

Proteomic identification of novel proteins associated with Lewy bodies

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1. ABSTRACT

The manifestation of Lewy bodies (LB) in the brain is a hallmark of Parkinson's disease. Here, we present a comprehensive analysis of protein elements in Lewy bodies by comparative mass spectrometry. Cortical LB inclusions were enriched by sucrose gradient centrifugation from postmortem brains, and a negative control sample was prepared from specimen without LB pathology. Whereas ~550 proteins were identified in the LB-enriched sample by mass spectrometry, quantitative comparison with the control sample revealed that ~40 proteins were co-enriched with α -synuclein, the major component in Lewy bodies. As expected, the list of proteins included previously reported constituents, such as those involved in protein folding, membrane trafficking and oxidative stress. More interestingly, we discovered in the LB-enriched sample several kinases (MAPKK1/MEK1, protein kinase C, and doublecortin-like kinase), a novel deubiquitinating enzyme (otubain 1), and numerous ubiquitin ligases (KPC and SCF). The proteomic studies provide enzyme candidates to investigate the regulation of α -synuclein and/or other LB proteins, which may contribute to the formation of Lewy bodies and the toxicity of α -synuclein in the related neurodegenerative disorders.

2. INTRODUCTION

Parkinson's disease (PD) is characterized by selective degeneration of dopaminergic neurons of the substantia nigra in the brain, and by neuronal inclusions called Lewy bodies - proteinaceous aggregates primarily composed of α -synuclein (1, 2). Lewy bodies have also been found in cases of dementia with Lewy bodies (DLB), the Lewy body variant of Alzheimer's disease, Down's syndrome, and several other neurological diseases, collectively under the common denomination of synucleinopathies (3). α -synuclein in Lewy bodies has been found to undergo a number of posttranslational modifications including phosphorylation (4), ubiquitination (5, 6) and oxidative nitration (7). These modifications are proposed to modulate the equilibrium among multiple forms of α -synuclein (i.e. monomer, soluble oligomers and aggregates) and the assembly of Lewy bodies, which may eventually modulate neuronal toxicity of α -synuclein, although it is arguable whether the toxicity stems from the soluble forms or the aggregates. Identification of proteins associated with Lewy bodies may offer molecular clues to underlying mechanisms.

Traditionally, LB-associated components have been identified by immunohistochemistry. This approach

relies on speculation or a priori knowledge of proteins expected to be present in LB and the availability of specific antibodies to the protein of interest. Recent advances in mass spectrometry-based proteomics approaches provide an opportunity to directly sequence proteins from minute amounts of isolated Lewy bodies, as hundreds of proteins can be identified from a mixture containing a few micrograms of proteins (8, 9). Utilizing this unbiased method, we identified approximately 550 proteins in a LB-enriched fraction, in which potential LB proteins were distinguished from unrelated contaminants by quantitative comparison with a control sample containing no LBs. The statistical significance was evaluated by G-test and probability values, resulting in approximately 40 proteins thought to be enriched in LBs, including a number of novel kinases and ubiquitin enzymes. Their potential involvement in the formation of LBs and the pathogenesis of neurodegeneration are discussed.

3. MATERIALS AND METHODS

3.1. Enrichment of Lewy bodies from postmortem brains

Lewy bodies were purified from brain tissues of patients diagnosed with Lewy body variant of Alzheimer's disease, whereas cases without LB pathology were used as negative controls, according to a previously reported protocol (10). Cortical tissues from both cases were dissected to remove white matter and then homogenized in a lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 M sucrose, 5 mM EDTA, 2 mM DTT and protease inhibitors, 10 ml of the buffer for 1 g of tissue). The lysates were then centrifuged at $100,000 \times g$ for 30 min, and the pellets were resuspended in the lysis buffer and loaded on a sucrose gradient composed of three layers (2.2 M, 1.5 M and 1.2 M). After $200,000 \times g$ centrifugation for 2.5 hours, all interfaces were collected. An aliquot of each collected sample was smeared onto a glass slide and examined by immunostaining with α -synuclein antibodies. In the LB cases, the fractions expected to contain LBs were enriched for round, α -synuclein-immunoreactive structures ranging in diameter from 5 μ m to 30 μ m; these structures were absent in the corresponding fractions from the control cases.

