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Axonal proteome analysis and western blot via APEX2 labeling V.1

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Chuyu Chen[1], Yevgenia Kozorovitskiy[2], Loukia Parisiadou[1]

[1]Northwestern University, Aligning Science Across Parkinson's (ASAP) Collaborative Research Network, Chevy Chase, MD 20815;
[2]Department of Neurobiology, Northwestern University, Evanston, IL, USA

Chuyu Chen
Northwestern University, Aligning Science Across Parkinson's...

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Abstract

We and others previously reported that knock-in mice expressing the pathogenic hyperactive Lrrk2^{G2019S} mutation, exhibit deficits in dopamine release within the striatum. To investigate the underlying molecular mechanisms, we conducted axonal proteome analysis of a subcluster within vulnerable Aldh1a1+ dopamine axons, defined by Annexin 1 (Anxa1+), confirmed subtle proteomic changes across comparisons.

Safety warnings

These protocols need prior approval by the users' Institutional Animal Care and Use Committee (IACUC) or equivalent ethics committee

APEX2 biotinylation in acute brain slices and tissue dissections

1 1) a pure injection of the AAV5-CAG-DIO-APEX2-NES (Addgene plasmid #79907, virus packaged by VectorBiolabs; titer: 3.8×10^{12} GC/ml), delivered bilaterally for APEX2 proximity labeling.

- 2)The homozygous Cre-dependent APEX2 mouse line (DIO-APEX2.NES-P2A-EGFP) was characterized in prior studies (PMID: **36239373**) and was a generous gift from Dr. Kozorovitskiy. These APEX2-EGFP mice were crossed with the Dat^{Cre} line to achieve expression of APEX2 in dopamine neurons of the substantia nigra compacta (SNc). All mice were backcrossed for several generations and maintained on a C57BL/6J background with either the $Lrrk2^{WT}$ or $Lrrk2^{G2019S}$ allele.

2 Mice were anesthetized with ketamine and underwent transcardial perfusion with 10 ml of ice-cold cutting solution consisting of 110 mM choline, 2.5 mM KCl, 1.25 mM monosodium phosphate, 10 mM glucose, 7 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1.3 mM NaH_2PO_4, and 25 mM sodium bicarbonate, all saturated with 95% O_2 and 5% CO_2.

2.1 The brains were quickly extracted and placed in ice-cold cutting solution also saturated with 95% O_2 and 5% CO_2.

3 Coronal slices of 300 µm were prepared using a Leica VT1200S.

4 Slices were transferred to a glass dish containing artificial cerebrospinal fluid (aCSF), which included 2.5 mM KCl, 10 mM glucose, 125.2 mM NaCl, 0.3 mM NaH_2PO_4, 1.3 mM $MgCl_2$, 2.4 mM $CaCl_2$, 26 mM $NaHCO_3$, and 0.3 mM KH_2PO_4, supplemented with 0.5 mM biotin-phenol. The aCSF was continuously saturated with 95% O_2 and 5% CO_2. The slices were allowed to recover at room temperature for 60 minutes.

5 APEX2 labeling was initiated by adding 1 mM H_2O_2 to the aCSF at room temperature for 5 minutes.

6 To quench the labeling, the slices were rapidly transferred to a separate glass dish containing quenching aCSF, which consisted of the aforementioned aCSF supplemented with 10 mM Trolox, 20 mM sodium ascorbate, and 10 mM sodium azide, for 5 minutes.

7 The slices were then rapidly dissected in ice-cold
quenching aCSF. The tissues were flash-frozen on dry ice and stored at -80°C until further western blot
or proteomic experiments.

5. Protein extraction, and streptavidin enrichment of APEX2 samples

8 Frozen APEX2-labeled tissues were homogenized on ice using a glass Dounce homogenizer with 30 strokes of both A and B pestles.

8.1 The lysis was performed in 0.75 ml of ice-cold tissue lysis buffer, which contained 50 mM Tris (pH 8.0), 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, 5 mM Trolox, 10 mM sodium ascorbate, 10 mM sodium azide,
and Halt protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific).

