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# Rit GTPase regulates a p38 MAPK-dependent neuronal survival pathway

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## HIGHLIGHTS

- Loss of the Rit GTPase sensitizes cells to oxidative-stress.
- A transgenic mouse expressing active Rit ($Rit^{Q79L}$) in neurons was generated.
- Expression of active Rit does not alter CNS development.
- Neuronal expression of $Rit^{Q79L}$ promotes oxidative stress resistance.
- Inhibitor studies demonstrate a key role for p38 MAPK in Rit-mediated neuronal survival.

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## ABSTRACT

Rit, along with Rin and *Drosophila* Ric, comprises the Rit subfamily of Ras-related small GTPases. Although the cellular functions of many Ras family GTPases are well established, the physiological significance of Rit remains poorly understood. Loss of Rit sensitizes multiple mammalian cell lines and mouse embryonic fibroblasts (MEFs) derived from $Rit^{-/-}$ mice to oxidative stress-mediated apoptosis. However, whether Rit-mediated pro-survival signaling extends to other cell types, particularly neurons, is presently unknown. Here, to examine these issues we generated a transgenic mouse overexpressing constitutively active Rit ($Rit^{Q79L}$) exclusively in neurons, under control of the Synapsin I promoter. Active Rit-expressing hippocampal neurons display a dramatic increase in oxidative stress resistance. Moreover, pharmacological inhibitor studies demonstrate that p38 MAPK, rather than a MEK/ERK signaling cascade, is required for Rit-mediated protection. Together, the present studies identify a critical role for the Rit-p38 MAPK signaling cascade in promoting hippocampal neuron survival following oxidative stress.



## 1. Introduction

Ras-related small GTPases function as guanine nucleotide-regulated molecular switches, responding to a wide variety of cellular stimuli to control a number of critical cellular processes, including proliferation and differentiation, cell survival and apoptosis, and cytoskeleton dynamics [7]. To accomplish these varied functions, Ras GTPases regulate an array of intracellular signaling pathways, with mitogen-activated protein kinase (MAPK) cascades among the most intensively studied [5]. There are three major classes of MAPKs, with ERK activation associated with cell growth and survival, and the stress-activated p38 and JNK MAPK pathways shown to play major roles in the cellular adaption to stress, including the induction of cell death [17].

Rit, along with the mammalian Rin and *Drosophila* Ric GTPases, comprise the Rit subfamily of Ras-related small GTP-binding proteins [12,20,26]. Rit is expressed in the majority of adult and embryonic tissues, including a variety of primary neurons and the developing brain [20,26]. Despite its widespread expression within the nervous system, its cellular functions remain incompletely characterized. Expression of constitutively active Rit ($Rit^{Q79L}$) results in the development of highly branched neurites in pheochromocytoma cells [21,23], in a process that relies upon the activation of both ERK and p38 MAPK cascades [19,21,22]. Studies in primary neurons support a role for Rit in the regulation of axonal and dendritic growth. In cultured sympathetic and hippocampal neurons, expression of $Rit^{Q79L}$ has been found to promote axonal but inhibit dendritic growth whereas expression of a dominant-negative Rit mutant inhibits axonal but enhances dendritic growth [13]. Moreover, Rit has recently been found to contribute to IFN-γ-induced dendritic retraction [1].

We have recently demonstrated that Rit serves as a central regulator of stress-activated MAPK regulation and pro-survival

*Abbreviations:* ROS, reactive oxygen species; MAPK, mitogen-activated protein kinase; MEF, mouse embryonic fibroblast; PC6, pheochromocytoma cells; SCG, superior cervical ganglia; DIV, days *in vitro*; SynI, synapsin I; $Syn^{CA}Rit$, Syn-HA-$Rit^{Q79L}$; RT, room temperature; PFA, paraformaldehyde; PBST, PBS plus 0.1% Tween-20.

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signaling [4,22]. Rit silencing renders both cultured cells and primary embryonic fibroblasts susceptible to apoptosis and results in a disruption of stress-dependent p38 and Akt signaling [4,22]. However, while Rit loss sensitizes cells to stress-dependent cell death, it has not been established whether Rit activation promotes *in vivo* protective signaling.

To further explore the physiological function of Rit in the central nervous system, we generated a transgenic mouse line overexpressing constitutively active $Rit^{Q79L}$ under the control of the neuron-specific Synapsin I promoter (termed $Syn^{CA}Rit$ mice). We demonstrate that neuronal overexpression of $Rit^{Q79L}$ does not result in any discernible morphological or anatomical abnormalities within the central nervous system. However, cultured hippocampal neurons from $Syn^{CA}Rit$ mice display significantly enhanced survival compared to wild-type neurons following $H_2O_2$ exposure, supporting a pro-survival function for Rit following oxidative stress. Moreover, pharmacological inhibitor studies demonstrate that p38 MAPK, but not MEK/ERK signaling, is required for $Rit^{Q79L}$-mediated survival. Taken together, these data strengthen the notion that Rit-p38 signaling plays a critical role in promoting survival in neurons adapting to oxidative stress.

## 2. Experimental procedures

### 2.1. Reagents

Hydrogen peroxide (Sigma) and kinase specific inhibitors SB203580 (Tocris), and PD98059 (CalBiochem), mouse monoclonal anti-HA (12CA5) (Applied Science), mouse monoclonal anti-MAP2 (AP20) (Sigma), and fluorescein-conjugated anti-mouse IgG (H + L) (vector, Burlingame, CA) were purchased.