3.2. Protein identification by mass spectrometry

A pair of LB-enriched and control fractions were dissolved in a gel loading buffer containing 2% SDS at 95 °C for 5 min, and separated on an SDS gel followed by Coomassie staining. The whole gel lane of each sample was excised into 10 bands and in-gel digested. The resulting peptides were analyzed by reverse-phase liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) (11). Briefly, the peptide mixtures were dissolved in Buffer A (0.4% acetic acid, 0.005% heptafluorobutyric acid, and 5% acetonitrile), loaded onto a 75- μ m-inner diameter self-packed column (10 cm of 5- μ m C₁₈ resin), eluted with a gradient of Buffer B (0.4% acetic acid, 0.005% heptafluorobutyric acid, and 95% acetonitrile). The eluates were monitored in a MS survey scan followed by three data-dependent MS/MS scans on an LCQ-Deca XP-Plus ion trap mass spectrometer (Thermo Finnigan).

The acquired MS/MS spectra were searched against the human reference database of the National Center for Biotechnology Information using the SEQUEST algorithm (12), with the following parameters: parent ion mass tolerance (3 Da), oxidized methionine (+16 Da) and carboxymethylated cysteine (+57 Da). To remove false positives caused by random matching or spectra of poor quality, we adopted the target-decoy strategy (13, 14) to evaluate false discovery rate. Peptide matches were classified according to trypticity (fully, partial and non-tryptic) as well as precursor ion-charge state (1+, 2+, and 3+), and then filtered by XCorr and Δ Cn values to reduce the false discovery rate to near zero. Basically, the filtering cutoffs were adjusted to discard all peptide matches from the decoy database. According to the rule of parsimony, all accepted proteins sharing peptides were grouped together, in which only the top protein with highest spectral counts was selected to represent the group.

3.3. Quantitation by spectral counts: *p*-value based on G-test and χ^2 distribution

Among the total ~700 proteins identified in the LB and/or control fractions, numerous proteins were only identified in only one sample. A common practice is to assign the undetected proteins with an arbitrarily low number of spectral counts (i.e. 0.5) with the reasoning that those proteins might display the most dramatic changes in abundance.

The G-test (15) is a likelihood ratio test for discrete frequency data, and has been used to assess protein abundance based on spectral counts assuming a binomial distribution (16, 17). According to the null hypothesis, the expected spectral count is set to be equal to the average spectral count of the two samples:

$$\hat{f}_{lb} = \hat{f}_{ct} = \frac{f_{lb} + f_{ct}}{2} \quad (1)$$

where f_{lb} and \hat{f}_{lb} are the detected and expected spectral counts of a given protein in the LB fraction, respectively; similarly, f_{ct} and \hat{f}_{ct} are the corresponding values in the control sample. The G value of each protein is then calculated as

$$G = 2 \left[f_{lb} \cdot \ln \left(\frac{f_{lb}}{\hat{f}_{lb}} \right) + f_{ct} \cdot \ln \left(\frac{f_{ct}}{\hat{f}_{ct}} \right) \right] \quad (2)$$

By combining the above two equations, the following equation is used to derive the G value.

$$G = 2 \left[f_{lb} \cdot \ln \left(\frac{f_{lb}}{\frac{f_{lb} + f_{ct}}{2}} \right) + f_{ct} \cdot \ln \left(\frac{f_{ct}}{\frac{f_{lb} + f_{ct}}{2}} \right) \right] \quad (3)$$

Although theoretical distribution of the G values is complex, the distribution of the values approximately follows the χ^2 distribution with one degree of freedom, and

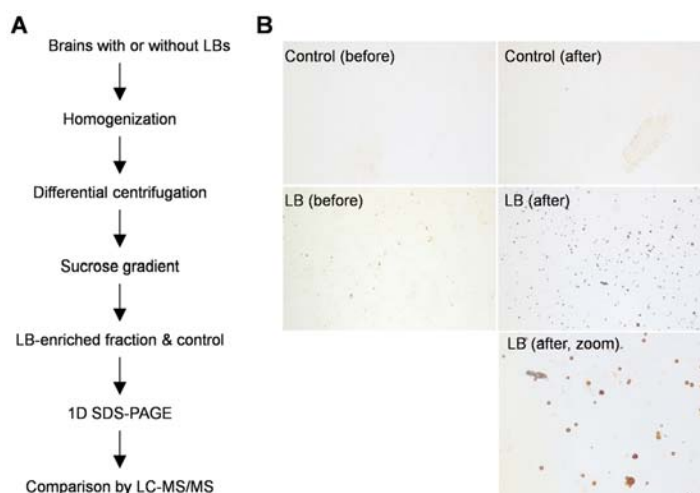


Figure 1. Preparation of the LB-enriched sample. A, flow chart for the experimental design. Brain tissues with or without LB were lysed, differentially centrifuged, and subsequently applied on sucrose gradient. LB-enriched fractions were collected and analyzed by geLC-MS/MS. B, immunostaining results showing the LBs before and after biochemical enrichment.

