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Dopamine elevates intracellular zinc concentration in cultured rat embryonic cortical neurons through the cAMP-nitric oxide signaling cascade

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ABSTRACT

Zinc ion (Zn^{2+}), the second most abundant transition metal after iron in the body, is essential for neuronal activity and also induces toxicity if the concentration is abnormally high. Our previous results show that exposure of cultured cortical neurons to dopamine elevates intracellular Zn^{2+} concentrations ($[Zn^{2+}]_i$) and induces autophagosome formation but the mechanism is not clear. In this study, we characterized the signaling pathway responsible for the dopamine-induced elevation of $[Zn^{2+}]_i$ and the effect of $[Zn^{2+}]_i$ in modulating the autophagy in cultured rat embryonic cortical neurons. N,N,N′,N′-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), a membrane-permeable Zn^{2+} chelator, could rescue the cell death and suppress the autophagosome puncta number induced by dopamine. Dopamine treatment increased the lipidation level of the endogenous microtubule-associated protein 1A/1B-light chain 3 (LC3 II), an autophagosome marker. TPEN added 1 h before, but not after, dopamine treatment suppressed the dopamine-induced elevation of LC3 II level. Inhibitors of the dopamine D1-like receptor, protein kinase A (PKA), and NOS suppressed the dopamine-induced elevation of $[Zn^{2+}]_i$. PKA activators and NO generators directly increased $[Zn^{2+}]_i$ in cultured neurons. Through cell fractionation, proteins with m.w. values between 5 and 10 kD were found to release Zn^{2+} following NO stimulation. In addition, TPEN pretreatment and an inhibitor against PKA could suppress the LC3 II level increased by NO and dopamine, respectively. Therefore, our results demonstrate that dopamine-induced elevation of $[Zn^{2+}]_i$ is mediated by the D1-like receptor-PKA-NO pathway and is important in modulating the cell death and autophagy.

1. Introduction

The zinc ion (Zn^{2+}) is essential for the structure and catalytic activity of many metalloproteins involved in gene expression, DNA synthesis, hormonal storage and neurotransmission (Yamasaki et al., 2007). In the brain, zinc is one of the most abundant transitional metals. At rest, the intracellular Zn^{2+} concentration ($[Zn^{2+}]_i$) is usually in the low nM to pM range; however, the total Zn^{2+} content is high and is primarily bound to metallothioneins (MTs) (Frederickson et al., 1983). Abundant cytosolic MTs and Zn^{2+} transporter proteins (SLC30s and SLC39s) in the plasma membrane and membranes of various organelles are effective in sequestering Zn^{2+} to maintain the $[Zn^{2+}]_i$ in the low nM range (Sekler et al., 2007).

Zn^{2+} is enriched in the synaptic vesicles (several hundred μM) of glutamatergic neurons and is co-released with glutamate into the synaptic cleft upon stimulation (Frederickson et al., 2000). During ischemia or waves of synaptic transmission, Zn^{2+} may reach a high concentration in the extracellular space; however, the high levels of Zn^{2+} binding proteins in the extracellular space sequester the Zn^{2+}. Therefore, the available free Zn^{2+} in the synaptic cleft is not known (Toth, 2011). Prolonged elevation of extracellular Zn^{2+} concentrations may be neurotoxic and responsible for neuronal cell death by damaging mitochondria or interfering with the ubiquitination pathway (Kambe et al., 2015; Sensi et al., 2011). These events may lead to abnormal protein degradation and induce autophagosome formation (Hung et al., 2013). The pathological effects of Zn^{2+} have been associated with Alzheimer's and Parkinson's diseases (Stelmashook et al., 2014). In addition, Zn^{2+} deficiency is a possible factor in autism spectrum disorder (Bjorklund, 2013). Therefore, maintaining an adequate intracellular and extracellular $[Zn^{2+}]$ is crucial for normal brain functions.

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Dopamine is a critical neurotransmitter that activates G-protein coupled receptors to modulate neuron excitability (Beaulieu and Gainetdinov, 2011). There are four main dopaminergic pathways projecting from the midbrain to various regions of brain, including the mesocortical, mesolimbic, nigrostriatal and tuberoinfundibular pathways (Anden et al., 1964; Dahlstroem and Fuxe, 1964). These pathways contribute to various behavioural responses, such as reward, motivation, emotion, drug addiction and movement (Beaulieu and Gainetdinov, 2011). Therefore, the release of dopamine from the dopaminergic neurons affects various cortical neurons.

In a previous study, we showed that dopamine treatment induces $[Zn^{2+}]_i$ elevation and autophagosome formation in cultured cortical neurons (Hung et al., 2013). To further characterize how dopamine treatment increases $[Zn^{2+}]_i$ and modulates the autophagic pathway, we applied various chemicals to illustrate that dopamine activates nitric oxide (NO) synthesis via the D1-like receptors and cAMP-dependent protein kinase (PKA); NO then dissociates Zn^{2+} from MTs. This dopamine-induced elevation of $[Zn^{2+}]_i$ was found to be a pre-requisite for increasing autophagosome formation and the level of the microtubule-associated protein 1A/1B-light chain 3 (LC3) in lipidation form. Therefore, other than the inhibitory effects of dopamine on the electric properties of the post-synaptic neurons, dopamine elevates $[Zn^{2+}]_i$ to modulate the autophagic pathway to support cell survival or enhance neuron death.

