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JNK3-Mediated Apoptotic Cell Death in Primary Dopaminergic Neurons

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Abstract

Investigation of mechanisms responsible for dopaminergic neuron death is critical for understanding the pathogenesis of Parkinson's disease, yet this is often quite challenging technically. Here, we describe detailed methods for culturing primary mesencephalic dopaminergic neurons and examining the activation of c-Jun N-terminal protein Kinase (JNK) in these cultures. We utilized immunocytochemistry and computerized analysis to quantify the number of surviving dopaminergic neurons and JNK activation in dopaminergic neurons. TUNEL staining was used to quantify apoptotic cell death. siRNA was used to specifically inhibit JNK3, the neural specific isoform of JNK. Our data implicate the activation of JNK3 in rotenone-induced dopaminergic neuron apoptosis.

Keywords

Dopaminergic neuron; JNK; Primary culture; Immunocytochemistry; TUNEL; siRNA

1. Introduction

Much effort has been put toward understanding mechanisms of cell death in neurodegenerative diseases. In Parkinson's disease, cell death of dopaminergic neurons is responsible for motor deficits; investigation of the underlying cell death mechanisms is important for a better understanding of Parkinson's disease pathogenesis (1). Many studies used model dopaminergic cell lines in the past. Although studies using model cell lines are clearly informative, it is important to confirm major observations in primary cultures because cell lines are immortalized and quite different from postmitotic, differentiated neurons. Primary dopaminergic neurons are most often prepared from embryonic day 14 (E14) embryos from rats or mice. However, the mesencephalon of an E14 mouse brain is very small; one can obtain approximately 150,000–300,000 cells from each embryo, of which only 1–5% are tyrosine hydroxylase (TH)-positive, dopaminergic neurons (2). Thus, this primary neuron culture system is technically challenging and it is important to develop techniques to maximize the use of these cells and identify cell-specific molecular responses to stimuli. The detailed methods for preparing and culturing primary dopaminergic neurons are described here. These methods can be adapted to prepare cultures from a single mouse embryo which can be extremely useful for studies involving transgenic knock-out animals (3, 4).

There are three major forms of cell death: autophagy, necrosis, and apoptosis. Autophagy relies on the cell's own lysosomes to breakdown the cell from inside out. Necrotic cells generally burst, releasing their cellular contents into the surrounding extracellular space triggering an inflammatory response. Apoptosis is an energy-dependant programmed form

of cell death and can be distinguished from necrosis, an uncontrolled form of cell death, in several ways. Characteristics of apoptosis include nuclear condensation, DNA fragmentation, membrane blebbing, and phagocytosis by neighboring cells (5). Unlike necrosis, apoptosis does not cause an immune response. Apoptosis can be identified by assaying nuclear morphology and Terminal dUTP Nick-End Labeling (TUNEL). Both of these methods can be used to identify apoptotic dopaminergic neurons (6, 7) and are described here.

There are several signaling pathways involved in apoptosis including c-Jun N-terminal protein kinase (JNK), a member of the mitogen-activated protein (MAP) kinases. The JNK signaling pathway is activated by phosphorylation and can work together with mitochondrial Bcl-2 family proteins to activate caspases, thus causing apoptosis (8, 9). JNK is of special interest since it is activated in response to toxicants and oxidative stress and is often associated with apoptotic cell death (10–12). Most importantly, JNK activation has been implicated in several Parkinson's disease models (7, 11, 13, 14). JNK signaling activation can be measured in dopaminergic neurons using immunocytochemistry (ICC) and quantification techniques described in this manuscript.

The ten JNK isoforms are encoded by three genes: *Jnk1*, *Jnk2*, and *Jnk3* (15). While *Jnk1* and *Jnk2* are expressed ubiquitously, *Jnk3* is the only neural-specific isoform of JNK. Though several drug inhibitors have been used to inhibit JNK, none is isoform specific. Small interfering RNAs (siRNAs) are 20–25 nucleotides of double-stranded RNA that interfere with specific gene expression by cleaving and degrading messenger RNAs with perfect matching complementary sequence or blocking translation of the target mRNA (16, 17). siRNAs have been widely used to silence the expression of a specific gene. Recently, specific siRNAs for *Jnk1*, *2* or *3* have been used in primary cultured cells to prevent the expression of each isoform of JNK (3, 18). The methods for siRNA transfection into primary cultured dopaminergic neurons and verification by PCR are described in this manuscript.