therefore allows the computation of p -value (15). The p -value is the probability of observing a random variable larger than G in the χ^2 distribution with one degree of freedom.

4. RESULTS

4.1. Biochemical isolation and proteomics analysis of Lewy bodies from postmortem brains

The purification and mass spectrometric analysis of Lewy bodies were achieved by sucrose density centrifugation and LC-MS/MS as illustrated in Figure 1A. Postmortem brain tissues with or without LBs were homogenized in the presence of 1M sucrose beneficial for the separation of myelin-rich layer during centrifugation. The LB-containing pellet was further resolved in a discontinuous sucrose gradient, and the resulting fractions were examined by immunostaining with α -synuclein antibodies to validate LB enrichment (Figure 1B). Judged by the number of LBs detected microscopically, the enrichment factor varied among samples in repeated analyses, and the LB inclusions were spread into different interfaces in the sucrose gradient, suggesting that the LBs are heterogeneous with respect to their densities (data not shown). For large-scale protein identification, we selected one pair of LB and control samples that were collected from the same interface of two cases, respectively. In the LB sample, LB particles were concentrated approximately 7-fold by the sucrose gradient (Figure 1B). By contrast, there were no LB inclusions detected in the control sample before and after the step of sucrose gradient.

To identify novel components in the LBs, the LB fraction was subsequently analyzed by 1D SDS gel electrophoresis followed by liquid chromatography-tandem mass spectrometry. We tested several buffers (containing Triton X-100, sarkosyl, SDS, urea or formic acid) to dissolve the LB pellet and found that the SDS buffer led to the best

yield of proteins. When the LB sample and the control were compared on a stained SDS gel (Figure 2), at least a few protein bands showed clear differences although the overall protein patterns in both samples were similar to each other, suggesting that the majority of proteins in the samples had comparable abundance, and therefore quantitative comparison was essential to identify LB-associated proteins in the noise of common contaminants. To reduce variations in key experimental parameters such as digestion activity, HPLC column performance, and ionization efficiency, we performed the analyses of the two samples in parallel under identical LC-MS/MS conditions. From the LC-MS/MS analysis, approximately 50,00 MS/MS spectra were acquired for each sample and searched against a human protein database. The matched peptides were further filtered rigorously, leading to the identification of ~700 proteins by combining the LB fraction and the control sample (Supplementary Table S1).

4.2. Quantitative comparison reveals novel proteins associated with Lewy bodies

To determine which proteins are significantly more abundant in the LB fraction than in the control, we used a label-free quantitation method based on spectral count (17, 18). The spectral count of a given protein is the number of its matching MS/MS spectra. As the peptide ions are selected for MS/MS sequencing based on ion intensity, the value of spectral count of a protein is capable of reflecting its abundance in the starting materials.

A series of statistical analyses were performed to evaluate the datasets. (i) The effect of spectral counts on the precision of the spectral count ratio was tested by plotting the sum of the spectral counts from two samples versus the ratio (Figure 3A). When the sum of spectral counts was too low (i.e. <3 at the \log_2 scale), the quantize effect was significant and the reliability of the ratio was greatly reduced (shown in the shaded area). Thus, these

