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Striatal synaptosome imaging and analysis V.1

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We use this protocol and it's working

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Abstract

Dopaminergic neuronal release sites have unique characteristics, as only about 25–30% of axonal varicosities contain active zones that support transmitter release. Rim1/2 proteins are important scaffolding molecules that organize these release sites. Our phosphoproteomic analysis showed that several pathways associated with the organization of presynaptic release sites are altered with changes in LRKK2 kinase activity, and we found a decrease in the interaction of phosphorylated Rab3a with various active zone proteins from mouse brain. Therefore, we next examined whether alterations in the number and/or organization of active zones may form the cellular basis for the observed deficits in dopamine release in Lrrk2^{G2019S} mice.

Safety warnings

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

Striatal synaptosome preparation

1 mouse striata were dissected and rapidly homogenized in 1ml iced-cold homogenizing buffer (4mM HEPES, 320mM Sucrose and Halt protease supplemented with phosphatase inhibitor cocktail (Thermo Fisher Scientific)) using a Teflon homogenizer (15 strokes).

2 Homogenized brain extract was centrifuged at 1000 g for 10 min at 4°C. The supernatant (S1) was taken and centrifuged at 12,500 g for 15 min at 4°C.

3 The supernatant (S2) was removed and the pellet (P2) was re-homogenized in 1 ml homogenizing buffer with 10 strokes in a Teflon homogenizer.

4 After the addition of 1 ml homogenizing buffer, the P2 homogenate was added to the top of a sucrose gradient made of 5ml 1.2M sucrose and 5ml 0.8M sucrose, and was centrifuged at 69,150 g (SW41) for 70 min at 4°C.

5 The synaptic plasma membrane fraction (SPM) in the interphase between two sucrose fractions was collected using a syringe and transferred to clean tubes.

6 Synaptosomes were diluted 5000 times in 1xPBS, spun down at 4000 rmp for 10 min at 4°C on #1.5
poly-d-lysine coated coverslips

7 fixed for 10 minutes in 4% PFA in PBS

Striatal synaptosomes staining and imaging

8 Synaptosomes were blocked in 5% donkey serum in PBS for 1 hour and permeabilized in 0.5% Triton X-100 in PBS for 10 minutes.

9 Incubation with primary antibodies (VAMP2 1:200, R&D; TH 1:500, SYSY; bassoon 1:300, Enzo) was carried out in 0.1% Triton X-100, 1% donkey serum PBS overnight at 4°C.

10 three 5-min washes in 1X PBS

11 synaptosomes were incubated with the secondary antibodies (Donkey anti-Rabbit Alexa Fluor™ 488; Donkey anti-goat Alexa Fluor™ 647,; Cy™3 AffiniPure™ Donkey Anti-Guinea Pig) in 0.1% Triton X-100, 1% donkey serum PBS for 90 min

12 mounted using ProLong™ Diamond Antifade Mountant

13 A single optical section for each area of synaptosomes was acquired with the confocal Nikon A1 laser scanning microscope system using a 100X 1.49NA objective.

Image analysis (ImageJ software)

14 Regarding intensity analysis, background was subtracted from all channels using the "rolling ball" ImageJ plugin with a radius of 33.0 pixels.

14.1 Automatic segmentation (threshold: default; analyze particles: size 0.04-0.40; circularity: 0.30-1.00) was exploited to define ROIs in VAMP2 channel. The intensity of the signal was measured within individual ROIs and only the ROIs positive for VAMP2 were kept.

14.2 a ROI is considered to contain the target protein when the average fluoresce intensity of that protein within the ROI is more than 2 times the average intensity of all pixels in the image and is considered as not containing the target protein when the intensity of the target protein within the ROI is below 2 times the average intensity of all pixels.

14.3 To identify VAMP2 containing synaptosomes that are also positive for TH, the intensity of TH signal was measured within VAMP2 positive ROIs. The intensity of the protein of interest signal (e.g. BSN, RIM) was measured in the double-positive ROIs to keep only the triple-positive ROIs and the mean intensity value of the protein of interest was calculated and plotted.

15 Regarding BSN area, automatic segmentation was used to define the ROIs set both for BSN and TH (threshold: default; analyze particles: size 0.04-0.40 for BSN and size 0,06-0,60 for TH; circularity: 0.30-1.00).

15.1 BSN and TH ROIs sets were used to define the overlapped ROIs exploiting "AND" function in the ROI Manager.

15.2 The intensity of VAMP2 signal was used to define the BSN-TH overlapped ROIs positive for VAMP2.

15.3 The average area value of BSN localized within TH and VAMP2 positive synaptosomes was determined using the "set measurement" and "mesure" ImageJ functions.

16 Regarding BSN %, automatic segmentation was used to define ROIs in TH channel (threshold: default; size 0,06-0,60; circularity: 0.30-1.00).

16.1 TH ROIs positive for VAMP2 were defined measuring the intensity signal of VAMP2.

16.2 BSN intensity signal was then measured within the double positive ROIs to obtain the number of ROIs that were triple positive.

16.3 Automatic segmentation was used to define the total number of ROIs in BSN channel (threshold: default; size 0,04-0,40; circularity: 0.30-1.00).

16.4 the % of BSN positive synaptosome localizing with VAMP2 and TH double positive synaptosomes was calculated.