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# miR-7-1 POTENTIATED ESTROGEN RECEPTOR AGONISTS FOR FUNCTIONAL NEUROPROTECTION IN VSC4.1 MOTONEURONS

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**Abstract—Protection of motoneurons is an important goal in the treatment of spinal cord injury (SCI). We tested whether neuroprotective microRNAs (miRs) like miR-206, miR-17, miR-21, miR-7-1, and miR-106a could enhance efficacy of estrogen receptor (ER) agonists such as 1,3,5-*tris* (4-hydroxyphenyl)-4-propyl-1H-pyrazole (PPT, ERα agonist), Way200070 (WAY, ERβ agonist), and estrogen (EST, ERα and ERβ agonist) in preventing apoptosis in the calcium ionophore (CI)-insulted ventral spinal cord 4.1 (VSC4.1) motoneurons. We determined that 200 nM CI induced 70% cell death. Treatment with 50 nM PPT, 100 nM WAY, and 150 nM EST induced overexpression of ERα, ERβ, and both receptors, respectively, at mRNA and protein levels. Treatment with ER agonists significantly upregulated miR-206, miR-17, and miR-7-1 in the CI-insulted VSC4.1 motoneurons. Transfection with miR-206, miR-17, or miR-7-1 mimic potentiated WAY or EST to inhibit apoptosis in the CI-insulted VSC4.1 motoneurons. Overexpression of miR-7-1 maximally increased efficacy of WAY and EST for down regulation of pro-apoptotic Bax and upregulation of anti-apoptotic Bcl-2. A search using microRNA database (miRDB) indicated that miR-7-1 could inhibit the expression of L-type $Ca^{2+}$ channel protein alpha 1C (CPα1C). miR-7-1 overexpression and WAY or EST treatment down regulated CPα1C but upregulated p-Akt to trigger cell survival signaling. The same therapeutic strategy increased expression of the $Ca^{2+}$/calmodulin-dependent protein kinase II beta (CaMKIIβ) and the phosphorylated cAMP response element binding protein (p-CREB) so as to promote Bcl-2 transcription. Whole cell membrane potential and mitochondrial membrane potential studies indicated that miR-7-1 highly potentiated EST to preserve functionality in the CI-insulted VSC4.1 motoneurons. In conclusion, our data indicated that miR-7-1 most significantly potentiated efficacy of EST for functional neuroprotection and this therapeutic strategy could be used in the future to attenuate apoptosis of motoneurons in SCI.**


**Key words: apoptosis, CI, CPα1C, ER agonists, miR-7-1, VSC4.1 motoneurons.**

## INTRODUCTION

Current understanding of the crucial mechanisms underlying progressive pathogenesis in spinal cord injury (SCI) is limited and traditional therapeutic agents cannot effectively prevent excessive neuroinflammation and nonstop neurodegeneration, especially in motoneurons. Effective treatment of SCI to protect motoneurons will require a multifaceted therapeutic strategy that can provide functional neuroprotection and promote cell regeneration. To accomplish this, it is imperative to uncover the upstream regulators responsible for coordinating apoptosis and inflammatory responses (Grau et al., 2004, 2006; Hook et al., 2009).

Calcium ionophore (CI) is extensively used to imitate the effects of physiological stimuli related to free $Ca^{2+}$ in the cells (Martina et al., 1994; Wang et al., 1994). CI induces synthesis of nitric oxide through the activation of calmodulin-dependent nitric oxide synthase in cell culture (Knowles and Moncada, 1992). It is also known to uncouple oxidative phosphorylation and inhibit ATPase activity in mitochondria (Reed and Lardy, 1972). CI potentiates response to glutamate receptors and induces apoptosis (Rodriguez-Tarduchy et al., 1990; Caron-Leslie et al., 1994).

MicroRNAs (miRs) may be critical to the pathogenesis of several neurodisorders (Eacker et al., 2009; Saugstad, 2010) including SCI (Yan et al., 2012). miRs are capable of regulating hundreds of genes simultaneously, either post-transcriptionally or through promoter interaction (Li et al., 2006; Breving and Esquela-Kerscher, 2010). It is highly likely that miRs play significant roles in induction of many secondary inflammatory processes following SCI and thus modulation of their expression can be an

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*Abbreviations:* CaMKIIβ, $Ca^{2+}$/calmodulin-dependent protein kinase II beta; CI, calcium ionophore; COX-4, cytochrome c oxidase subunit IV; CPα1C, L-type $Ca^{2+}$ channel protein alpha 1C; CREB, cAMP response element binding protein; DMEM, Dulbecco's Modified Eagle Medium; DMSO, dimethyl sulfoxide; ECL, enhanced chemiluminescence; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; EST, estrogen; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HEPES, hydroxyethyl piperazineethanesulfonic acid; IgG, Immunoglobulin G; miRDB, microRNA database; miRs, microRNAs; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PCR, polymerase chain reaction; p-CREB, phosphorylated cAMP response element binding protein; PI, propidiun iodide; PPT, 1,3,5-*tris* (4-hydroxyphenyl)-4-propyl-1H-pyrazole; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; SBDP, spectrin breakdown product; SCI, spinal cord injury; VSC4.1, ventral spinal cord 4.1; WAY, Way200070.

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attractive therapeutic strategy in SCI. Currently, miRs are considered to be a novel class of therapeutic tools for treatment of SCI, especially because of their small size. There are only scanty reports describing the potential roles of miRs in SCI, most of which have performed expression analysis. Deregulation of miR-206, miR-17, miR-21, miR-7-1, and miR-106a is known to be correlated with different pathophysiological processes like inflammation, oxidative stress, apoptosis, glial scar formation, and axonal degeneration (Kusuda et al., 2011; Liu and Xu, 2011; Yan et al., 2012).