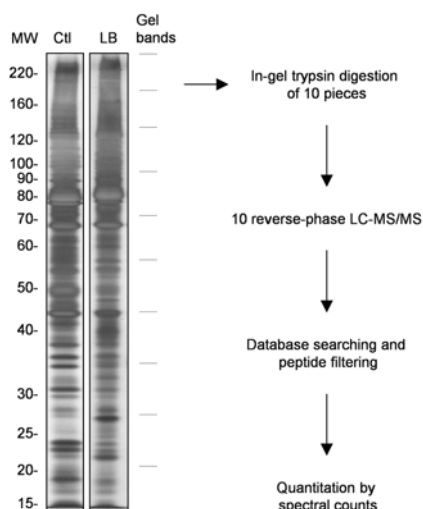


Figure 2. Sample processing and data analysis. Proteins in the LB and control fractions were extracted by a SDS-containing buffer, run in parallel on a SDS-PAGE gel. Each sample lane was cut into 10 pieces that were subjected to trypsin digestion and LC-MS/MS analysis. The gel excision pattern is shown on the right according to the marker. Database searching was performed with SEQUEST followed by peptide filtering. Finally, quantitation was carried out with protein spectral counts.

data points were excluded from further statistical analyses. (ii) The histogram of the dataset was fitted to the Gaussian distribution according to the null hypothesis (Figure 3B). The mean of zero indicated no detectable systematic error in the analysis, consistent with the gel picture that the two samples had very similar protein composites (Figure 2). Moreover, a quantile-quantile plot confirmed the Gaussian distribution of the log ratios of the spectral counts (Figure 3C). (iii) Additional statistical methods were adopted to derive *p*-values for each protein comparison (see Materials and Methods), which represented the statistical significance of the abundance change. The histogram of an expanded fraction of *p*-values from zero to 0.1 is shown in Figure 3D, and from the histogram, the selection of a *p*-value of 0.01 was deemed a reasonable cutoff to detect significant changes.

Based on the *p*-value criteria, a list of ~40 proteins was generated for the LB sample (Table 1). However, the *p*-value cutoff has the potential to exclude interesting proteins with biological relevance, especially for large-scale proteomics that are normally not repeated due to the significant effort required, thus resulting in insufficient data points for statistical evaluation. Therefore, the proteins below the cutoff were further examined manually, and some proteins were included in the table according to the involvement of the protein in neurodegeneration, the clustering of protein families, or the magnitude of protein changes (Table 1). To evaluate their potential roles in the formation of LBs, the proteins were classified into several functional groups, including chaperones, cytoskeleton components, oxidative stress, protein kinases, protein trafficking, ubiquitination, and lysosomal degradation, indicating that the cellular events governing LB formation are complex and diverse.

5. DISCUSSION

This work revealed ~40 proteins co-purified with LB inclusions in the background of several hundreds of proteins, primarily accomplished by quantitative comparison of the LB-enriched sample with the control fraction. As anticipated, many previously reported LB proteins were identified, such as α -synuclein (1, 2), UCH-L1 (19), and ubiquitin-activating enzyme E1 (20). More intriguing were a number of novel regulatory proteins involved in phosphorylation and ubiquitination that emerged from this large-scale proteomic analysis.

5.1. Comparison of different strategies for LB purification

As an alternative to the biochemical approach used here, the LB aggregates in brain specimens may be enriched by laser-capture microdissection (LCM) (21). The LCM strategy is capable of procuring and thereby concentrating specific microscopic structures from brain sections, and has been utilized for proteomic analysis of senile plaques in Alzheimer's disease in our group (22). During the preparation of this manuscript, another group has reported a qualitative analysis of LBs isolated by LCM, identifying a total of ~300 proteins (23). Without a negative control sample, however, it is not clear whether those proteins are present in the LBs themselves or are derived from the surrounding neuropil tissues. Indeed, close examination of our LCM preparations (data not shown) has suggested that it is difficult to capture LBs without introducing significant contamination, as the surrounding tissues are always co-isolated with the target structures during the procedure. This becomes a major concern when the targeted structure is small and near the size limit of the LCM (~5 μ m in diameter) (24). Further purification from LCM samples is, unfortunately, not practical due to the minute amount of tissues captured (~2 ng proteins from a plaque region of 60 μ m in diameter and 10 μ m in thickness) (25). Alternatively, LBs can be isolated by sucrose density gradient from brain homogenates (10). Using this method, we were able to enrich LBs by approximately seven-fold. To further distinguish the LB proteins from co-purified contaminants, control tissue without LB pathology was processed in parallel for quantitative comparison. This was an essential component of our analysis because less than 10% of the proteins we identified showed statistically significant enrichment in the LB sample.