2. Material and methods

2.1. Chemicals

KT5720, KT5823, GF109203X, N^{ω}-propyl-L-arginine hydrochloride (L-NPA), dihydrexidine (DHX), sulpiride and CY208-243 were purchased from Tocris Bioscience (Bristol, UK). The cell culture medium, FluoZin-3 AM and 4-amino-5-methylamino-2′,7′-difluorofluorescein (DAF-FM) diacetate, were purchased from Invitrogen (Waltham, MA, USA). 8-Br-cAMP was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA), and the Radio-Immunoprecipitation Assay (RIPA) buffer was purchased from Bioman, Inc. (New Taipei, Taiwan). Vinyl-L-NIO was purchased from Cayman Chemical Company, Inc. (Ann Arbor, MI, USA). Any chemicals not mentioned above were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise indicated.

2.2. Cell culture

We isolated the cortexes from E14.5 rat embryos and used collagenase to dissociate the neurons for culture using a previously published protocol (Wu et al., 2008). The procedure complied with the Animal Welfare Regulations and was approved by the Institutional Animal Care and Use Committee (Permit No. 103-30), National Taiwan University. During the procedure, the rat was anesthetized with a high-dose sodium pentobarbital injection (100 mg/kg) before dissection and was euthanatized with one or two sodium pentobarbital injections (100 mg/kg) after the operation to minimize suffering.

2.3. Western blot analysis

The Western blot assay was performed as described previously (Hung et al., 2013). In brief, we washed and lysed the cultured cortical neurons with PBS (142 mM NaCl, 2 mM KCl, 8 mM Na_2HPO_4, 1.5 mM NaH_2PO_4, pH 7.1 to 7.3) and ice-cold RIPA buffer (150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 7.5, containing Protease Inhibitor Cocktail Set III, which was EDTA-Free), respectively. We centrifuged the lysates at 20,000*g* for 10 min and collected the supernatant for the Western blot assay. The protein concentration was determined using the Bradford protein assay (Bradford, 1976). The proteins were separated with a Tris-glycine acrylamide gel (15%) and then transferred onto nitrocellulose membranes. To label the proteins, we incubated the membranes with a primary antibody against LC3 (NB600-1384, 1:3000 dilution, Novus Biologicals, LLC, St. Charles, MO, USA), or Atg7 (A2856, 1:1500 dilution, Novus Biologicals, LLC) at 4 °C overnight; after washing out the primary antibody, we used a horseradish peroxidase-conjugated antibody (GTX213110-01, 1:7500 dilution, GeneTex, Inc., Irvine, CA, USA) to visualize the targeted proteins and analysed the blot densitometry with ImageJ software (National Institutes of Health).

2.4. $[Zn^{2+}]$ imaging

To measure the $[Zn^{2+}]_i$ in cultured neurons, we incubated the cells in Hank's balanced salt solution (HBSS: 138 mM NaCl, 4 mM $NaHCO_3$, 5.33 mM KCl, 1.26 mM $CaCl_2$, 0.3 Na_2HPO_4, 0.44 mM KH_2PO_4, 0.5 mM $MgCl_2$, 0.41 mM $MgSO_4$, 5.6 mM glucose, pH 7.2) containing 1 μM of FluoZin-3 AM for 1 h at 37 °C. After washing the cells 3 times with HBSS, we placed the cells on the stage of a Nikon inverted microscope (Eclipse Ti-E Inverted Microscope System; Japan) for fluorescence recording. We excited the FluoZin-3 with the light provided from a DG4 system (Sutter Instrument Co., Novato, CA, USA) through an excitation filter centered at 485 nm (Chroma Technology Corp., Rockingham, VT, USA); the emitted light (510–540 nm) was captured through a ×40 objective and directed to a cooled CCD camera (Evolve™ 512 EMCCD Camera; Photometrics, Tucson, AZ, USA). The background fluorescence was subtracted, and the images were acquired every second.

To measure the Zn^{2+} concentration in a buffer containing the cell lysate, we added 1 μM of FluoZin-3 acid in the buffer and monitored the fluorescence intensity using a 96-well plate reader (FlexStation 3 Multi-Mode Microplate Reader; Molecular Devices, Sunnyvale, CA, USA) with the excitation and emission set at 494 and 516 nm, respectively. The cell lysates were collected in a RIPA buffer as described above.

To convert the measured fluorescence to $[Zn^{2+}]$, we used the following equation: $[Zn^{2+}] = K_d \times (F - F_{min}) / (F_{max} - F)$; where F is the fluorescence intensity measured and K_d is the dissociation constant (15 nM in this report). After the experiment, we sequentially added N,N,N′,N′-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) (50 μM) and zinc pyrithione (50 μM) plus $ZnCl_2$ (200 μM) into the recording chamber to obtain the maxima (F_{max}) and minimum (F_{min}) fluorescence signals, respectively.

2.5. Cell fractionation

To collect protein fractions of different m.w. from the neurons, we used a Vivaspin® 500 Centrifugal Concentrator (Vivaproducts, Inc., Littleton, MA, USA) and centrifuged the columns at 15,000*g* for 15 min. The lysates were first spun through a separation column with a m.w. cut off of 10 kD to collect the suspension (referred as >10 kD) and filtrate (referred as <10 kD). The filtrate was then applied to another separation column with a m.w. cut off of 5 kD to collect the fractions with a m.w. below 5 kD (referred as <5 kD) and above 5 kD but below 10 kD (referred as 5–10 kD). The protein concentrations in these fractions were determined using a Bradford protein assay; some fractions may have a concentration below the detection limit. To measure the Zn^{2+} concentration, 10 μL of each fraction was added into the testing buffer; for stimulation, 100 μL of the chemicals dissolved in HBSS were added into each well using the autopipette installed in the reader. The final volume in each well was 200 μL.

2.6. NO imaging

To monitor the NO generated in cultured neurons, we incubated the cells in an HBSS contaning 5 μM DAF-FM diacetate for 45 min at 37 °C. The cells were then washed three times with HBSS and plated on the microscope stage. The setting for fluorescence monitoring was the same as that used for FluoZin-3 measurement.