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JNK inhibition of VMAT2 contributes to rotenone-induced oxidative stress and dopamine neuron death

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ABSTRACT

Treatment with rotenone, both *in vitro* and *in vivo*, is widely used to model dopamine neuron death in Parkinson's disease upon exposure to environmental neurotoxicants and pesticides. Mechanisms underlying rotenone neurotoxicity are still being defined. Our recent studies suggest that rotenone-induced dopamine neuron death involves microtubule destabilization, which leads to accumulation of cytosolic dopamine and consequently reactive oxygen species (ROS). Furthermore, the c-Jun N-terminal protein kinase (JNK) is required for rotenone-induced dopamine neuron death. Here we report that the neural specific JNK3 isoform of the JNKs, but not JNK1 or JNK2, is responsible for this neuron death in primary cultured dopamine neurons. Treatment with taxol, a microtubule stabilizing agent, attenuates rotenone-induced phosphorylation and presumably activation of JNK. This suggests that JNK is activated by microtubule destabilization upon rotenone exposure. Moreover, rotenone inhibits VMAT2 activity but not VMAT2 protein levels. Significantly, treatment with SP600125, a pharmacological inhibitor of JNKs, attenuates rotenone inhibition of VMAT2. Furthermore, decreased VMAT2 activity following *in vitro* incubation of recombinant JNK3 protein with purified mesencephalic synaptic vesicles suggests that JNK3 can inhibit VMAT2 activity. Together with our previous findings, these results suggest that rotenone induces dopamine neuron death through a series of sequential events including microtubule destabilization, JNK3 activation, VMAT2 inhibition, accumulation of cytosolic dopamine, and generation of ROS. Our data identify JNK3 as a novel regulator of VMAT2 activity.

1. Introduction

Parkinson's disease is a common neurodegenerative disorder characterized by selective and progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) of the midbrain (Olanow and Tatton, 1999). Although molecular mechanisms underlying this dopaminergic neuron death are not well understood, one of the long-held theories is that impairment of mitochondrial complex I is a key factor (Abou-Sleiman et al., 2006). However, our recent studies using gene targeting strategy suggest that inhibition of mitochondrial complex I by itself is not sufficient to induce dopamine neuron death (Choi et al., 2008, 2011b).

Most cases of Parkinson's disease are sporadic. Exposure to environmental toxicants including pesticides may increase the risk of developing Parkinson's disease (Costello et al., 2009; Mouradian 2002; Ramsden et al., 2001). Interestingly, administration of rotenone, a natural pesticide widely used worldwide, induces many key features of Parkinson's disease in rodents, including motor deficits, loss of dopaminergic neurons, and the presence of α-synuclein – containing inclusion bodies (Betarbet et al., 2000; Inden et al., 2007; Pan-Montojo et al., 2010; Sherer et al., 2003b). Rotenone is a well-known inhibitor of mitochondrial complex I. A number of studies have suggested that rotenone induces dopamine neuron death by inhibiting mitochondrial complex I activity (Marella et al., 2008; Richardson et al., 2007; Seo et al., 2006; Sherer et al., 2003a, 2007). However, recent studies using cultured neurons prepared from Ndufs4$^{-/-}$ mouse embryos, which have no detectable complex I activity, questioned this theory (Choi et al., 2008, 2011b). Moreover, alternative mechanisms have been

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suggested underlying rotenone-induced dopaminergic cell death (Choi et al., 2011b; Ren et al., 2005). Nevertheless, rotenone treatment still provides a useful model to study mechanisms of dopaminergic cell death associated with Parkinson's disease, and it is important to elucidate molecular mechanisms underlying rotenone toxicity.

There is a general consensus that rotenone-induced dopamine neuron death is mediated through oxidative stress (Choi et al., 2011b; Sherer et al., 2002, 2003a, 2007; Testa et al., 2005). Activation of the stress-activated JNK, a member of the mitogen-activated protein (MAP) kinases, has also been implicated (Chen et al., 2008; Choi et al., 2010; Kalivendi et al., 2010; Klintworth et al., 2007; Newhouse et al., 2004; Reinhardt et al., 2013). In addition, rotenone causes microtubule destabilization in dopamine neurons, which contributes to rotenone toxicity (Choi et al., 2011b; Ren et al., 2005). We also reported that rotenone-induced microtubule destabilization leads to accumulation of the cytosolic dopamine and ROS (Choi et al., 2011b). However, the signaling pathways leading from microtubule destabilization to accumulation of cytosolic dopamine and oxidative stress have not been identified. In this study, we report that rotenone inhibits the activity of VMAT2, the primary transporter that packages dopamine into presynaptic vesicles in dopamine neurons (Guillot and Miller, 2009). Furthermore, JNK activation occurs downstream from microtubule destabilization, and contributes to VMAT2 inhibition.

2. Materials and methods

2.1. Animals

Generation and characterization of the JNK3$^{-/-}$ mice was described (Yang et al., 1997). For primary culture, the JNK3 heterozygotes (*JNK3*$^{+/-}$) were bred to generate littermates of *JNK3*$^{+/+}$*JNK3*$^{+/-}$, and *JNK3*$^{-/-}$ embryos. PCR genotyping of the embryos was performed as described (Yang et al., 1997) and the results were matched to each single embryo culture at the end of the experiment.