5.2. Potential roles of identified kinases and ubiquitin ligases

The main LB protein, α -synuclein, has been found to be modified by a number of posttranslational events, as exemplified by phosphorylation (4, 26) and ubiquitination (5). In LBs, α -synuclein has been shown to be highly phosphorylated at Ser 129, and this phosphorylation facilitates the assembly of fibril formation *in vitro*. Mutation of this Ser residue causes the suppression of dopaminergic neuron loss in the fly model of Parkinsonism (27). Moreover, ubiquitination of α -synuclein occurs primarily on the phosphorylated form of α -synuclein, suggesting that the addition of ubiquitin might be dependent on prior phosphorylation of α -synuclein (26). In this study, we detected MAPKK1/MEK1, protein kinase

Novel proteins associated with Lewy bodies

Table 1. The list of proteins enriched in the Lewy body sample

| Protein names | GenBank TM accession number | Spectra counts (LB) | Spectra counts (control) | log ₂ (LB/control) ¹ | p value ² |
|--|--|---------------------|--------------------------|--|----------------------|
| Chaperones | | | | | |
| Heat shock protein 90kda | NP_031381.2 | 59 | 30 | 1.0 | 1.9E-03 |
| Cytoskeleton components | | | | | |
| Spectrin, alpha | NP_003118.1 | 100 | 31 | 1.7 | 6.2E-10 |
| Spectrin, beta | NP_003119.1 | 84 | 8 | 3.4 | 0.0E+00 |
| Plectin 1 | NP_958786.1 | 44 | 5 | 3.1 | 2.4E-09 |
| Gelsolin | NP_000168.1 | 9 | 1 | 3.2 | 6.7E-03 |
| WD repeat-containing protein 1 | NP_059830.1 | 9 | 1 | 3.2 | 6.7E-03 |
| Oxidative stress | | | | | |
| Carbonyl reductase 1 | NP_001748.1 | 21 | 2 | 3.4 | 1.9E-05 |
| Peroxiredoxin 5 ³ | NP_036226.1 | 4 | 0 | 3.0 | 7.8E-02 |
| Protein disulfide isomerase-associated 3 | NP_005304.3 | 8 | 0 | 4.0 | 4.7E-03 |
| Kinases | | | | | |
| Mek1, mitogen-activated protein kinase kinase 1 | NP_002746.1 | 12 | 0 | 4.6 | 2.9E-04 |
| Protein kinase c, beta 1 | NP_002729.2 | 9 | 1 | 3.2 | 6.7E-03 |
| Doublecortin-like kinase ³ | NP_004725.1 | 4 | 0 | 3.0 | 7.8E-02 |
| Protein trafficking | | | | | |
| Adaptor-related protein complex 2, alpha 1 | NP_570603.2 | 10 | 0 | 4.3 | 1.2E-03 |
| Adaptor-related protein complex 2, beta 1 | NP_001273.1 | 8 | 0 | 4.0 | 4.7E-03 |
| Coatomer protein complex, subunit alpha | NP_004362.1 | 12 | 0 | 4.6 | 2.9E-04 |
| Calcium-dependent secretion activator | NP_003707.2 | 13 | 0 | 4.7 | 1.4E-04 |
| Clathrin heavy chain 1 | NP_004850.1 | 70 | 0 | 7.1 | 0.0E+00 |
| Dynactin 1 | NP_004073.2 | 10 | 0 | 4.3 | 1.2E-03 |
| Dynammin-like protein | NP_036193.1 | 11 | 0 | 4.5 | 5.8E-04 |
| Dynein, heavy polypeptide 1 | NP_001367.2 | 29 | 6 | 2.3 | 5.0E-05 |
| Protein ubiquitination | | | | | |
| Ubiquitin ³ | NP_003324.1 | 11 | 3 | 1.9 | 2.7E-02 |
| Ubiquitin-activating enzyme E1 | NP_695012.1 | 26 | 0 | 5.7 | 1.7E-08 |
| E3 ubiquitin ligase kpc1, ring finger protein 123 ³ | NP_071347.2 | 5 | 0 | 3.3 | 3.9E-02 |
| Cullin 1 ³ | NP_003583.2 | 2 | 0 | 2.0 | 3.3E-01 |
| F-box protein 2 ³ | NP_036300.2 | 4 | 2 | 1.0 | 4.1E-01 |
| Ubiquitin c-terminal hydrolase 11 | NP_004172.2 | 10 | 0 | 4.3 | 1.2E-03 |
| Ubiquitin-specific protease otubain 1 ³ | NP_060140.1 | 6 | 1 | 2.6 | 4.7E-02 |
| Proteasome 26s non-atpase subunit 2 ³ | NP_002799.3 | 2 | 0 | 2.0 | 3.3E-01 |
| Lysosomal degradation | | | | | |
| Vacuolar protein sorting 35 | NP_060676.2 | 18 | 0 | 5.2 | 4.5E-06 |
| Atpase, lysosomal 70 kd, v1 subunit alpha | NP_001681.2 | 18 | 1 | 4.2 | 1.7E-05 |
| Others | | | | | |
| Alpha-synuclein | NP_009292.1 | 9 | 0 | 4.2 | 2.4E-03 |
| Aconitase 2 | NP_001089.1 | 18 | 3 | 2.6 | 5.7E-04 |
| Glucose phosphate isomerase | NP_000166.2 | 22 | 0 | 5.5 | 2.8E-07 |
| Glyceraldehyde-3-phosphate dehydrogenase | NP_002037.2 | 9 | 1 | 3.2 | 6.7E-03 |
| Imp cyclohydrolase | NP_004035.2 | 11 | 0 | 4.5 | 5.8E-04 |
| Calgranulin beta | NP_002956.1 | 9 | 0 | 4.2 | 2.4E-03 |
| Collapsin response mediator protein HCRMP-2 | NP_001377.1 | 20 | 1 | 4.3 | 4.4E-06 |
| Guanine nucleotide binding protein alpha | NP_620073.1 | 21 | 7 | 1.6 | 6.8E-03 |