Estrogen (EST) and other EST receptor (ER) agonists provide neuroprotection in traumatic brain injury, SCI, and ischemic injury and also in many neurodegenerative diseases (Sribnick et al., 2003; Nilsen and Brinton, 2004; Gerstner et al., 2009). EST and other ER agonists act as powerful anti-oxidant and also as anti-inflammatory agent (Sribnick et al., 2005). Recent studies indicated that ER agonists could prevent increases in intracellular free $Ca^{2+}$, activation of calpain, and apoptosis (Sur et al., 2003; Sribnick et al., 2004). The protective effects of EST and other ER agonists are mediated via ER alpha (ERα) and ER beta (ERβ). As demonstrated in a number of earlier studies, EST and its structural analogs acted as promising neuroprotectants due to their anti-oxidant properties in a number of model systems (Behl et al., 1997; Green and Simpkins, 2000). Other previous studies demonstrated that EST or ER agonists could protect hippocampal neurons (Wu et al., 2005; Zhao et al., 2005) and primary cortical neurons (Cordey and Pike, 2005) after the activation of mitogen-activated protein kinase (MAPK) signaling cascades.

The ventral spinal cord 4.1 (VSC4.1) motoneuron cell line was generated by fusion of embryonic rat ventral spinal cord neuron with mouse N18TG2 neuroblastoma cell (Crawford et al., 1992; Smith et al., 1994). It has not yet been investigated whether overexpression of neuroprotecive miR can enhance efficacy of any ER agonist or EST in VSC4.1 motoneurons. Our results demonstrated that transfection with miR-7-1 followed by treatment with EST could upregulate cell survival factors and inhibit apoptosis to protect functionality of VSC4.1 motoneurons from supraphysiological CI toxicity. Therefore, miR-7-1-mediated augmentation of efficacy of EST can be a novel therapeutic strategy for functional protection of motoneurons in SCI.

## EXPERIMENTAL PROCEDURES

### Cell culture

The VSC4.1 motoneurons were grown in monolayer to subconfluency in 75-$cm^2$ flasks containing 10 ml of Dulbecco's Modified Eagle Medium (DMEM)/F12 medium with 15 mM HEPES, pyridoxine, and $NaHCO_3$ (Sigma Chemical, St. Louis, MO, USA), supplemented with 2% Sato's components, 1% penicillin, and 1% streptomycin (Invitrogen, Carlsbad, CA, USA), and 10% heat-inactivated fetal bovine serum (FBS). The media and FBS were purchased from BioAbChem (Ladson, SC, USA). CI, 1,3,5-*tris* (4-hydroxyphenyl)-4-propyl-1H-pyrazole (PPT, the ERα agonist), Way200070 (WAY, the ERβ agonist), and EST (the ERα and ERβ agonist) were procured from Sigma Chemical. All anti-miR and miR mimics were purchased from Dharmacon (Chicago, IL, USA). Cells from all treatment groups were used to determine cell viability, levels of mRNA and protein of specific factors regulating apoptosis, and biochemical features of apoptosis.

### Determination of residual cell viability using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

The VSC4.1 motoneurons were seeded into 96-well microculture plates at $1 \times 10^4$ cells/well and allowed to attach overnight. The next day, cells were exposed to different concentrations (25, 50, 100, 200 and 500 nM) of CI in DMEM/F12 medium supplemented with 2% FBS and incubated for 24 h. The medium was replaced with fresh medium containing MTT (0.2 mg/ml) and the plates were incubated for another 3 h. Then, dimethyl sulfoxide (DMSO) was added to dissolve the MTT formazan crystals and absorbance of the color was measured at 570 nm with background subtraction at 630 nm. Final concentration of DMSO in each treatment was maintained at $<0.01\%$ that did not affect cell viability or death. Cell viability was calculated as percentage of viable cells in the total population. Another set of cell viability studies was performed to optimize neuroprotective efficacy of ER agonists (PPT, WAY, and EST) following exposure of VSC4.1 motoneurons to CI insult. First, cells were exposed to 200 nM CI for 24 h and post-treated with different doses (ranging from 0 to 175 nM) of PPT, WAY, and EST. After 24-h incubation with ER agonists, the MTT assay was performed as described above.

### Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) for mRNA

The VSC4.1 motoneurons were grown in 6-well plates for 48 h and then exposed to 200 nM CI and incubated for another 24 h. The old medium was replaced with fresh medium and cells were treated with 50 nM PPT, 100 nM WAY, or 150 nM EST for another 24 h. Total RNA was extracted from all treatment groups using TRIzol reagent as per manufacturer's protocol (Invitrogen). The levels of mRNA expression of ERα, ERβ, Bax, Bcl-2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were examined using semi-quantitative RT-PCR. Primers for ERα, ERβ, Bax, Bcl-2, and GAPDH genes (Table 1) were designed using Oligo software (National Biosciences, Plymouth, MN, USA). Total RNA (300 ng) was used for each set of primers for transcription and amplification using a single-step RT-PCR kit (Invitrogen) on a PCR thermal cycler (Eppendorf, Westbury, NY, USA), as we reported recently (Chakrabarti et al., 2013). The RT-PCR products were resolved on 1.5% agarose gels by electrophoresis, stained with ethidium bromide (1 μg/ml), and visualized on a UV (303 nm) transilluminator, and photographed digitally using the UVDI Compact