RhoA/Rho kinase mediates neuronal death through regulating cPLA$_2$ activation

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This is the author's manuscript of the article published in final edited form as:

Wu, X., Walker, C. L., Lu, Q., Wu, W., Eddelman, D. B., Parish, J. M., & Xu, X.-M. (2016). RhoA/Rho Kinase Mediates Neuronal Death Through Regulating cPLA2 Activation. Molecular Neurobiology, 1–11. https://doi.org/10.1007/s12035-016-0187-6

Abstract

Activation of RhoA/Rho kinase leads to growth cone collapse and neurite retraction. Although RhoA/Rho kinase inhibition has been shown to improve axon regeneration, remyelination and functional recovery, its role in neuronal cell death remains unclear. To determine whether RhoA/Rho kinase played a role in neuronal death after injury, we investigated the relationship between RhoA/Rho kinase and cytosolic phospholipase A_2 ($cPLA_2$), a lipase that mediates inflammation and cell death, using an *in vitro* neuronal death model and an *in vivo* contusive spinal cord injury model performed at the 10^{th} thoracic (T10) vertebral level. We found that co-administration of TNF-α and glutamate induced spinal neuron death, and activation of RhoA, Rho kinase and $cPLA_2$. Inhibition of RhoA, Rho kinase and $cPLA_2$ significantly reduced TNF-α/glutamate-induced cell death by 33%, 52% and 43%, respectively ($p < 0.001$). Inhibition of RhoA and Rho kinase also significantly down-regulated $cPLA_2$ activation by 66% and 60%, respectively ($p < 0.01$). Furthermore, inhibition of RhoA and Rho kinase reduced the release of arachidonic acid, a downstream substrate of $cPLA_2$. The immunofluorescence staining showed that $ROCK_1$ or $ROCK_2$, two isoforms of Rho kinase, was co-localized with $cPLA_2$ in neuronal cytoplasm. Interestingly, co-immunoprecipitation (Co-IP) assay showed that $ROCK_1$ or $ROCK_2$, bonded directly with $cPLA_2$ and phospho-$cPLA_2$. When the Rho kinase inhibitor Y27632 was applied in mice with T10 contusion injury, it significantly decreased $cPLA_2$ activation and expression, and reduced injury-induced apoptosis in the lesion area. Taken together, our results reveal a novel mechanism of RhoA/Rho kinase-mediated neuronal death through regulating $cPLA_2$ activation.

Keywords: RhoA; Rho kinase; cytosolic phospholipase A_2; cell death; neuroprotection; spinal cord injury.

Introduction

A spinal cord injury (SCI) refers to any damage to the spinal cord that is caused by a trauma instead of disease [1]. SCI includes a primary mechanic injury followed by a much more complicated secondary injury process involving inflammation, oxidation and excitotoxicity [1]. Both injuries inevitably cause neuronal and glial cell death at and surrounding the site of injury, which contributes to long-lasting functional deficits [1].

Rho kinase (including $ROCK_1$ and $ROCK_2$) is one of the AGC families of serine-threonine kinases [2]. Rho kinase is a downstream effector of the small GTPase RhoA, which is a major cytoskeleton regulator. The RhoA/Rho Kinase signaling pathway contributes to diverse neuronal functions ranging from cell migration, survival, axonal guidance, to axonal regeneration [3-5]. This pathway has also been shown to play an important role in the pathophysiology of SCI [6,4,7]. For example, upregulation of RhoA was observed after SCI [8]. Rho kinase has also been shown to be involved in cell death and signaling [9]. It not only regulates morphological apoptotic events such as membrane blebbing [10], fragmentation and phagocytosis of apoptotic cells [11-13], but also mediates production of reactive oxygen species and inflammatory cytokines [14]. However, the mechanism by which RhoA/Rho kinase mediates cell death remains unclear.