2. Materials

2.1. Primary E14 Ventral Mesencephalic Culture

1. Embryonic day 14 (E14) Sprague-Dawley rats or mice.
2. Aclar sheets (Electron Microscopy Sciences, Hatfield, PA).
3. Poly-D-lysine (PDL) and laminin aliquoted for single use and stored at -80°C .
4. Dissection tools: large, medium, and small scissors, and fine forceps.
5. Dissection scope.
6. Phosphate-buffered saline (PBS), pH 7.2.
7. Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 $\mu\text{g/mL}$ streptomycin, 25 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], and 30 mM glucose. Supplements are aliquoted and stored at -20°C (see Note 1). Fresh media is made for each culture.

¹All water is filtered through Millipore filtration system (Milli-Q Plus PF, Millipore, Billerica, MA) and is tested to have a resistance of 18.2 M Ω cm. In addition, all water used for tissue culture purposes is autoclaved at 121°C for 40 min.

8. DMEM-F12 supplemented with 1% N2 supplement and 10 µg/mL bovine serum albumin (BSA). Supplements are aliquoted and stored at −20°C. Fresh media is made for each culture.
9. Rotenone is dissolved in dimethylsulfoxide (DMSO) to 1 mM and paraquat is dissolved in water to 400 mM. Both solutions were aliquoted and stored at −20°C. Aliquots are diluted in culture medium to desired concentration before drug treatment.

2.2. ICC and Quantification of JNK Activation in Dopaminergic Neurons

1. Fixing solution: 4% paraformaldehyde/4% sucrose in PBS, pH 7.5. Heat 70 mL water with 0.3 ml of 0.5% Phenol-red to 60°C in fume hood. Add 4 g paraformaldehyde and stir. Add 5N sodium hydroxide drop wise until solution is clear to help dissolve. Add 4 g sucrose and stir until dissolved. Add 10 mL of 10× PBS. Adjust water to about 100 mL. Adjust pH to 7.5 and bring to a final volume of 100 mL. Solution can be aliquoted and stored at −20°C. A 1 L stock of 10× PBS can be prepared by dissolving 80 g NaCl, 2 g KCl, 14.4 g Na₂HPO₄, and 2.4 g KH₂PO₄ in 0.8 L of distilled water and bringing the final volume to 1 L.
2. 10 mM Glycine in 0.1% Triton X-100 in 1× PBS (PBST). For 1 L of 1× PBS buffer dissolve 8.00 g NaCl, 0.20 g KCl, 1.44 g Na₂HPO₄, 0.24 g KH₂PO₄ in 800 mL of water and adjust the pH to 7.4. Add water to bring the final volume to 1 L.
3. Permeabilization solution: 0.5% IPEGAL in 1× PBS.
4. Blocking solution: 2.5% BSA, 5% horse serum, and 5% goat serum in PBST. Serum stored in small aliquots at −20°C.
5. Humidified container: This can consist of a 10-cm petri dish lined with damp filter paper or paper towel.
6. Primary antibodies: mouse monoclonal antibody against tyrosine hydroxylase (TH; 1:500), *gamma*-Aminobutyric acid (GABA; 1:1,000), and rabbit polyclonal antibody against TH (1:5,000), phospho-JNK (1:100), active caspase-3 (0.2 µg/mL).
7. Secondary antibodies: Alexa Fluor 488, 568, 594, or 660 goat anti-rabbit IgG and Alexa Fluor 488, 568, 594, or 660 goat anti-mouse IgG (1:200).
8. Nuclear stain: 2.5 µg/µL Hoechst 33258 in PBST. Solution can be stored at 4°C for several months.
9. Aqua-Poly/Mount.
10. Fluorescent microscope equipped with a digital camera.
11. NIH Image J program (<http://rsb.info.nih.gov/ij/>).

