisms associated with the causation of PD. Although several cellular defects, including oxidative stress, mitochondrial dysfunction, and impairment of the ubiquitin-proteasome system have been widely proposed to be associated with PD, the cause and effect relationships remain elusive.[3,48] Recently, Lindquist and co-workers demonstrated that protein transportation from the endoplasmic reticulum to the Golgi apparatus was blocked in α-syuclein expression associated PD using three independent animal models.[49] This is a newly proposed cellular defect associated with PD pathogenesis. Our data suggest that comprehensive study of the proteins that were disturbed with the progression of the disease, especially those at day 1, may provide clues for understanding the relationships among the cellular malfunctions and exploring new strategies for early diagnosis and neuron protection. For example, one of the interesting dysregulated proteins at day 1 is GTP cyclohydrolase I (encoded by *Punch*). Low expression levels of GTP cyclohydrolase I have been observed in nigrostriatal dopamine neurons.[50] GTP cyclohydrolase I is the first enzyme in the biosynthesis of tetrahydrobiopterin, which is a cofactor of tyrosine hydroxylase in the synthesis of catecholamines (e.g., dopamine, norepinephrine, and epinephrine).[51] Tetrahydrobiopterin in the brains of PD patients has been found at ~50% the level of that in age-matched controls.[52] It is likely that a decrease in GTP cyclohydrolase I leads to less synthesized tetrahydrobiopterin and/or dopamine. At the transcript level, GTP cyclohydrolase I was also down-regulated in 1-day-old A30P α-synuclein PD-like flies compared to age-matched controls.[18] Down-regulation of GTP cyclohydrolase I at both the mRNA and protein levels warrants further studies to understand the role of GTP cyclohydrolase I in PD and to explore new strategies for early diagnosis and prevention of PD.

Summary and Conclusions

The present work presents a semiquantitative comparison of the proteome of an A30P α-synuclein transgenic *Drosophila* model of PD with age-matched controls at seven ages across the lifespan (days 1, 10, 20, 30, 40, 50, and 60; note that a comparison of days 1, 10, and 30 were described previously).[24] From the experimental approach employed in this study, it appears that the majority of the dysregulated proteins were identified at a specific age, indicating that perturbed proteins in PD-like flies compared with age-matched controls may exist only over a narrow distribution of ages. Because the *Drosophila* PD model develops the human PD-like symptoms after day 10, the early changes associated with the 28 proteins at day 1 may provide key insights to the understanding of the molecular basis causing the dopaminergic neuron degeneration.[24] Down regulation of a group of actin cytoskeletal proteins at day 1 suggests that perturbation of actin cytoskeletal proteins at the presymptomatic stage may be responsible for the onset of PD-like symptoms in transgenic flies. Mitochondrial dysfunction is known to be a pathological feature of human PD.[3] The variation in mitochondrial proteins between control and PD-like animals reported here (two of which, ATP synthase subunit b and ATP synthase γ chain, are consistent with reported work in human PD patients. This finding is interesting and we are planning to pursue further studies) suggests that the molecular mechanisms associated with neurodegeneration may have similarities. Thus, comprehensive understanding of the pathogenesis and etiology of the *Drosophila* model may shed light on human PD.

In addition, differential analysis of the proteome at later ages (days 40, 50, and 60) provides a more comprehensive look at protein expression profiles as the *Drosophila* PD model develops human PD-like symptoms and proceeds to advanced disease stages. While studies across many time points appear to be important for characterizing disease state, an understanding of molecular changes at the youngest ages should be most important for addressing causation. Finally, the aim of the present study was to survey proteome changes in an A30P α-synuclein *Drosophila* model of Parkinson's disease at different disease states to provide useful directions for PD research. Further studies (e.g., incorporation of independent measurements using Western blot analysis and/or isotopic labeling approaches) are warranted to validate these findings. Overall, this report demonstrates the utility of the *Drosophila* model for the study of the molecular mechanisms associated with neurodegeneration in human PD.

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Supporting Information Available: The complete list of unique peptides, corresponding proteins, and the number of peptide hits identified for PD-like flies and age-matched controls. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Martin, J. B. *New Engl. J. Med.* **1999**, *340*, 1970–1980.
(2) Fearnley, J. M.; Lees, A. J. *Brain* **1991**, *114*, 2283–2301.
(3) Olanow, C. W.; Tatton, W. G. *Annu. Rev. Neurosci.* **1999**, *22*, 123–144.
(4) Maries, E.; Dass, B.; Collier, T. J.; Kordower, J. H.; Steece-Collier, K. *Nature Rev. Neurosci.* **2003**, *4*, 727–738.
(5) Lees, A. J. *Mov. Disord.* **1992**, *7*, 110–116.
(6) Goetz, C. G.; Poewe, W.; Rascol, O.; Sampaio, C. *Mov. Disord.* **2005**, *20*, 523–539.
(7) Lang, A. E.; Lozano, A. M. *New Engl. J. Med.* **1998**, *339*, 1130–1143.