2.2. Primary mesencephalic neuron cultures and drug treatments

Primary cultured dopamine neurons were prepared from E14 mouse or rat embryos as described (Choi et al., 2010, 2013, 2011a,b, 2008), either as single embryo cultures (for JNK3$^{+/+}$ and *JNK3*$^{-/-}$ cultures) or as pooled cultures of C57Bl/6 mouse or Sprague Dawley rat embryos (Charles Rivers, Wilmington, MA). Briefly, we dissected the mesencephalon of each embryo in phosphate-buffered saline (PBS, pH 7.2, Invitrogen, Carlsbad, CA) on ice. The tissue was washed with Dulbecco's modified Eagle medium (DMEM, Sigma, St Louis, MO) and incubated at 37 °C for 10 min. The medium was replaced with culture media consisting of DMEM supplemented with 4 mM glutamine, 10 mM HEPES buffer, 30 mM glucose, 100 IU/ml penicillin, 0.1 mg/ml streptomycin, and 10% heat-inactivated fetal bovine serum (FBS, Invitrogen). The tissue was then dissociated with a narrow pipet tip (Cat # P-3207, ISC BioExpress, Kaysville, UT) and plated ($3–5 \times 10^4$ cells/100 μl) on 9-mm diameter Aclar embedding film (Electron Microscopy Sciences, Fort Washington, PA) pre-coated with 100 μg/ml poly-D-lysine and 4 μg/ml laminin (BD Bioscience, Bedford, MA). The cultures were maintained at 37 °C in a humidified 7% CO_2 atmosphere. After overnight incubation, fresh culture media were added. Thereafter, half of the media was changed at every 48 h.

Rotenone and taxol (Sigma) were dissolved in dimethyl sulfoxide (DMSO) as 10 mM stock solutions. Drugs were diluted in N2 media (Invitrogen) right before the drug treatment, and the final concentration of DMSO did not exceed 0.0001%. All drug treatments were performed in defined serum-free N2 medium. Half of the media was replaced with N2 medium on the day before drug treatment, and then again at the time of drug treatment. Cultures treated with vehicle were used as controls.

2.3. Transient transfection of JNK3 plasmid

Primary neurons were cultured from E14 embryos as described above and co-transfected with GFP and JNK3 cDNA or empty vector as a control on DIV5, using FuGENE6 (Life Technologies, Rockville, MD). Two days after the transfection, transfected cells were identified and quantified by GFP autofluorescence. Total GFP+ cells were counted on each coverslip (9 mm diameter) and presented as cell number/area.

2.4. siRNA

JNK1, 2 or 3 siRNA and non-silencing siRNA as scrambled control were from Qiagen (Heidelberg, Germany). *Jnk1* siRNA sequence is 5′ GAAGCUCAGCCGGCCAUUUdTdT 3′; *Jnk2* siRNA 5′ GCCUUGCGCCACCCGUAUAdTdT 3′; *Jnk3* siRNA 5′ GCCAGGGACUUGUUGUCAAdTdT 3′; Scrambled siRNA 5′ UUCUCCGAACGUGUCACGUdTdT 3′ (QIAGEN, Valencia, CA) (Wang et al., 2007). Primary neurons were cultured from E14 Sprague Dawley rat mesencephalons and plated on the 24-well or 48-well plate. At 80% confluency, cells were transfected with siRNA in combination with 1/4 amount of EGFP expression vector using TransMessenger Transfection Reagent (QIAGEN) according to the manufacturer's protocol. The final concentration of siRNAs was 2.5 μg/ml.

2.5. Immunocytochemistry

Cells were fixed with 4% paraformaldehyde/4% sucrose for 30 min at room temperature and incubated for 1 h in blocking buffer (PBS containing 5% BSA, 5% normal goat serum, and 0.1% Triton X-100). The cells were then incubated with primary antibodies in blocking buffer at 4 °C overnight. Primary antibodies included mouse monoclonal antibody against tyrosine hydroxylase (TH; 1:500; Sigma), rabbit polyclonal antibody against TH (1:50,000; Pel-Freez, Rogers, AR), rabbit polyclonal antibody against phospho-JNK (1:100; Cell Signaling), and rabbit polyclonal antibody against cleaved caspase 3 (1:1000; Cell Signaling). After three washes with PBS, the cells were incubated at room temperature for one hour with appropriate secondary antibodies: Alexa Fluor 488 (or 568) goat anti-rabbit IgG and Alexa Fluor 568 (or 488) goat anti-mouse IgG (1:200; Molecular Probes, Eugene, OR). Stained cells were monitored under a fluorescence microscope (Leica, Heidelberg, Germany).

2.6. Quantitation of TH$^+$ or total neurons

Cells immunostained positive for TH antibody and having neurites twice the length of the soma were scored as TH$^+$ cells. All TH$^+$ cells on a 9-mm diameter Aclar embedding film were scored.

2.7. Preparation of free and complex tubulin

Free or polymerized tubulin was extracted from cells as described (Choi et al., 2011a; Jiang et al., 2006). Briefly, cells were maintained in 24-well plates and washed twice at 37 °C with 1 ml of Buffer A (0.1 M MES (pH 6.75), 1 mM $MgSO_4$, 2 mM EGTA, 0.1 mM EDTA, and 4 M glycerol). The cells were then incubated at 37 °C for 5 min in 300 μl of free tubulin extraction buffer (Buffer A plus 0.1% (v/v) Triton X-100 and protease inhibitors). The extracts were removed and centrifuged at 37 °C for 2 min at 16,000 × g. The supernatant fractions were used as free cytosolic tubulin samples.