Phospholipase A_2 (PLA_2) is a family of enzymes that hydrolyze the acyl bond of glycerophospholipids at the sn-2 position to produce free fatty acid and lysophospholipids [15-17]. The metabolic products of PLA_2s play important roles in cell death [18]. Among different PLA_2 isoforms, cytosolic PLA_2 ($cPLA_2$) participates in mediating inflammation and cytotoxicity [17]. A main product of $cPLA_2$ is arachidonic acid (AA)[19], which can give rise to eicosanoids that mediate inflammation, necrosis and apoptosis [19,20]. Our prior research has demonstrated significant increases of PLA_2 activity and $cPLA_2$ expression following SCI [21,22]. $cPLA_2$ is highly expressed in neurons, axons and glial cells and peaked at day 1 in axons, day 3 in glial cells, and day 7 in neurons after SCI [21]. Activation of $cPLA_2$ is sufficient to induce spinal cord

neuronal death *in vitro.* Genetic ablation of $cPLA_2$ reduces cell loss and tissue damage, and improves behavioral recovery after SCI [22].

After contusive spinal cord injury, neurons and glial cells die acutely caused by primary mechanical force and later by multiple injury mechanisms such as inflammation, oxidation and excitotoxicity [23,24]. A previous study showed that apoptosis occurred in neurons in the lesion area between 4 to 24 hours post-injury and reached the peak at 8 hours [25]. The apoptosis in glial cells were found at all stages from 4 hours to 14 days, with a maximum presence in lesion area 24 hours after injury [25]. This study indicates that neuronal and glial cell apoptosis occurred at the lesion site within 1 day after contusive SCI.

In the present study, we sought to determine potential mechanisms of RhoA/Rho kinase-mediated cell death in both *in vitro* and *in vivo* SCI models. We examined the relationship between RhoA/Rho kinase and $cPLA_2$ activation in which spinal cord neuronal death was induced by a combined insult of TNF-α and glutamate, the two major mediators of damage in the progression of secondary SCI. Then we confirmed the RhoA/Rho kinase modulation on $cPLA_2$ activationby injecting Y27631, an inhibitor of Rho kinase, into the lesion site of a T10 contusive SCI produced by the Louisville System Injury Apparatus (LISA) device in adult mice.

Materials and Methods

Reagents

TNF-α (Sigma-Aldrich, T5944), L-glutamic acid (Sigma-Aldrich, G1626), CT04 (Cytoskeleton. Inc., Rho inhibitor I), Y27632 (Enzo Life Sciences, ALX-270-333), arachidonyl trifluoromethyl ketone (ATK) (Cayman Chemical, 62120).

Rats and mice

Sprague-Dawley (SD) E13 pregnant rats and C57BL/6J mice (12 weeks, 18-24g) were purchased from Charles River (USA). Rats and mice were housed at Indiana University School

of Medicine Laboratory Animal Resource Center. All surgical and animal handling procedures were performed as approved under the Guide for the Care and Use of Laboratory Animals (National Research Council) and the Guidelines of the Indiana University School of Medicine Institutional Animal Care and Use Committee.

Cell culture and treatments

Rat primary spinal cord neurons were obtained from SD rat embryonic E15 pups according to a previously established protocol [26]. In brief, E15 rat spinal cords were isolated and placed in L15 medium. Meninges were carefully removed and spinal cords were cut into small pieces, dissociated by incubation in 0.05% trypsin/EDTA 15 min at 37°C and triturated every 5 min. The dissociated cells were washed with and triturated in 10% heat-inactivated fetal bovine serum (FBS), 5% heat-inactivate horse serum (HS), 2mM glutamine-DMEM (all from Gibco, USA) and cultured in 10 cm plate for 30 min at 37°C to eliminate glial cells and fibroblasts. The supernatant which contains neurons was collected and seeded on poly-L-lysine coated 48 well plates and incubated in a humidified atmosphere containing 5% CO_2 at 37°C with 10% FBS + 5% HS + 2 mM glutamine DMEM. After 16 hours, medium was replaced with Neurobasal medium with 2% B27 (Gibco), 1% N2 (Gibco) and 2 mM glutamine. On day 3, 5 μM cytosine-β-D-arabinofuranoside (Sigma-Aldrich) was added for 24 hours to inhibit glia cell proliferation. With this culture protocol, a purity of greater than 94% spinal cord neuronal population was obtained on day 7. All experiments were performed between 7-10 days. Hela cells (ATCC, USA) were cultured in 10% FBS-DMEM (Gibco).