¹ & ² The values were derived from spectral counts of identified proteins in the Lewy body sample and the control, ³ Proteins may be of interest but have *p* values >0.01.

C and doublecortin-like kinase with higher abundance in the LB fraction than in the control, suggesting that these kinases may participate in the modification of α -synuclein and/or other LB components. Indeed, both MEK1 and α -synuclein are upregulated in a human neuroblastoma cell line treated with MPP⁺, a toxin that can induce Parkinsonism in human, indicating that the MAP kinase pathway is activated under the toxic insult (28).

In addition to ubiquitin-activating enzyme E1, UCH-L1, and proteasome subunits, a number of novel ubiquitin enzymes were found to be co-purified with LBs (Table 1 and supplemental Table S1). Those are KPC, SCF (skip-cullin-F-box), Ube4b, and otubain 1. The KPC complex is a ring-type ubiquitin ligase composed of KPC1 and KPC2; and it functions to promote the degradation of p27 during the cell cycle (29). SCF is a family of cullin-type ligases that also

regulate the timing of the cell cycle. Recently, many ubiquitin ligases that are well studied in cell cycle control have been found to play critical roles in postmitotic neurons (30). Moreover, Ube4b may directly modulate axonal degeneration, as it is overexpressed as a chimeric protein in the C57BL/6JdS mouse that exhibits protection of Wallerian degeneration of injured axons (31). Otubain 1 is a member of a newly discovered ubiquitin protease family that removes ubiquitin from its modified targets (32). In those ligases, two proteins (KPC2 and Ube4b) were sequenced only once in the LB sample but not in the control, which cannot be viewed as reliable evidence, indicated by the high *p* value. Nevertheless, the discovery of these regulatory proteins provides molecular clues for the follow-up studies. Given that the ubiquitin enzymes that modify LB components are still elusive, the pathological involvement of those enzymes in synucleinopathies awaits future investigation.