2.3. TUNEL

1. Fixing solution: 4% paraformaldehyde in PBS, pH 7.5. Heat 70 mL water with 0.3 ml of 0.5% Phenol-red to 60°C in fume hood. Add 4 g paraformaldehyde and stir. Add 5N sodium hydroxide drop wise until solution is clear to help dissolve. Add 10 mL of 10× PBS. Adjust water to about 100 mL. Adjust pH to 7.5 and bring to a final volume of 100 mL.
2. 1× PBS.

3. Permeabilization solution: 1× PBS containing 0.1% Triton X-100 and 0.1% sodium citrate.
4. Terminal deoxynucleotidyl Transferase with 5× buffer (TdT, Promega, Madison, WI).
5. Biotin-11-dUTP.
6. Streptavidin-FITC.
7. Fluorescent microscope.

2.4. Transfection of JNK siRNAs into Primary Cultured Neurons

1. siRNA against *Jnk1*, 2, or 3 and scrambled control, non-silencing siRNA (18) (QIAGEN, Valencia, CA).
Jnk1 siRNA sequence is 5' GAAGCUCAGCCGGCCAU UUdTdT 3';
Jnk2 siRNA 5' GCCUUGCGCCACCCGUAUAdTdT 3';
Jnk3 siRNA 5' GCCAGGGACUUGUUGUCAAdTdT 3';
 Scrambled siRNA 5' UUCUCCGAACGUGUCACGUdTdT 3'.
2. TransMessenger™ Transfection Reagent (QIAGEN) with Enhancer R and Buffer EC-R included.

3. Methods

3.1. Primary E14 Ventral Mesencephalic Culture

1. Two days prior to culture, punch out Aclar coverslips 1 cm in diameter (for 24-well plates). Collect coverslips in a 50-mL conical tube and add 70% ethanol (EtOH) to completely cover the coverslips. Vortex several times and soak overnight. Coverslips can be kept in EtOH until ready to use.
2. Wash coverslips five times in autoclaved water. To do this, aspirate EtOH. Add water to completely cover coverslips, vortex, and aspirate water. Repeat five times.
3. Place coverslips in 24-well plate and coat plates with 50 µg/mL PDL and 5 µg/mL laminin in water, 600 µl per well. Ensure that coverslips are completely covered in solution.
4. Incubate plates at 37°C overnight.
5. On day of culture, wash plates twice with autoclaved H₂O, ensuring that coverslips are immersed with each wash. Allow to dry completely in tissue culture hood with lid off.
6. Sanitize all dissection tools in 70% EtOH.
7. Euthanize dam in accordance with Institutional Animal Care and Use.
8. Extract embryos by making a longitudinal cut from the sternum to the tail and two lateral cuts across the lower abdomen using large scissors. Cut through the muscle wall to expose the uterine horn. Remove both sides of the uterine horn and carefully cut away the embryonic sacs from each embryo using medium to small scissors. Decapitate the embryo with small scissors and store on ice for dissection.
9. Using a dissecting microscope, excise ventral mesencephalon (VM) on ice using fine forceps in cold PBS, pH 7.2. To isolate VM, make two vertical cuts, one separating the diencephalon from the midbrain and one at the isthmus separating

the midbrain from the hindbrain. Next make one horizontal cut separating the dorsal mesencephalon from the ventral portion. See diagram in Fig. 1 for details.