(8) Basso, M.; Giraudo, S.; Corpillo, D.; Bergamasco, B.; Lopiano, L.; Fasano, M. *Proteomics* **2004**, *4*, 3943−3952.
(9) Leenders, K. L. *Eur. Neuropsychopharmacol.* **2002**, *12*, 575−580.
(10) Zarranz, J. J.; Alegre, J.; Gómez-Esteba, J. C.; Lezcano, E.; Ros, R.; Ampuero, I.; Vidal, L.; Hoenicka, J.; Rodriguez, O.; Atarés, B.; et al. *Ann. Neurol.* **2004**, *55*, 164−173.
(11) Spillantini, M. G.; Schmidt, M. L.; Lee V. M.-Y.; Trojanowski, J. Q.; Jakes, R.; Goedert, M. *Nature* **1997**, *388*, 839−840.
(12) Feany, M. B.; Bender, W. W. *Nature* **2000**, *404*, 394−398.
(13) Matsuoka, Y.; Vila, M.; Lincoln, S.; McCormack. A.; Picciano, M.; LaFrancois, J.;Yu. X.; Dickson, D.; Langston, J. W.; McGowan, E.; Farrer, M.; Hardy, J.; Duff, K.; Przedborski, S.; Di Monte, D. A. *Neurobiol. Dis.* **2001**, *8*, 535−539.
(14) Klein, R. L.; King, M. A.; Hamby, M. E.; Meyer, E. M. *Hum. Gene Ther.* **2002**, *13*, 605−612.
(15) Kirik, D.; Annett, L. E.; Burger, C.; Muzyczka, N.; Mandel, R. J.; Bjorklund, A. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 2884−2889.
(16) Drysdale, R. A.; Crosby, M. A. The FlyBase Consortium. *Nucleic Acids Res.* **2005**, *33*, D390−D395. http://flybase.org/.
(17) Adams, M. D.; Celniker, S. E.; Holt, R. A.; Evans, C. A.; Gocayne, J. D.; Amanatides, P. G.; Scherer, S. E.; Li, P. W.; Hoskins, R. A.; Galle, R. F.; George, R. A.; Lewis, S. E.; Richards, S.; Ashburner, M.; et al. *Science* **2000**, *287*, 2185−2195.
(18) Scherzer, C. R.; Jensen, R. V.; Gullans, S. R.; Feany, M. B. *Hum. Mol. Genet.* **2003**, *12*, 2457−2466.
(19) Taraszka, J. A.; Kurulugama, R.; Sowell, R. A.; Valentine, S. J.; Koeniger, S. L.; Arnold, R. J.; Miller, D. F.; Kaufman, T.; Clemmer, D. E. *J. Proteome Res.* **2005**, *4*, 1223−1237.
(20) Taraszka, J. A.; Gao, X. F.; Valentine, S. J.; Sowell, R. A; Koeniger, S. L.; Miller, D. F.; Kaufman, T. C.; Clemmer, D. E. *J. Proteome Res.* **2005**, *4*, 1238−1247.
(21) Chen, L.; Feany M. B. *Nat. Neurosci.* **2005**, *8*, 657−663.
(22) Ghosh, S.; Feany, M. B. *Hum. Mol. Genet.* **2004**, *13*, 2011−2018.
(23) Myung, S.; Lee, Y. L.; Moon, M. H.; Taraszka, J. A.; Sowell, R.; Koeniger, S. L.; Hilderbrand A. E.; Valentine, S. J.; Cherbas, L.; Cherbas, P.; Kaufmann, T. C.; Miller, D. F.; Mechref, Y.; Novotny, M. V.; Ewing, M.; Clemmer, D. E. *Anal. Chem.* **2003**, *75*, 5137−5145.
(24) Xun, Z.; Sowell, S. A.; Kaufman, T. C.; Clemmer, D. E. *J. Proteome Res.* **2007**, *6*, 348−357.
(25) Qian, W. J.; Jacobs, J. M.; Camp, D. G.; Monroe, M. E.; Moore, R. J.; Gritsenko, M. A.; Calvano, S. E.; Lowry, S. F.; Xiao, W. Z.; Moldawer, L. L; Davis, R. W.; Tompkins, R. G.; Smith, R. D. *Proteomics* **2005**, *2*, 572−584.
(26) Gao, J.; Friedrichs, M. S.; Dongre, A. R.; Opiteck, G. J. *J. Am. Soc. Mass Spectr.* **2005**, *16*, 1231−1238.
(27) Gao, J.; Opiteck, G., J.; Friedrichs, M. S.; Dongre, A. R.; Hefta, S. A. *J. Proteome Res.* **2003**, *2*, 643−649.
(28) Fach, E. M.; Garulacan, L. A.; Gao, J.; Xiao, Q.; Storm, S. M.; Dubaquie, Y. P.; Hefta, S. A.; Opiteck, G. J. *Mol. Cell. Proteomics* **2004**, *3*, 1200−1210.