9 After adding 39 µl of 10% SDS to achieve a final concentration of 0.5%, the lysates were rotated for 15 minutes at 4°C.

10 The lysates were then clarified by centrifugation at 21,000 × g for 10 minutes at 4°C.

11 The supernatants were transferred to a new prechilled Eppendorf tube for trichloroacetic acid (TCA)
precipitation (for LC-MS) or stored at −80°C (for western blot).

12 To precipitate proteins from the lysates, an equal volume of ice-cold 55% trichloroacetic acid (TCA) was added. The samples were incubated on ice for 30 minutes, followed by centrifugation at 21,000 × g for
10 minutes at 4°C.

13 The protein pellets were then resuspended in 1 ml of acetone prechilled to -20°C and centrifuged again under the same conditions. This resuspension and centrifugation process was repeated three additional times for a total of four washes

14 After removing any residual acetone, the protein pellets were resuspended in Urea Dissolve Buffer, which contained 8 M urea, 1% sodium dodecyl sulfate (SDS), 100 mM sodium phosphate (pH 8), and 100 mM
ammonium bicarbonate (NH_4HCO_3).

15 The pellets were dissolved by sonication for 1 minute, followed by gentle agitation on an orbital shaker for 1 hour at room temperature.

16 A small aliquot (5%) of the resuspended protein was flash-frozen and stored at -80°C. The samples were
diluted with 0.87 volumes of water to achieve a final concentration of 4 M urea and 0.5% SDS.

17 Streptavidin magnetic beads (Thermo Fisher #88817) were resuspended and washed three times in Urea Detergent Wash Buffer (4 M urea, 0.5% SDS, 100 mM sodium

phosphate, pH 8) for 15 minutes at 4°C.

18 The streptavidin beads were resuspended in ice-cold Urea Detergent Wash Buffer, and 50 µl containing 0.5 mg of beads was added to each sample.

19 Proteins were incubated with the streptavidin beads overnight on a rotary wheel at 4°C for 14-18 hours.

20 The unbound supernatant was discarded, and the beads were resuspended in 1 ml of Urea Detergent Wash Buffer and transferred to a new tube. The beads were washed three times for 10 minutes in 1 ml of Urea Detergent Wash Buffer at room temperature.

21 The beads were resuspended in 1 ml of Urea Wash Buffer (4 M urea, 100 mM sodium phosphate, pH 8) and transferred to a new tube. This washing step was repeated three times for 10 minutes in 1 ml of Urea
Wash Buffer at room temperature.

22 The beads were then resuspended in 200 µl of Urea Wash Buffer and transferred to a new tube. The buffer was removed using a magnetic stand, and the beads were flash-frozen and stored at -80°C for western blot or LC-MS.

Western blot analysis

23 The protein concentration of frozen tissue lysates was determined using the BCA assay (Thermo Scientific) and 10 µg APEX2 labeled tissue lysate were loaded into 4–12% NuPAGE Bis-Tris PAGE (Invitrogen)
as input.

24 Frozen streptavidin beads were resuspended in ~20 µl of 1× SDS sample buffer supplemented with 20 mM DTT and 2 mM biotin. Samples were boiled for 5 min at 95°C to elute biotinylated proteins.

25 Beads were immediately placed immediately onto a magnetic rack and the entire sample was immediately loaded into 4–12% NuPAGE Bis-Tris PAGE (Invitrogen) and transferred to membranes using the iBlot nitrocellulose membrane blotting system (Invitrogen) according to the manufacturer's protocol.

26 The membranes were incubated with primary antibodies specific for streptavidin-HRP (1:1000, Abcam), LRRK2/Dardarin clone N137/6 (1:1000, NeuroMab), Th (1:1000, Millipore), Rab3a (1:1000, Sigma), Rab3c (1:1000, Proteintech) overnight at 4°C

27 Following this, the membranes were incubated with secondary anti-mouse or anti-rabbit antibodies (1:2000, Thermo Scientific) for 1 hour at room temperature.