### 2.2. Generation of $Syn^{CA}Rit$ transgenic mice

To generate a transgenic mouse line the rat Synapsin I promoter was fused to 3× HA-human $Rit^{Q79L}$ followed by a Simian Virus (SV40) RNA splice donor/splice acceptor sequence and an SV40 polyadenylation sequence in the pZero-2 vector (Invitrogen). The linear fragment was released by NsiI digestion and used for microinjection at the University of Kentucky Transgenic Facility. Two lines positive for the human Rit transgene were identified and crossed back to C57BL/6 line. No differences were observed between these two lines in pilot studies, and further characterization was only carried out with Line 2.

### 2.3. Mouse genomic DNA extraction and genotyping PCR

Genomic DNA was extracted from tail-snips by incubation in tail lysis buffer (100 mM Tris–HCl (pH 8.8), 5 mM EDTA, 0.2% SDS and 200 mM NaCl) containing 0.4 mg/mL proteinase K (Invitrogen) at 55 °C overnight, followed by incubation with 60 μg/mL RNaseA (Invitrogen) at 37 °C for 1 h. DNA was precipitated, and resuspended in 10 mM Tris–HCl (pH 8.0) prior to genotyping. Primers used for PCR analysis were as follows: forward primer (Human Rit 36-51) 5′-TAGCAGCCCCGCTGGG-3′; reverse primer (Human Rit 471-453) 5′-GCTGAATTCTCGGGCCAAG-3′.

### 2.4. Morphological analysis of brain structure

Adult $Syn^{CA}Rit$ mice and wild-type littermates were anesthetized (130 mg/kg sodium pentobarbital, intraperitoneally) and perfused intracardially with heparinized saline (1000 units/L) followed by 60 mL of 10% neutral-buffered formalin (Sigma). 24 h post-fixation, brains were removed from the skull, postfixed in formalin for 3–4 h, cryprotected in 30% sucrose, and frozen using 2-methylbutane at −40 °C. Serial 40 μm coronal sections were cut using a sliding microtome (HM400, Microm, Walldorf, Germany). The sections were subsequently stained for Nissl substance and examined with an AX80 light microscope (Olympus, Melville, NY). Images were captured using 4× and 10× objectives.

### 2.5. Primary cell cultures from post-natal mouse pups

Primary cultures of hippocampal neurons were prepared from C57BL/6 wild-type or $Syn^{CA}Rit$ transgenic pups within 12 h after birth. Isolated hippocampi were dissociated by treatment for 30 min at 37 °C in a solution of DMEM supplemented with 4 mg/mL papain (Sigma) and 2.5 μg/mL DNase I (Sigma). Cells were then triturated in DMEM plus 10% FBS and plated at $5 \times 10^5$ cells per 35 mm poly-D-lysine (0.1 mg/mL in 5 mM Tris (pH 8.0)) coated tissue culture dishes or equivalent density in other dishes. After 4 h incubation at 37 °C, the medium was replaced with serum-free Neuobasal medium (Invitrogen) supplemented with 0.5 mM Glutamine (Invitrogen), B27 (Invitrogen) and 100 μg/mL streptomycin and 100 U/mL penicillin (Invitrogen) (Complete Neurobasal). Cells were incubated in a humidified incubator at 37 °C and 5% $CO_2$. After 3 days *in vitro* (DIV3), half the medium was removed and replaced with fresh complete neurobasal supplemented with 2 μM AraC to prevent the proliferation of non-neuronal cells. At DIV7, the cells were re-fed with Complete Neurobasal medium. All the experiments were performed at DIV8 unless noted.

Primary cultures of mixed glial cells were prepared from C57BL/6 wild type and $Syn^{CA}Rit$ transgenic pups within 24 h after birth. Cerebral hemispheres were dissociated by treatment for 30 min at 37 °C in 0.25% Trypsin–EDTA (Invitrogen) plus DNase 1 (2.5 μg/mL). Tissue was triturated in DMEM containing 10% FBS, 100 μg/mL streptomycin, 100 U/mL penicillin, and plated in 10 cm dishes at the density of one pup/dish. Cells were then incubated in a humidified incubator at 37 °C and 5% $CO_2$. Every 2–3 days thereafter, cells were re-fed with DMEM supplemented with 10% FBS, 100 μg/mL streptomycin, and 100 U/mL penicillin.

### 2.6. Treatment of isolated primary hippocampal neurons

At DIV8, hippocampal neurons were left untreated or exposed to 60 μM $H_2O_2$ for 2 h at 37 °C. Cells were fixed with fresh 4% paraformaldehyde (PFA, Sigma) for 15 min. For pharmacological inhibitor studies, cultures were incubated for 30 min prior to $H_2O_2$ exposure with either 0.1% DMSO (vehicle), 10 μM PD98059 (MEK inhibitor) suspended in DMSO or 10 μM SB203580 (p38 MAPK inhibitor) suspended in distilled $H_2O$.

### 2.7. Immunofluorescence of primary neuronal cultures

Cultures fixed with 4% PFA were washed three times with PBS and permeabilized for 5 min at room temperature (RT) in 0.1% Triton X-100. Cultures were blocked for 2 h at RT in 5% BSA, incubated overnight at 4 °C with MAP-2 antibody (1:1000) in 5% BSA to label neuronal dendrites and cell bodies, washed three times with PBS, and incubated overnight at 4 °C with Fluorescein-conjugated anti-mouse IgG (H + L) (1:1000, Vector, Burlingame, CA) diluted in 5% BSA. The cultures were rinsed three times with PBS, incubated in 1 μg/mL Hoechst 33258 (Sigma) for 10 min to label nuclei, and washed three times with PBS. Apoptotic neurons were determined by condensed and fragmented nuclei using a Zeiss Axiovert 200M fluorescence microscope and representative images were captured with an Orca ER camera using a 32× objective.

### 2.8. Immunoblotting

Protein concentrations of cell lysates were determined with the Bio-Rad protein assay reagent. Equal amounts of total protein were