(29) Gao, J.; Garulacan, L. A.; Storm, S. M.; Hefta, S. A.; Opiteck, G. J.; Lin, J. H.; Moulin, F.; Dambach, D. M. *Toxicol. in Vitro* **2004**, *18*, 533−541.
(30) Pang, J. X.; Ginanni, N.; Dongre, A. R.; Hefta, S. A.; Opiteck, G. J. *J. Proteome Res.* **2002**, *1*, 161−169.
(31) Zhang, B.; VerBerkmoes, N. C.; Langston, M. A.; Uberbacher, E.; Hettich, R. L.; Samatova, N. F. *J. Proteome Res.* **2006**, *5*, 2909−2918.
(32) Chakraborty, A.; Regnier, F. E. *J. Chromatogr. A* **2002**, *949*, 173−184.
(33) Kindy, J. M.; Taraszka, J. A.; Regnier, F. E.; Clemmer, D. E. *Anal. Chem.* **2002**, *74*, 950−958.
(34) http://www.matrixscience.com.
(35) http://www.ncbi.nlm.nih.gov.
(36) Analytical Methods Committee. *Analyst.* **1988**, *113*, 1469−1471.
(37) Qian, W. J.; Monroe, M. E.; Liu, T.; Jacobs, J. M.; Anderson, G. A.; Shen, Y. F.; Moore, R. J.; Anderson, D. J.; Zhang, R.; Calvano, S. E.; Lowry, S. F.; Xiao, W. Z.; Moldawer, L. L.; Davis, R. W.; Tompkins, R. G.; Camp, D. G.; Smith, R. D.; Injury, I. H. R. *Mol. Cell. Proteomics* **2005**, *4*, 700−709.
(38) Liu, H. B.; Sadygov, R. G.; Yates, J. R. *Anal. Chem.* **2004**, *76*, 4193−4201.
(39) Zhang, B.; VerBerkmoes, N. C.; Langston, M. A.; Uberbacher, E.; Hettich, R. L.; Samatova, N. F. *J. Proteome Res.* **2006**, *5*, 2909−2918.
(40) Sokal R. R.; Rohlf, F. J. *Biometry*; W. H. Freeman and Company: New York, 1995; pp 239−240.
(41) Ashburner, M.; Ball, C. A.; Blake, J. A.; Botstein, D.; Butler, H.; Cherry, J. M.; Davis, A. P.; Dolinski, K.; Dwight, S. S.; Eppig, J. T.; Harris, M. A.; Hill, D. P.; Issel-Tarver, L.; Kasarskis, A.; Lewis, S.; Matese, J. C.; Richardson, J. E.; Ringwald, M.; Rubin, G. M.; Sherlock, G.; Consortium, G. O. *Nat. Genet.* **2000**, *25*, 25−29.
(42) Winder, S. J.; Ayscough, K. R. *J. Cell Sci.* **2005**, *118*, 651−654.
(43) Featherstone, D. E.; Rushton, E.; Broadie, K. *Nat. Neurosci.* **2002**, *5*, 141−146.
(44) Periz, G.; Fortini, M. E. *Embo. J.* **1999**, *18*, 5983−5993.
(45) DeSouza, L.; Diehl, G.; Rodrigues, M. J.; Guo, J. Z.; Romaschin, A. D.; Colgan, T. J.; Siu, K. W. M. *J. Proteome Res.* **2005**, *4*, 377−386.
(46) Smith-Beckerman, D. M.; Fung, K. W.; Williams, K. E.; Auersperg, N.; Godwin, A. K.; Burlingame, A. L. *Mol. Cell. Proteomics* **2005**, *4*, 156−168.
(47) Kim, S. W.; Hwang, H. J.; Cho, E. J.; Oh, J. Y.; Baek, Y. M.; Choi, J. W.; Yun, J. W. *J. Proteome Res.* **2006**, *5*, 2966−2976.
(48) McNaught, K. S. P.; Olanow, C. W.; Halliwell, B.; Isacson, O.; Jenner, P. *Nat. Rev. Neurosci.* **2001**, *2*, 589−594.
(49) Cooper, A. A.; Gitler, A. D.; Cashikar, A.; Haynes, C. M.; Hill, K. J.; Bhullar, B.; Liu, K. N.; Xu, K. X.; Strathearn, K. E.; Liu, F.; Cao, S. S.; Caldwell, K. A.; Caldwell, G. A.; Marsischky, G.; Kolodner, R. D.; LaBaer, J.; Rochet, J. C.; Bonini, N. M.; Lindquist, S. *Science* **2006**, *313*, 324−328.
(50) Hirayama, K.; Kapatos, G. *J. Neurochem.* **1998**, *70*, 164−170.
(51) Nagatsu, T.; Levitt, M.; Udenfriend, S. *J. Biol. Chem.* **1964**, *239*, 2910−2917.
(52) Lovenberg, W.; Levine, R. A.; Robinson, D. S.; Ebert, M.; Williams, A. C.; Calne, D. B. *Science* **1979**, *204*, 624−626.

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