Cell death assessment

A non-radioactive cytotoxicity assay (Promega, USA) was used to measure lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis. Released LDH

can convert tetrazolium salt into a red formazan product. On day 7 in culture, the medium was replaced by fresh medium and the neurons were treated by TNF-α, glutamate or combined TNF-α and glutamate for 24 hours. In the inhibition groups, the inhibitors, CT04, Y27632 and ATK pretreated neurons at 40 min before the application of TNF-α, glutamate or the combination. Fifty microliters of supernatant were removed for LDH assay following the vendor's instruction. The LDH level was measured by a 96 well plate reader (1420 multilabel counter, PerkinElmer) at 490 nm wavelength.

Propidium iodide (PI)/Hoechst staining was also used as a measurement of cell death. Hoechst 33342 (Sigma-Aldrich) 5 μg/ml was added to the cells at 37^{o}C for 15 min to label all cell nuclei, followed by incubation with 5 μg/ml PI (Sigma-Aldrich) at room temperature for 10 min to stain dead cell nuclei. After staining, the medium was removed and cells were washed with 0.01M PBS, followed by 10 min of 4% PFA for cell fixation. The fixed cells were then washed 3 times with PBS and ready for imaging using an Olympus IX71 inverted fluorescence microscope (Olympus America Inc.). Three wells were assigned as one group. The images of three views per well were taken. The blue stained cells and the red stained cells were counted using ImageJ software (NIH) and the percentage of death cell was calculated.

Immunofluorescence staining

Immunofluorescence staining was performed as previously described [27]. Briefly, spinal cord neurons on coverslips on day 7 were fixed by 4%PFA followed by washing with 0.01M PBS, blocking 1 hour with 5% goat serum-0.3% Triton X-100-PBS, and staining with anti-rabbit βIII tubulin(1:1000, Cell Signaling, USA), anti-mouse NeuN (1:100, EMD Millipore, USA), anti-rabbit $ROCK_1$ (1:100) or anti-rabbit $ROCK_2$ (1:100, Santa Cruz, USA) [27], anti-mouse $cPLA_2$ (1:50, Santa Cruz, USA) antibodies at 4^{o}C overnight. The next day, the cells were washed 3 times with PBS. The anti-rabbit FITC (1:200) or anti-mouse rhodamine (1:200) secondary antibodies (ICN Biochemicals, Aurora, OH) were applied 1 hour at room temperature followed by 3 PBS

washes. The coverslips were mounted by Vectashield mounting medium containing DAPI (Vector Laboratory, Inc.). Images were taken with Olympus BX61 fluorescence microscope (Olympus America Inc.) or by phase invert microscope (Olympus CK2, Japan) at 20x, 40x, and 60x magnifications.

