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# Stress hormone potentiates $Zn^{2+}$-induced neurotoxicity via TRPM7 channel in dopaminergic neuron

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ABSTRACT

Zinc toxicity is one of the key factors responsible for the neuronal injuries associated with various neurological conditions. Zinc accumulation in some cells is accompanied by the increase of blood stress hormone levels, which might indicate a functional connection between stress and zinc toxicity. However, the cellular mechanism for the effect of stress on zinc toxicity is not known. Recently, it was reported that the zinc permeable transient receptor potential melastatin 7 (TRPM7) channel may represent a novel target for neurological disorders where zinc toxicity plays an important role. To investigate the effect of stress hormone on zinc-induced cell death, neuroblastoma SH-SY5Y cells were pretreated with urocortin, a corticotropin releasing factor (CRF)-related peptide. Urocortin potentiated zinc-induced cell death at μM range of extracellular zinc concentrations. It significantly increased TRPM7 channel expression, and zinc influx into cytosol. Moreover, application of TRPM7 channel blockers and RNA interference of TRPM7 channel expression attenuated the zinc-induced cell death in urocortin-pretreated cells, indicating that TRPM7 channel may serve as a zinc influx pathway. These results suggest that TRPM7 channel may play a critical role for zinc toxicity associated with stress.



## 1. Introduction

The ionic form of zinc ($Zn^{2+}$) is highly enriched in many brain areas. It is one of the most crucial trace metals required for the function of broad range of enzymes involved in transcription, protein synthesis, and signal transduction [1]. $Zn^{2+}$ is released upon neuronal activity and is critical to the control of physiological and pathological brain function at some types of glutamatergic synaptic terminal [2]. $Zn^{2+}$ is also a potent toxin that plays an important role in triggering neuronal death and is associated with various neurological conditions [3,4]. In neuronal cells, prolonged exposure to $Zn^{2+}$ induces cell death and short exposures to several hundred μM $Zn^{2+}$ also destroys cultured cortical, cerebellar granular, and hippocampus neurons [5–7]. The alteration of zinc homeostasis in brain is implicated in the etiology of neurodegenerative disorders such as Alzheimer's disease, amyotrophic lateral sclerosis and Parkinson's disease (PD) [8–11].

Corticotrophin releasing factor (CRF) is a key mediator hormone of the stress response [12]. CRF levels are elevated in the cortex, amygdala, and hypothalamus of rats exposed to brain injury [13,14], which may indicate that the endogenous CRF contributes to neuronal cell death. Moreover, chronic exposure to stress hormone reduces the number of hippocampal neurons [15,16]. However, the cellular mechanism for the stress effect on neuronal cell death is unknown.

Transient receptor potential melastatin 7 (TRPM7) is a member of the large TRP channel superfamily expressed in almost every tissue and cell type [17–19]. It is implicated in the regulation of $Mg^{2+}$ and $Ca^{2+}$-homeostasis [17,20,21], and is associated with anoxic neuronal death, and neurodegenerative disease [22]. TRPM7 is the only known zinc permeable channel among the TRP channel family [18,20]. Zinc permeability for TRPM7 channels is four fold higher than that of $Ca^{2+}$ [20]. Recently, TRPM7 channel is reported to play an important role for $Zn^{2+}$-mediated neuronal injury, and may represent a novel target for neurological disorders where $Zn^{2+}$ toxicity plays an important role [23]. From these results, we hypothesized that the elevated stress hormone level may cause neuronal cell death by the increased intracellular zinc concentration via TRPM7 channel.

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## 2. Materials and methods

### 2.1. Cell culture and pharmacological treatments

Human neuroblastoma SH-SY5Y cells were cultured at 37 °C in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum and 1% antibiotics in an atmosphere of 95% air and 5% $CO_2$. Cells were exposed to urocortin for 24 h. After zinc treatment, cells were incubated for 2 h. Antibody against TRPM7 was purchased from Abcam. Waixenicin A was from Huiena Chemical Co. FluoZin-3, AM was from Molecular Probes. All other chemicals were purchased from Sigma–Aldrich.

### 2.2. Cell viability

Cell viability was measured using a conventional 3-(4,5-dimethyl thiazoly)-2,5-diphenyl-tetrazolium bromide (MTT)-based assay. SH-SY5Y cells were seeded at a density of $1 \times 10^4$ onto 96 well plates and incubated with zinc as a described above for 2–4 h at 37 °C. Optical density was measured at 450 nm on an enzyme-linked immunosorbent assay (ELISA) plate reader. Each experimental data point was the average value obtained from six replicates. All values were average from at least three wells from the independent experiments.

### 2.3. RNA interference

A TRPM7-specific interfering shRNA (TRPM7-shRNA) based on human TRPM7 corresponding to coding region 2204–2228 (AAC AAT GGC TAA AGC ATT AGT TGC, GenBank accession number NM_017672.3) was obtained from Integrated DNA Technologies. The control shRNA (control-shRNA) was a non-specific RNA construct that did not affect any endogenous genes. The shRNAs were transfected into SH-SY5Y cells using RNAiFect transfection reagent (Qiagen). Forty eight hours later, cells were harvested and Western blotting was performed.