10. Mechanically cut tissue blocks with fine forceps into smaller pieces.
11. Wash with DMEM. Allow tissue pieces to settle and draw off excess medium with pipet.
12. Add 5 ml of DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin, 25 mM HEPES, and 30 mM glucose.
13. Dissociate tissue into single cells with 1-mL pipet tip by pipetting up and down 5–7 times.
14. Suspend cells at a density of 10^6 cells/mL; plate 100 µL in one drop per well to form microislands (i.e., 100,000 cells/well). Use care to ensure drop stays on a clear coverslip.
15. Incubate overnight at 37°C, 7% CO₂.
16. Add 400 µL DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin, 25 mM HEPES, and 30 mM glucose next morning (Day in vitro 1, DIV1). Always pre-warm media to 37°C in a water bath before adding to cells. Always add media carefully to the side of the tissue culture well as to not disturb cells.
17. Change 1/2 media at DIV3. Remove 250 µL of media and discard. Add 250 µL of fresh pre-warmed (37°C) media.
18. At DIV5, change 1/2 media to DMEM-F12 supplemented with 1% N2 supplement and 10 µg/mL BSA.
19. Change 1/2 media on DIV7 with DMEM-F12 supplemented with 1% N2 supplement and 10 µg/mL BSA with appropriate drug treatments. In experiments described here, rotenone or paraquat was used to induce apoptosis specific to dopaminergic neurons.

3.2. ICC

1. After drug treatment, aspirate media. Fix cells with 4% paraformaldehyde/4% sucrose in PBS, pH 7.5 at room temperature for 30 min. If cells are not well attached, 8% paraformaldehyde/8% sucrose in PBS, pH 7.5 can be added directly to the media in a 1:1 ratio.
2. Wash cells three times for 5 min each with 10 mM glycine in PBST (0.1% Triton X-100 in 1× PBS). Cells can be kept in last wash and stored at 4°C till staining.
3. To permeabilize cells, add 0.5% IPEGAL in 1× PBS at room temperature for 30 min.
4. Rinse once with PBST.
5. Block with 2.5% BSA, 5% horse serum, 5% goat serum in PBST 1–4 h at room temperature or overnight at 4°C.
6. Remove coverslips from tissue culture dish and lay on para-film in a humidified container. Add 40 µL of primary antibody diluted in blocking solution per coverslip. For double immunostaining, dilute two desired antibodies of different species origin in blocking buffer. For example, Fig. 2c uses mouse monoclonal TH and rabbit polyclonal active caspase-3 primary antibodies (see Note 2). Incubate for 2 h at room temperature or overnight at 4°C.

7. Wash three times for 10 min each with 500 μ L of PBST per coverslip.
8. Add 40 μ L of secondary antibody diluted in blocking solution per coverslip. For double immunostaining, dilute two appropriate secondary antibodies in blocking buffer. For example, Fig. 2c uses goat anti-mouse Alexa 660 and goat anti-rabbit 594 antibodies. Incubate for 1 h at room temperature.
9. Wash three times for 10 min each with 500 μ L of PBST per coverslip.
10. To stain nuclei, incubate with 2.5 μ g/ μ L Hoechst 33258 in PBST for 10 min.
11. Wash for 10 min with 500 μ L of PBST per coverslip.
12. Mount coverslips on slides with one drop per coverslip of Aquapoly mount (see Note 3). Air dry in dark.
13. Immunostaining is monitored using fluorescence microscopy. See Figs. 2–5 for examples.

3.3. Quantification of JNK Activation in Dopaminergic Neurons

1. Fix and double stain the cells with anti-TH and anti-phosphorylated JNK antibody.
2. Capture images of eight random fields from each well using a fluorescent microscope equipped with a digital camera.
3. Open the images using NIH Image J program.
4. Using freehand selection tool, draw the margin of phosphorylated JNK and TH double-stained cells.
5. Measure the mean staining intensity of p-JNK using Analyze tools.
6. Score all transfected TH⁺ neurons in captured images and calculate the average p-JNK level per TH⁺ neuron.
7. Normalize the intensity of p-JNK staining to vehicle control-treated group as relative intensity. See Fig. 4 for an example.