Western blot analysis

Western blotting analysis was performed as described previously [26]. Whole cell lysates were prepared with RIPA buffer and protease/phosphatase inhibitors cocktail (Thermo Scientific). Protein concentration was measured with a Bio-Rad protein assay (Bio-Rad). The amount of 60 μg protein was loaded and separated by 12% or 8% SDS-polyacrylamide gel and transferred onto polyvinylidene difluoride membrane (PVDF) (Li-COR Biosciences). After 1 hour blocking with LI-blocking buffer (LI-COR Biosciences), the membranes were incubated with primary antibodies overnight at 4^{o}C. Rabbit antibodies specific for phospho-cPLA$_2$ (1:800), RhoA (1:1000), and cleaved-caspase 3 (1:800) were from Cell Signaling Tech. Inc., and ROCK$_1$ and ROCK$_2$ (1:800), GAPDH (1:2000) were purchased from Santa Cruz Biotech. Inc. Mouse antibody specific for cPLA$_2$ (1:100) was from Santa Cruz Biotech, Inc. and β-tubulin antibody (1:2000) was from Sigma-Aldrich. Following incubation, membranes were washed and incubated with a secondary fluorescence conjugated antibodies, IRDye800, IRDye680 anti-rabbit or IRDye800, IRDye680 anti-mouse (1:10000, Rockland Immunochemicals) for 1 hour at room temperature. After removal of secondary antibody solution via subsequent PBS washes, the membranes were scanned using a LI-COR Odyssey infrared scanner. The images were captured and the densitometries were measured and analyzed by Image Studio2.0 software (LICOR).

Measurement of AA release

Arachidonic acid was measured by an AA ELISA kit (Bluegene Biotech, China) according to the manufacturer's instructions. Briefly, 150 μL fresh 2%B27-1%N2-2mM Glutamine-Neurobasal medium was replaced in 48 well plates with cultured neurons. 2.5 μM ATK, 5 μM Y27632 and 4.0 μg/ml CT04 were applied to the medium for 40 min, followed by TNF-α and glutamate treatment for 16 hours. Next, 100 μL supernatant was collected for detection of released AA by ELISA. Following the given instructions, the densitometry of the reaction was measured by a 96 well plate reader (1420 multi-label counter, PerkinElmer) on wavelength 450 nm.

RhoA activity Assay

Following the manual for a RhoA activation assay Biochem kit (Cytoskeleton, Inc.), 600 μg whole cell lysate was incubated with 50 μg rhotekin-RBD beads at 4^{o}C, 1 hour. The beads were then washed with wash buffer and treated by 20 μl 2x Laemmli sample buffer (Bio-Rad, CA, USA) then denatured at 95^{o}C for 5 min. The samples were then ready for Western blot assay.

Co-immunoprecipitation assay

The assay was performed using a Universal Magnetic Co-IP kit (Active Motif Inc.) according to manufacturer's instructions. In brief, 500 μg cell lysate was combined with 5 μg $ROCK_1$ or $ROCK_2$ antibody, and underwent shaking at 4^{o}C for 4 hours. 25 μl protein G Magnetic Beads was then added in the reaction and incubated for 1 hour at 4^{o}C. Next, the tube with the beads was placed on a magnetic stand to pellet beads. The supernatant was carefully removed and 500 μl Co-IP/wash buffer was added to wash the beads 4 times. Next, the bead pellet was suspended in 20 μl of 2xLaemmli sample buffer and heated at 95^{o}C for 5min, the samples were ready for Western blot assay.

T10 contusive spinal cord injury and treatment

Fifteen C57BL/6J mice (12 weeks, 18-24g) were divided into 3 groups: sham with laminectomy only, T10 SCI with saline, and T10 SCI with Y27632, an inhibitor of Rho kinase. The mouse was anesthetized with a mixture of 17.2 mg/ml Ketamine, 0.475 mg/ml Xylazine and 0.238 mg/ml Acepromazine. The spinal cord contusive injury was performed at the 10^{th} thoracic (T10) vertebral level with the LISA device at a displacement of 0.5 mm from the dorsal surface of the spinal cord, and a velocity of 1.00 M/Sec as previously described [28]. A total of 4 microinjections of Y27632 (1 microliter 100 μM/injection) were made into the spinal cord immediately after the injury in bilateral and rostrocaudal directions according to the following coordination: 1.0 mm rostrocaudally from the injury epicenter, 0.24 mm laterally from the midline and 0.5 mm ventrally from the dorsal cord surface. After the injury and Y27632 injection, the muscles and skin were closed in layers and the animal received intraperitoneal injection of 1 ml saline. After 24 hours, mice were re-anesthetized and perfusion with 0.01M PBS and 1 cm spinal cord segment containing the lesion center was harvested. The protein was isolated and analyzed by Western Blot assay.