28 Membranes were incubated with Immobilon ECL Ultra Western HRP Substrate (Millipore) for 3 minutes before image acquisition

29 Chemiluminescent blots were imaged using the iBright imaging system (Thermo Fisher Scientific).

LC-MS (This analysis was performed in Biognosys AG (Schlieren, Switzerland).)

30 Samples were solubilized and digested overnight with sequencing grade trypsin (Promega) in a urea-containing denaturation buffer. Each sample is the eluted proteins from one mouse

31 Beads were collected on a magnetic rack, supernatant was transferred to a new tube and used for the clean-up.

32 Purification for mass spectrometry was carried out using Oasis HLB µElution Plate 30µm plate (WATERS) according to the manufacturer's instructions.

33 Peptides were dried down to complete dryness using a SpeedVac system and dissolved in LC solvent A (1 % acetonitrile in water with 0.1 % formic acid (FA)) containing Biognosys' iRT-peptide mix for retention time calibration.

34 Peptide concentrations in mass spectrometry ready samples were measured using the mBCA assay (Thermo Scientific Pierce).

35 For DIA LC-MS/MS measurements, peptides were injected on an in-house packed reversed phase column on a Thermo Scientific EASY-nLC 1200 nano-liquid chromatography system connected to a Thermo Scientific Orbitrap Exploris 480 mass spectrometer equipped with a Nanospray Flex ion source and a FAIMS Pro ion mobility device (Thermo Scientific).

35.1 LC solvents were A: water with 0.1 % FA; B: 80 % acetonitrile, 0.1 % FA in water. The nonlinear LC gradient was 1 – 50 % solvent B in 171 minutes followed by a column washing step in 90 % B for 7 minutes, and
a final equilibration step of 1 % B for 0.5 column volumes at 64 °C with a flow rate set to a ramp between 450 to 271 nl/min (min 0: 450 nl/min, min 172: 271 nl/min, washing at 400 nl/min).

MS Data analysis (This analysis was performed in Biognosys AG (Schlieren, Switzerland).)

36 The DIA mass spectrometric data were analyzed using Spectronaut software (Biognosys, version 19.0). The false discovery rate on peptide and protein level was set to 1 %.

37 A mouse UniProt .fasta database (Mus musculus, 2024-07-01) was used for the search engine, allowing for 2 missed cleavages, carbamidomethylation of cysteine as fixed modification and up to 5 variable
modifications (N-terminal acetylation, methionine oxidation, deamidation of asparagine or glutamine and phosphorylation at serine or threonine or tyrosine).

38 HRM mass spectrometric data were analyzed using Spectronaut software (Biognosys, version 19.0). The false discovery rate on peptide and protein level was set to 1 %, data was filtered using row-based
extraction.

39 The directDIA+ library generated in this project was used for the analysis. The HRM measurements analyzed with Spectronaut were normalized using global median normalization.

40 Proteins that were not enriched (enriched: q-value < 0.05, log2fc > 0) in $Anxa1^{Cre}$; $Lrrk2^{G2019S}$ vehicle
vs. non-transgenic were removed from the dataset of samples from SNc and striatum, respectively. The filtered dataset was used for further analysis.

41 For testing of differential protein abundance, protein intensities for each protein were analyzed using a two-sample Student's t-test. The following thresholds were applied for candidate identification: p-value < 0.05; absolute average log2 ratio > 0.58 (fold-change > 1.5).

42 Distance in heat maps was calculated using the "manhattan" method, the clustering using "ward.D" for both axes. Principal component analysis was conducted in R using prcomp and a modified ggbiplot
function for plotting. Functional analysis was performed using String-db (string-db.org, version 12.0)

43 Phospho-site summarization was carried out using a PTM localization probability cutoff of > 0.75 and linear model as aggregation type.

44 For testing of differential phospho-site abundance, phosphosite intensities for each protein were analyzed using a two sample Student's t test. The following thresholds were applied for candidate identification: p- value <0.05; absolute average log2 ratio > 0.58 (fold change > 1.5).