3.4. TUNEL

1. Aspirate media. Fix cells with 4% paraformaldehyde for 30 min.
2. Wash cells two times with 1 $\times$ PBS 10 min each.
3. Permeabilize cells in 1 $\times$ PBS containing 0.1% Triton X-100 and 0.1% sodium citrate at 4°C for 2 min.
4. Wash cells two times with 1 $\times$ PBS 10 min each.
5. Incubate with 20 μ L per coverslip of a TUNEL labeling mixture containing 0.5 U/ μ L of TdT, 1 $\times$ of provided 5 $\times$ buffer and 0.2 nmol biotin-11-dUTP at 37°C for 45 min.
6. Wash two times with 1 $\times$ PBS containing 1% BSA.

²It is important to include controls for background immunofluorescence of primary and secondary antibodies. Therefore, some slides should be set aside and stained with only primary antibody or only secondary antibody.

³Avoid air bubbles when mounting coverslips as this may interfere with imaging. Apply drop of mounting media to slide and ensure no bubbles appear. Then carefully place coverslip on top of mounting media. If needed, very gently apply pressure to coverslip to guide any remaining air bubbles to the edge.

7. Incubate with 50 μ l per coverslip of 3 μ g/mL streptavidin-FITC in 1 $\times$ PBS with 1% BSA at RT for 30 min in the dark.
8. Wash two times with 1 $\times$ PBS.
9. Either mount coverslips onto slides or incubate in blocking solution for 1 h to continue protocol for ICC at step 5.
10. TUNEL staining is monitored using fluorescence microscopy. See Figs. 2 and 3 for examples.

3.5. Transfection of JNK siRNAs into Primary Cultured Neurons

1. Plate E14 Sprague–Dawley rat mesencephalic primary neurons on 24-well plates at a density of 300,000 cells/well in 300 μ L medium.
2. Add 300 μ L of pre-warmed medium on the next day.
3. Incubate cells for 2 more days.
4. Dilute 4 μ L Enhancer R in Buffer EC-R (final volume 100 μ L). Add 2.5 μ g RNA and mix by vortexing for 10 s.
5. Incubate at room temperature (15–25°C) for 5 min.
6. Add 8 μ L TransMessenger Transfection Reagent to the mixture and mix by vortexing for 10 s.
7. Incubate the mixture for 10 min at room temperature.
8. Remove 300 μ L of medium from the cells and add 1 mL of pre-warmed DMEM.
9. Repeat step 8 and remove 1 mL of medium.
10. Add 400 μ L of DMEM to the mixture from step 7 and mix by pipetting in and out twice.
11. Add 300 μ L of mixture to each well.
12. Incubate for 4 h.
13. Remove 300 μ L of medium and add 300 μ l pre-warmed N2 medium.
14. After 24 h, remove 300 μ L of medium and add 300 μ L pre-warmed N2 medium.
15. After another 24 h, treat cells with drugs as desired. See Figs. 5 and 6 for an example.
16. (Optional) If the siRNA has no label, eGFP expression vector could be cotransfected to identify transfected cells (4:1 for siRNA:eGFP).

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Fig. 1. Diagram of E14 rodent brain. **(a)** To dissect out the mesencephalon, make two diagonal cuts shown as *dashed lines*. **(b)** Separate ventral from dorsal mesencephalon by cutting along *dashed line*. *DI* diencephalon, *VM* ventral mesencephalon, *DM* dorsal mesencephalon, *RH* rhombencephalon, *Arrow* isthmus.

Fig. 2.

Rotenone induces dopaminergic neuron apoptosis in E14 ventral mesencephalic primary cultures. Primary ventral mesencephalic cultures were prepared from E14 rats and treated with rotenone for 24 h. **(a)** Representative photomicrographs of cells treated with 5 nM rotenone or vehicle control for 24 h and immunostained for tyrosine hydroxylase (TH), a marker for dopaminergic neurons. **(b)** Rotenone induces a dose-dependant cell loss of TH+ neurons. All TH+ cells on each coverslip were counted. Vehicle control represents 100% survival, which normally has approximately 100 TH+ neurons per coverslip. **(c)** Representative deconvolution photomicrographs of cell treated with 2 nM rotenone or vehicle control for 18 h. Cells were TUNEL labeled followed by immunostaining for active caspase-3 and TH. Data are representative of at least two independent experiments with duplicate determinations. *Error bars* are SEM. *** $p < 0.001$ (ANOVA); compared with vehicle control-treated group. (Reproduced from ref. 6 with permission from the Society for Neuroscience).