Statistical Analysis

One-way ANOVA was used to determine statistical significance between multiple groups and a Student's *t*-test was utilized to determine significant value differences between 2 groups. Data are reported as mean ± s.e.m. A p value < 0.05 was considered statistically significant.

Results

Combined insults of TNF-α and glutamate induced neurotoxicity *in vitro*.

To mimic secondary neuronal injury after the initial spinal cord trauma, we established an *in vitro* spinal neuronal injury model treated with an inflammatory mediator TNF-α or/and an excitatory mediator glutamate. Rat spinal cord neurons at 7 days of culture in our preparation reached a near pure population of spinal cord neurons (Fig.1 a). Using these neurons, we first

investigated the effect of TNF-α on spinal neuronal death. We tested different concentrations of TNF-α from 20 to 200 ng/ml and different treatment time points from 24 to 72 hours. Regardless of the concentration or treatment time point, TNF-α alone did not induce neuronal death (data not shown). We then tested glutamate neurotoxicity at a concentration range between 3 and 200 μM for 24 hours. We found that 5 μM glutamate was the minimum concentration sufficient to induce cell death ($p < 0.05$). The glutamate excitotoxicity peaked at 50 μM, and increasing concentration of glutamate to 100 or 200 μM did not induce further increasing of cell death (data not shown). Next we combined TNF-α with a sub-lethal dose of 3 μM glutamate. We found that TNF-α at 50 and 100 ng/ml significantly increased spinal neuronal death by 45% and 51%, respectively. When combined with 5 μM of glutamate, TNF-α induced additional 27% (50 ng/ml) and 21% (100 ng/ml) neuron death (Fig. 1 b, c). These data indicated that TNF-α potentiated glutamate neurotoxicity on rat spinal cord neurons, which are in agreement with other reports [29,30].

TNF-α and glutamate induced activation of both RhoA/Rho kinase and $cPLA_2$

To dissect out the mechanism of neurotoxicity induced by TNF-α and glutamate, we examined RhoA/Rho kinase and $cPLA_2$ activities. The data showed that, following TNF-α and glutamate treatment, the active form of RhoA (RhoA-GTP), which was precipitated by Rhotekin beads, was increased significantly at 5 minutes (Fig. 2 a, c), decreased at 10 and 30 minutes and elevated considerably by 3 hours post-treatment. Meanwhile, Rho kinase ($ROCK_1$, $ROCK_2$) and $cPLA_2$ were activated as well at 10 min and 30 min and remained highly elevated up to 3 hours post-treatment (Fig. 2 b, d). These results indicate that TNF-α and glutamate treatment activated RhoA, Rho kinase and $cPLA_2$.

Inhibition of RhoA, Rho kinase and $cPLA_2$ activation reduced spinal cord neuronal death

To investigate whether RhoA, Rho kinase and $cPLA_2$ are involved in neuronal death, we applied a RhoA inhibitor CT04, a Rho kinase inhibitor Y27632, and a $cPLA_2$ inhibitor arachidonyl trifluoromethyl ketone (ATK), to spinal cord neurons at 40 min prior to the combined administration of TNF-α and glutamate. We found that with combined TNF-α and glutamate treatment, neurons showed morphological degenerative changes including neurite retraction and soma condensation as compared to the control; such changes, however, were effectively blocked by treatments of ATK, Y27632 or CT04, respectively (Fig. 3 a-e). LDH releasing assay showed that ATK, Y27632 and CT04 significantly decreased cell death by 19%, 18% and 16% ($p < 0.05$) (Fig. 3 f). Likewise, Propidium iodide (PI)/Hoechst staining showed a significant increase in neuronal death after receiving TNF-α and glutamate treatment as compared to the non-treated; such death, however, were significantly reduced by 43%, 52% and 33%, respectively, by treatments of ATK, Y27632 or CT04 (Fig. 3 a-1-e-1, g). These data indicate that RhoA, Rho kinase and $cPLA_2$ activation contribute to neuronal pathology and death induced by TNF-α and glutamate.