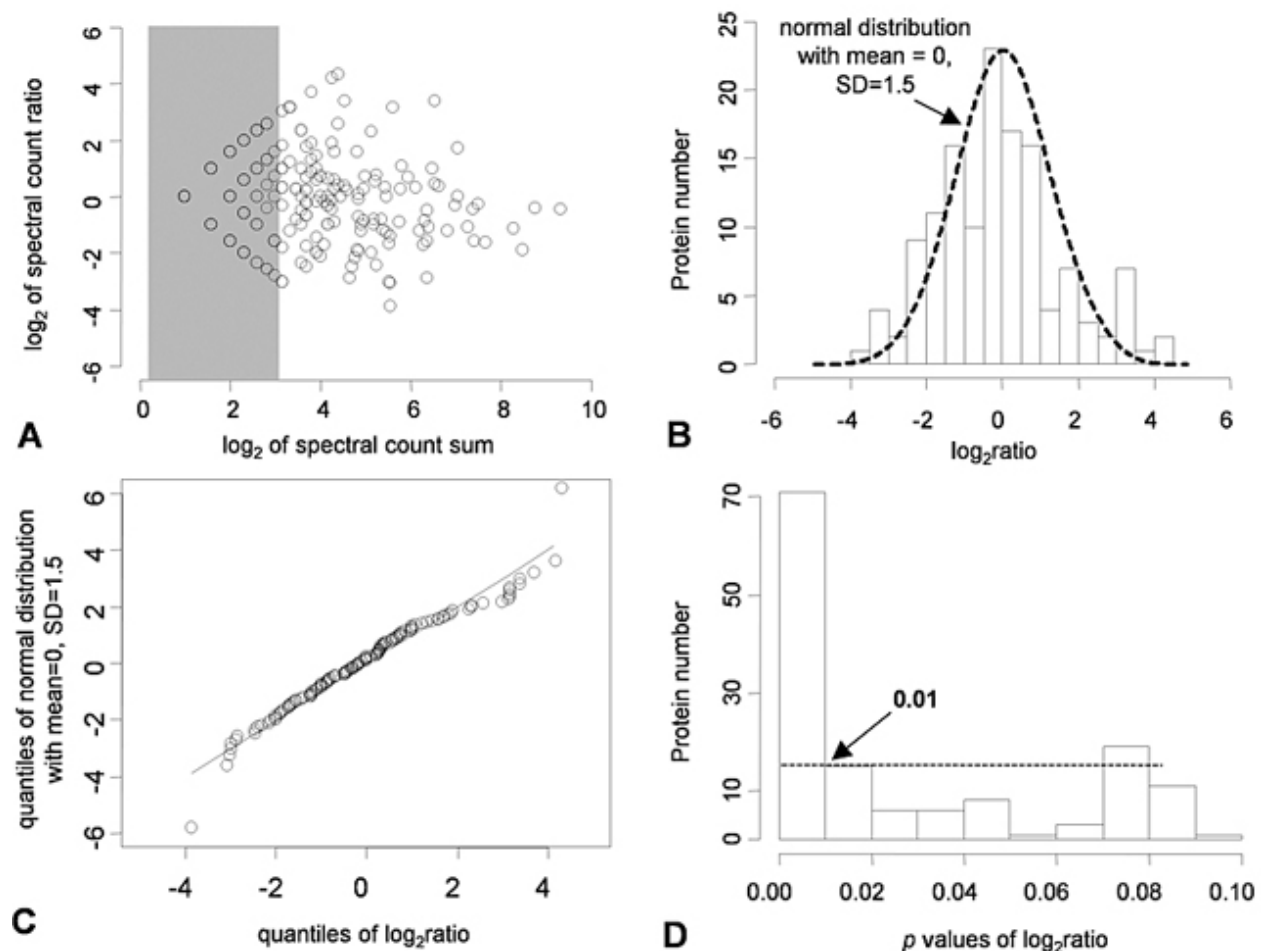


Figure 3. Statistical data analyses. A, the scatter plot of log₂ values of the sum versus the ratio of spectral counts in the LB and control fractions. B, the histogram of log₂ transformed spectral count ratios superimposed with the density plot of a Gaussian distribution. C, quantile-quantile plot (q-q plot) of log₂ of spectral count ratios with a simulated normal distribution. D, the histogram of an expanded fraction of *p*-values from zero to 0.1. The dashed horizontal line shows the level under which the frequency of *p*-values start to flatten, which indicates the closeness to uniform distribution due to randomness.

The study described here is a pilot quantitative proteomics analysis of a LB-enriched fraction. The evolution of mass spectrometry instrumentation and the advancement of quantitative strategies will enable more extensive characterization of LBs in the future. The results of this study provide a list of candidates of interest, which are instructive for further hypothesis-driven research of the molecular mechanisms of neurodegeneration.

6. ACKNOWLEDGEMENTS

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Abbreviations: LB: Lewy body; PD: Parkinson's disease; AD: Alzheimer's disease; DLB: dementia with Lewy bodies; LCM: laser capture microdissection; LC-MS/MS: liquid chromatography coupled with tandem mass spectrometry

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