### 2.4. Western blotting

Cells were rinsed twice with ice-chilled phosphate buffered saline (PBS), harvested, and lysed in a solution containing 25 mM Tris–HCl (pH 7.4), 5 mM EDTA, 1 mM dithiothreitol and 1% protease/phosphatase inhibitors for 1 h. Lysates were centrifugated for 30 min at 4 °C (10,000 × g). Proteins were separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the resolved proteins were transferred to a nitrocellulose membrane (Potran). The membrane was blocked with 5% skim milk in Tris-buffered saline (TBS) containing 0.1% Tween-20 for 1 h at 22–25 °C, and incubated with the primary antibody at 4 °C overnight, and then incubated with peroxidase labeled secondary antibody against immunoglobulin at room temperature for 1 h. Chemiluminescence western detection system was used (Amersham Pharmacia Biotech).

### 2.5. Electrophysiology

TRPM7 currents were recorded using whole-cell mode of patch clamp technique as described previously [24]. Data acquisition was controlled by pClamp 7.0 software (Axon Instrument). Extracellular $Na^+$-based divalent cation free solution was used, which contained (in mM) 130 Na·methanesulfonate, 5 NaCl, 10 HEPES, 10 EGTA, 0.1 EDTA, 0.5 $CaCl_2$, 5 glucose (pH was adjusted to 7.2 with NaOH). Pipette solution (internal solution) contained (in mM) 135 Cs·methanesulfonate, 10 BAPTA, 10 HEPES, 3.17 $CaCl_2$ (pH was adjusted to 7.4 with CsOH).

### 2.6. Measurement of intracellular reactive oxygen species (ROS)

ROS visualization was performed using 5-(and-6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate (CM-$H_2$DCFDA), a cell membrane permeable fluorescent dye sensitive to hydrogen peroxide-related ROS. SH-SY5Y cells were suspended in PBS and incubated in the dark at 37 °C for 1 h. About $5 \times 10^4$ cells were placed in each well in a black 96-well plate with 10 μM CM-$H_2$DCFDA, which was cleaved into fluorescent dichlorodihydrofluorescein (DCF) in the presence of ROS. DCF-fluorescence was measured by a FLUOstar spectrofluorometer microplate reader at 520 nm. Each experiment was repeated at least three times in triplicates.

### 2.7. Intracellular zinc imaging

The intracellular zinc levels from SH-SY5Y cells were imaged using a zinc sensitive fluorescent dye, FluoZin-3, AM in normal PBS solution for 30 min at 37 °C. FluoZin-3 was excited at a wavelength of 490 nm, and emitted light was filtered with a 500–550 nm band pass filter. Fluorescence intensities ($\Delta F$) were normalized to the resting values. Cells were perfused with (in mM) 140 NaCl, 5.4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 33 glucose, 20 HEPES (pH was adjusted to 7.4 with NaOH).

### 2.8. Data analysis

Analysis of data was performed using Clamfit 7.0. To analysis the inhibition of TRPM7 currents via various TRPM7 channel specific blockers, peak value of the obtained currents from −120 mV was used. Values are expressed as mean ± SE. Statistical significances between the means of groups were analyzed by paired or unpaired Student's t-test.

## 3. Results and discussion

### 3.1. Urocortin potentiates zinc-induced SH-SY5Y cell death

We investigated whether stress hormone affected zinc-induced cell death. To this end, SH-SY5Y cells, a dopaminergic neuronal cell line, were pretreated with 10 nM urocortin for 24 h. Then, cells were exposed to 100 μM $Zn^{2+}$ for 2 h. Microscopic views are shown in Fig. 1A from control cells (con) and urocortin-pretreated cells (URO). When MTT assay was performed to measure the cell viability, most of the control cells was viable even in the presence of 30 μM $Zn^{2+}$ (98.7 ± 1.8%, n = 10) or 100 μM $Zn^{2+}$ (93.9 ± 1.2%, n = 10) (Fig. 1B). Urocortin itself did not affect cell viability. In urocortin-pretreated cells, however, cell viability was significantly decreased to 84.7 ± 3.9% by 30 μM $Zn^{2+}$ (n = 10) or to 73.0 ± 3.3% (n = 10) by 100 μM $Zn^{2+}$. As the extracellular $Zn^{2+}$ concentration was increased further, cell death increased both in control and urocortin-pretreated cells (Fig. 1B). Thus, there was no difference in the cell viability between these cells at 1000 μM $Zn^{2+}$. These results indicated that urocortin potentiated the zinc-induced cell death at μM range of extracellular zinc concentrations.

Since CRF receptor (CRFR) is endogenously expressed in SH-SY5Y cells [25], we pretreated cells with the specific CRFR antagonist, astressin, together with urocortin for 24 h. As shown in Fig. 1C, astressin itself did not affect cell viability (URO + at, n = 5). However, it recovered the cell viability from zinc-induced cell death (91.6 ± 2.3%, n = 5), which indicated that the effect of urocortin was via the activation of CRFR.