RhoA or Rho kinase inhibition down-regulated $cPLA_2$ activation and AA release

To investigate whether RhoA or Rho kinase regulated $cPLA_2$ activation, inhibitors of Rho kinase (Y27632) and RhoA (CT04) were administered and phospho-$cPLA_2$ was analyzed by Western blot. The data showed that phospho-$cPLA_2$ significantly decreased by 60% and 66% by these inhibitors, respectively ($p < 0.01$; Fig. 4 a, b). Moreover, the $cPLA_2$ inhibitor, ATK, also decreased activation of $cPLA_2$ by 38% ($p < 0.01$; Fig 4 a, b). To confirm these results, we further measured the levels of AA, which is a primary product of $cPLA_2$. The data showed that inhibiting either Rho kinase or RhoA decreased AA release by 26% and 26%, respectively ($p < 0.01$; Fig.4 c). Additionally, direct inhibition of $cPLA_2$ reduced AA release by 30% ($p < 0.01$; Fig. 4 c). These data indicate that RhoA and Rho kinase may regulate $cPLA_2$ activation.

$ROCK_1$ and $ROCK_2$, two isoforms of Rho kinase, co-localized and bound with $cPLA_2$

Since inhibition of RhoA and Rho kinase down-regulated $cPLA_2$ activation, we then asked whether Rho kinase had the same distribution as $cPLA_2$ and whether it could directly interact with $cPLA_2$. We stained spinal cord neurons with Rho kinase ($ROCK_1$ or $ROCK_2$) and $cPLA_2$ antibodies and immunofluorescent secondary antibodies. The images showed that $ROCK_1$ or $ROCK_2$ existed in cytoplasm, the same distribution as cytosolic PLA_2 ($cPLA_2$) (Fig. 5 a). Next, we used a co-immunoprecipitation assay to address the second question and found that both $ROCK_1$ and $ROCK_2$ directly bound with either p-$cPLA_2$ or $cPLA_2$ in two cell types, i.e., spinal cord neurons and Hela cells (Fig. 5 b).

Rho kinase inhibitor Y27632 downregulated $cPLA_2$ expression and activation following T10 contusive SCI in adult mice

Finally, we confirmed Rho kinase inhibition on $cPLA_2$ expression and activation *in vivo* in a mouse T10 contusive SCI model using a Louisville Injury System Apparatus (LISA) impactor. This model has been used reliably in our previous studies [28]. It produces consistent and reliable injuries due to its precise determination of the “0” point of the spinal dorsal surface by a laser sensor and precise determination of displacement parameters of injury. Immediately after the injury, four injections of 1 μl Y27632 (100μM), were made into the rostral and caudal host spinal cord on both sides close to the lesion epicenter. At 24 hours post-injury, 1 cm of spinal cord segment containing the injury epicenter was harvested and analyzed by Western Blot. The data showed that at 24 hours post-SCI, the expression of Rho kinase and $cPLA_2$ was significantly increased. In addition, the apoptotic cell death marker cleaved-caspase 3 was also remarkably increased. Notably, Y27632 administration significantly inhibited expression of Rho kinase, $cPLA_2$, and phorspho-$cPLA_2$. As anticipated, cleaved-caspase 3 was also significantly reduced (Fig. 6A, B). These data indicate that Rho kinase regulates $cPLA_2$ activation and subsequently apoptotic cell death.

