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PROCEDURE TO INDUCE MITOCHONDRIAL DEPOLARISATION AND LYSING OF MOUSE CORTICAL NEURONS

In 1 collection

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ABSTRACT

Mutations in PINK1 cause early-onset Parkinson's disease. PINK1 becomes stabilised and active upon mitochondrial depolarisation. This leads to phosphorylation of ubiquitin and Parkin via Serine 65 residues and a feed forward mechanism whereby PINK1 phosphorylates newly formed polyubiquitin chains, generating phospho-ubiquitin, which further promotes Parkin recruitment and activation. Once activated, Parkin ubiquitylates proteins at the outer face of the outer mitochondrial membrane (OMM) and then initiates a downstream pathway that eventually leads to mitophagy, a mitochondria-specific type of autophagy. Notably, much of previous investigation into PINK1/Parkin activity has been performed in non-neuronal human cancer cells where Parkin and/or PINK1 is over-expressed. Here we report a protocol for generation of mouse embryonic cortical neuronal cultures that produce high cell yields and can be used for studying endogenous PINK1 and Parkin signalling by biochemical methods and proteomics

ATTACHMENTS

[Mouse PINK1 pathway protocol \(166 - 337\).pdf](#)

MATERIALS

Please refer PROCEDURE TO ISOLATE AND CULTURE NEURONS FROM EMBRYONIC MOUSE CORTEX for materials and methods.

Mitochondrial depolarisation

12h

1

12h

Note

◇TIMING ⌚ 01:00:00 - ⌚ 09:00:00 , day of experiment in this section.

To depolarize or uncouple mitochondrial membrane potential in neurons, cultures were treated for ⌚ 03:00:00 to ⌚ 09:00:00 with a combination of 🧪 10 undetermined Antimycin A and 🧪 1 undetermined Oligomycin dissolved in DMSO at 🌡️ 37 °C .

Lysing of mouse neuronal cultures

1h

2

Note

◇TIMING ⌚ 01:00:00 - ⌚ 01:30:00 in this section.

Gently aspirate the medium from wells which neurons are plated.

3

Wash twice by adding 🧪 1 mL of warmed DPBS containing protease inhibitors, phosphatase inhibitors and 🧪 200 undetermined Chloroacetamide.

Note

Note: Chloracetamide is added to the PBS and the lysis Buffer to inactivate deubiquitinase enzymes and enhance the detection of Ubiquitin signal.

4

Place the 6 well multidishes 🌡️ On ice and add 🧪 50 µL of Lysis Buffer. Carefully scrape the cells and collect the lysate in a 🧪 1.5 mL microcentrifuge tube.

5

Sonicate the cell lysate with a probe sonicator 5 seconds 20% amplitude and incubate for

30m

⌚ 00:30:00

🌡️ On ice

6

Centrifuge the cell lysate at **17000 x g** in a refrigerated centrifuge for **00:30:00**.

30m

Supernatants were collected and protein concentration was determined by using the Coomassie Protein Assay.

7

Cell lysates are stored at **-80 °C**.

Immunoblotting of mouse neuronal cultures

7h 13m

8

Note

⌚ TIMING ⌚ 05:00:00 - ⌚ 48:00:00 in this section.

40 µg of cell lysates are diluted 4X into 4X LDS loading buffer supplemented with fresh 10% 2-mercaptoethanol.

9

Samples are boiled for **00:03:00** at **97 °C** or for **00:20:00** at **37 °C**.

23m

Note

Do not boil the sample for an extended time, ubiquitin can refold on membrane and obscure epitope

10

Analyse samples by running on Nu-page Bis-Tris 4-12% gels for a better resolution of ubiquitin chains, at 120 V for **~ 02:00:00**.

2h

11

Transfer gel on PVDF membrane for phospho-ubiquitin and ubiquitin signal and nitrocellulose

1h 30m

membrane for phospho-Parkin and Parkin signal. Transfer in Towbin buffer at 80 V for

01:30:00 On ice or in cold room.

Note

Note: Prepare only 1 membrane per transfer tank -avoid multiple membranes for transfer in same tank as this reduces ubiquitin transfer.

12

Incubate membrane with blocking buffer 5% milk in 0.1% TBS-Tween for 01:00:00 at

Room temperature .

1h

13

Remove blocking buffer, if primary antibodies are in 5% BSA, rinse twice with 0.1% TBS-Tween to remove any traces of milk, add primary antibodies and incubate Overnight at 4 °C .

Note

Note: Prepare phospho-Ubiquitin (1:2000), Ubiquitin (1:1000), GAPDH (1:5000) and Parkin (1:1000) in 5% BSA (TBS-Tween). Prepare phosphoParkin (1:2000) in 5% milk (TBS-Tween). To avoid non-specific signal, it is recommended to preincubate phospho-Parkin antibody with a membrane for 2 days before using it.

1h

14

Remove primary antibody and wash 3 times with 0.1%TBS-Tween for 00:10:00 .

10m

15

Add HRP-conjugate secondary antibodies for 01:00:00 at RT diluted 1:5000 in 1% BSA (0,1% TBS-Tween).

1h

16

Remove secondary antibody and wash 3 times with 0.1%TBS-Tween for 00:10:00 .

10m

17 Develop signal using ECL western Blotting reagents and analysing with Chemidoc.

Note

Note: Depending on signal, film can be best for sensitivity.