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SIM imaging and analysis for active zone release sites in vulnerable dopamine axons V.1

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We use this protocol and it's working

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Abstract

Vulnerable dopamine neurons show higher LRRK2 expression, we wondered whether active zone changes may be prominent in vulnerable Aldh1a1+ as compared to resilient Aldh1a1- dopaminergic axons in Lrrk2^{G2019S} mice. To accomplish this, we used a intersectional/subtractive approach that allows for simultaneous labeling of Aldh1a1+ with EYFP and Aldh1a1- neuronal with mCherry, respectively. Striatal brain sections were then stained with antibodies against bassoon to analyze the number and organization of active zone sites in Aldh1a1+ (EYFP+) or Aldh1a1- (mCherry+) axons from the same mouse (Figure 6B). Due to the high signal of bassoon in the striatum, we used structured illumination microscopy (SIM) for improved resolution, followed by 3D reconstruction analysis.

Safety warnings

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

Stereotactic injections and viruses

1 For the intersectional labeling of Aldh1a1 and Anxa1 populations, adult mice expressing Cre and FlpO were anesthetized with 3% isoflurane, which was maintained at approximately 1.5% during the procedure.

1.1 Pain management included subcutaneous injections of Rimadyl (3 mg/kg) at the beginning of the surgery, followed by a slow-release injection of Buprenex (0.25 mg/kg) after the surgery, and an additional dose of
Rimadyl (3 mg/kg) the following day.

2 The surgery commenced with the exposure of the skull, using bregma as the reference point. A 0.5-1 mm hole was drilled into the skull at the stereotactic coordinates of the substantia nigra pars compacta (SNc) (RC: -3.16 mm, ML: -1.50 mm, DV: -4.00 mm).

3 A 0.5 µl Hamilton Neuros syringe was employed for precise delivery, with the needle left in place for 5 minutes after reaching the target depth.

4 A volume of 0.3 µl of the viral vector was slowly injected into the SNc over a period of 5 minutes, followed by another 5-minute wait to allow for proper viral diffusion within the brain parenchyma.

5 a 1:1 mixture of AAV8 hSyn-Con/Fon-YFP (Addgene #55650, titer: 2.4×10^{13} GC/ml) and AAV8 Ef1a-Coff/Fon-mCherry (Addgene #137134, titer: 2.2×10^{13} GC/ml) delivered unilaterally for imaging experiments

SIM imaging and analysis

6 Striatal sections (30 µm) were incubated in 5% goat serum with 0.2% Triton X-100/PBS for 2 hours. Antigen retrieval for Lrrk2 staining was performed by incubating the sections in antigen unmasking solution
(Vector, H-3300) at 100°C for 30 minutes, followed by cooling to room temperature for another 30 minutes.

7 The sections were then washed with PBS for 5 minutes

8 The sections were probed with the primary antibodies anti-EGFP (1:1000, Invitrogen), anti-mCherry (1:1000, Invitrogen), and anti-bassoon (1:300, Enzo). 48 to 72 hours at 4°C

9 The sections were then washed with PBS for 5 minutes

10 The sections were incubated in the same buffer used for primary antibodies with secondary antibodies—Alexa Fluor™ 488, Alexa Fluor™ 568, and Alexa Fluor™ 647 (1:300, Invitrogen)

11 Multichannel SIM images were obtained with a Nikon N-SIM Structured Illumination super-resolution microscope in the Center for Advanced Microscopy at Nikon Imaging Center at Northwestern University. The imaging was conducted with a 100x objective lens with a numerical aperture (NA) of 1.4.

12 The acquisition settings were set to 10 MHz, and 16 bit depth with electron multiplication (EM) gain and no binning. Exposure times ranged between 200 and 900 ms, with the EM gain multiplier kept below 1500. The conversion gain was maintained at 1x. The laser power was adjusted to keep LUTs within the first quarter of the scale. LUTS were kept similar across comparisons.

13 Single images were processed and analyzed using Nikon Elements software. 3D reconstructions in a single plane used nine images captured with 2D SIM and reconstructed with 3D SIM to increase xy and z resolution were generated by Nikon Element, and the illumination modulation contrast was set automatically by the software.

14 Images were cropped using 3D crop function to remove the edges, and a resolution of 1950×1950 was applied for further analysis with Imaris 10.1.

15 The segmentation of bassoon particles and axons was conducted using the surface creation tool. In the surface wizard, the source channel for axons was selected, corresponding to either Alexa488 (EGFP) or
Alexa568 (mCherry), with object-object statistics activated.

16 To create a clean surface of axon surface, the smoothing surface detail option was enabled (Surface Grain Size = 0.0643µm). Background subtraction was performed using local contrast with a diameter of "Largest Sphere" set to 0.241µm for better separation of the background.

17 The auto-threshold value was applied, with user adjustments made as required. The segments were then filtered by setting the “Number of Voxels Img=1”, above the lower automatic threshold. The settings were saved and manually checked and validated with 10 different images before they were applied to all the images in the dataset.

18 The fluorescence signal outside the surface was removed by using the “Mask channel” function, selecting the corresponding channel for either the Alexa488 (EGFP) or Alexa568 (mCherry), and voxel intensity outside the surface was set to 0.

19 Axonal terminal segments' length was measured with filaments tool. In the tool wizard, the source channel for axonal terminal segments was again selected corresponding to the masked Alexa488 (EGFP) or masked Alexa568 (mCherry), with the seed points threshold set at 6,000.

20 Machine learning pixel classification was performed by training Imaris to discard unwanted segments (not overlapping with surface) while keeping segments that overlapped with the surface until they covered over 99% of visible surface. The settings were again saved and verified with 10 different randomly chosen images before being applied across all images without further user adjustments.

21 The source channel for bassoon particles was selected based on Alex647 with object-object statistics on. To create a clean surface of bassoon particles, the smoothing surface detail was activated (Surface Grain Size = 0.06µm).

22 Background subtraction (local contrast) (Diameter of Largest Sphere =0.15µm) was employed to separate the background.

23 An auto-threshold value was applied with user adjustments as required. Additionally, the Region Growing Estimated Diameter was set to 0.100 µm based on intensity to help split touching objects.

24 The segments were then filtered by “quality” between lower and upper automatic thresholds, followed by a further “volume” filter between 0.003-0.04 μm^3.

25 Bassoon clusters within axonal terminals were filtered based on “overlapped volume ratio to surface”, where the ratio of EGFP/mCherry was set above 1.0. These settings were saved and, as before, checked with 10 different randomly chosen images before being applied to all the images.

26 The number of bassoon objects, their volume, and the length of axon terminal segments were automatically measured and calculated.Density calculations were performed in Excel using the number of bassoon objects relative to the total length of axonal terminal segments, with bassoon object number and axonal terminal segments length determined in Excel.