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Western blot and phostag gels 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, and a dopaminergic neuron-specific knockout of Rim1/2 leads to the structural disorganization of active zones and almost completely abolishes evoked dopamine release in the nigrostriatal pathway.

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

Brain extracts

1 Tissues were extracted and homogenized in 1x cell lysis buffer (Cell Signaling Technologies) supplemented with the Halt protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific) using
pellet pestles for 30 seconds

2 Homogenized brain extract was centrifuged at 13800 g for 10 min at 4°C. Keep the supernatant.

3 The protein concentration was determined using BCA Protein Assays (Thermo Scientific).

4 Equal amounts of protein (30 µg of total tissue lysate, were separated by 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

Synaptosomes

5 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).

6 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.

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

8 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.

9 The synaptic plasma membrane fraction (SPM) in the interphase between two sucrose fractions was collected using a syringe and transferred to clean ultracentrifuge tubes and the samples were centrifuged in a swinging bucket rotor (SW55) at 200,000 g for 30 min at 4°C.

10 The pellet was resuspend SDS lysis buffer (1% SDS, 10mM EDTA, 50mM Tris) supplemented with the Halt protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific)

11 The protein concentration was determined using BCA Protein Assays (Thermo Scientific) and 5µg were separated by 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.

Phos Tag gels

12 SNc tissues extracted and homogenized in EDTA-free Lysis buffer (1% Triton X-100, 1% glycerol, 150 mM NaCl, 25 mM HEPES, 1.5 mM $MgCl_2$) using pellet pestles (30 seconds)The lysis buffer was supplemented with Halt protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific).

13 Homogenized brain extract was centrifuged at 13800 g for 10 min at 4°C. Keep the supernatant.

14 The protein concentration was determined using BCA Protein Assays (Thermo Scientific).

15 A total of 10 µg of lysate were separated by 12.5% SuperSep™ Phos-tag™ (Wako).

16 Gels were washed 3 times with 1x TG transfer buffer (Fisher BioReagents) supplemented with 10 % Methanol and 10 mM EDTA, with each wash lasting 10 minutes

17 The gels were incubated with 1x TG transfer buffer with 10% Methanol for an additional 10 minutes

18 The gels were transferred to Nitrocellulose membranes (Thermo Scientific) using the Mini Gel Tank and Blot Module (ThermoFisher) at 15V for 3 hours

Image acquisition

19 The membranes were incubated in 3% BSA in 1x Tris buffered saline (Sigma) supplemented with 0.1% Tween 20 for 1 hour

20 The membranes were incubated with primary antibodies specific for LRRK2/Dardarin clone
N137/6 (1:1000, NeuroMab), LRRK2/Dardarin clone N241A/34 (1:1000, NeuroMab), Th (1:1000, Millipore), Rab3a (1:1000, Sigma), Rab3c (1:1000, Proteintech), synaptophysin (1:1000, Cell Signaling), Vmat2 (1:1000, R&D Systems), Snap25 (1:1000, Proteintech), DAT

(1:1000, Millipore), pS1292 LRRK2 (1:100, Abcam), pS935 LRRK2 (1:100, Abcam), Rim1 (1:1000, Proteintech), GDI1 (1:1000, Sigma), GDI2 (1:1000, Novus), phospho-Rab12 (1:1000, Abcam), total Rab12 (1:1000, Proteintech), and β-actin (1:3000, Sigma) overnight at 4°C.

21 The membranes were washed 3 times with TBS/Tween-20 for 10 mins

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

23 The membranes were washed 3 times with TBS/Tween-20 for 10 mins

24 The membranes were incubated with Immobilon ECL Ultra Western HRP Substrate (Millipore) for 3 minutes prior to image acquisition.

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