Fig. 3.

Rotenone did not induce cell loss of GABAergic neurons or the general population. Primary ventral mesencephalic cultures were prepared from E14 rats, and treated with 0–5 nM rotenone for 24 h. **(a)** Cells were immunostained for GABA, and the number of GABA+ cells in ten randomly selected fields was counted. Vehicle control represents 100% survival. **(b)** Representative photomicrographs of cells treated with rotenone or vehicle control for 24 h and stained for TUNEL, GABA, and Hoechst. Arrows point to GABA+ neurons that are negative for TUNEL. **(c)** Representative photomicrographs of nuclear morphology of the entire population, revealed by Hoechst staining. Cells were treated with rotenone as in **(a)**. Arrows point to condensed or fragmented nuclei, characteristic of apoptosis. **(d)** Rotenone treatment did not affect survival of the general population based on nuclear morphological changes revealed by Hoechst staining as in **(c)**. Data are representative of two (panel A) or three (panel D) independent experiments with duplicate determinations. At least 2,000 cells were counted for each condition. (Reproduced from ref. 6 with permission from the Society for Neuroscience).

Fig. 4. Treatment of paraquat and rotenone induces JNK phosphorylation in dopaminergic neurons. Primary ventral mesencephalic cultures were prepared from E14 mice and treated with paraquat, rotenone, or vehicle control. **(a)** Paraquat (40 μ M, 8 h) and rotenone (5 nM, 8 h) induce JNK phosphorylation in dopaminergic neurons. Images are representative photomicrographs of cells immunostained for tyrosine hydroxylase (TH), a marker for dopaminergic neurons, and for phosphorylated JNK (p-JNK). *Arrows* point to dopaminergic neurons with JNK phosphorylation, indicative of JNK activation. *Scale bar*: 20 μ m. **(b)** Quantification of data in panel A for JNK phosphorylation in TH⁺ dopaminergic neurons. ** $p < 0.01$. (Reproduced from ref. 3 with permission from Lippincott, Williams and Wilkins).

Fig. 5.

JNK3 is the major isoform of JNKs in dopaminergic neurons activated by paraquat or rotenone. **(a)** Transfection of siRNA specific for *Jnk3* reduces *Jnk3* mRNA levels. Primary cultured E14 mesencephalic neurons were transfected with *Jnk3* siRNA or control siRNA. Total mRNA was purified 24 h after transfection and analyzed by semiquantitative reverse transcription-polymerase chain reaction (RT-PCR). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. **(b)** Images of representative cells transfected with *Jnk3* siRNA or control siRNA and immunostained for phosphorylated JNK (p-JNK). **(c, d)** The *Jnk3* siRNA reduced JNK activation induced by paraquat **(c)** or rotenone **(d)** in dopaminergic neurons. Twenty-four hours after transfection of siRNA, E14 mesencephalic cultures were treated with 40 μ M paraquat, 5 nM rotenone, or their respective vehicle controls for another 8 h. Cells were fixed and stained for TH and p-JNK. The intensity of JNK phosphorylation in TH⁺ dopaminergic neurons was quantified. * $p < 0.05$. (Reproduced from ref. 3 with permission from Lippincott, Williams and Wilkins).

Fig. 6. siRNA silencing of JNK3 protects dopaminergic neurons from paraquat and rotenone toxicity. Twenty-four hours after transfection of siRNA, primary cultured E14 mesencephalic neurons were treated with 40 μ M paraquat (**a**), 5 nM rotenone (**b**), or their respective vehicle control for another 24 h. The cells were fixed and stained for TH. The number of TH⁺ neurons was normalized to vehicle control-treated groups. * $p < 0.05$. (Reproduced from ref. 3 with permission from Lippincott, Williams and Wilkins).