Discussion

During the secondary SCI process, inflammation, glutamate excitotoxicity and free radical-mediated oxidative stress are major events that contribute to cell death [31,32]. Glutamate is a common mediator of excitotoxicity in the CNS disease and trauma [33]. The inflammatory cytokine TNF-α is another important mediator which participates in inflammatory and cell death post-SCI [34,35,24]. Recently, both *in vitro* and *in vivo* studies demonstrated that TNF-α does not induce neuronal death on its own [30,36]; however, it can potentiate glutamate excitotoxicity by inhibiting glutamate uptake [37], and elevating NF-κB [37,38] and cFOS activation [36]. Our data are consistent with these reports. Regardless of the dose (up to 200ng/ml) or duration of treatment (up to 72 hours), TNF-α did not induce neuronal loss in the present study. However, when combined with a sub-lethal dose of glutamate, TNF-α significantly increased neuronal death. These data indicate that TNF-α and glutamate act synergistically to induce neuronal death in this model.

Previous studies have demonstrated that TNF-α or glutamate can activate RhoA and Rho kinase [39-41]. RhoA is an essential component of the excitotoxicity pathway, and is sufficient to induce the activation of such pathway [42]. Rho kinase also has been shown to be important in the regulation of cell death [9]. Rho kinase-phosphorylated myosin light chain which stimulates actomyosin contractility that controls apoptosis cell membrane blebbing [11], nuclear disintegration [12] and cellular fragmentation [11]. Rho kinase activation also stimulates phosphatase and tensin homologue (PTEN) and inhibits insulin receptor substrate 1 (IRS1) signaling to inactivated Akt which plays an important role in cell survival[43,44]. In addition, $ROCK_2$ can promote apoptosis by increasing ezrin phosphorylation, which increases Fas, death receptor clustering and expression[45]. Furthermore, Rho kinase mediates inflammatory cell infiltration and cytokine production [46,47], and mediates reactive oxygen species (ROS) production [48]. Our data also demonstrate that RhoA/Rho kinase activation is involved in cell

death and that inhibiting RhoA or Rho kinase activation significantly reduces neuronal cell death induced by TNF-α and glutamate.

$cPLA_2$ is an 85kDa protein enzyme that preferentially produces arachidonic acid (AA) by hydrolyzing the sn-2 position of glycerophospholipids[49]. Our lab has previously shown that $cPLA_2$ activation increased as early as 8 hours and peaked at 7 days post-SCI [22,21]. Activation of $cPLA_2$ by its activator, ceramide-1-phosphate or A23187, is sufficient to induce neuronal death, whereas $cPLA_2$ inhibition reduces cell loss *in vitro[22].* Following acute SCI, $cPLA_2$ inhibition reduces inflammation, cell death, tissue damage and improves functional recovery [22]. TNF-α or glutamate have also been shown to activate $cPLA_2$ in several non-neuronal cell types [50,51]. Our results demonstrate that $cPLA_2$ can be activated by combined stimulation of TNF-α and glutamate, and that inhibition of $cPLA_2$ significantly decreases neuronal death. These results further confirm that $cPLA_2$ activation can induce neuronal death.

Recent studies indicate that $cPLA_2$ can be activated by two distinct pathways, one involves Ca^{2+}, and another involves multiple kinases. Elevated Ca^{2+} binds with $cPLA_2$ amino-terminal C2 domain then activates $cPLA_2$ [52]. Multiple kinases including p42/p44 mitogen-activated protein kinase ($ERK_{1/2}$ MAPKs) [53], p38 MAPK and MNK1-related protein kinases [54,55] phosphorylate $cPLA_2$ at serine 505 or 727. Other studies showed that N-terminal kinase (JNK) also activates $cPLA_2$ [56]. After activation, phospho-$cPLA_2$ translocate from the cytosol to the nuclear envelope, endoplasmic reticulum and Golgi membranes [57,58] and hydrolyzes glycerophospholipids to produce free fatty acids and lysophospholipids [19]. *In vivo* research showed that $ERK_{1/2}$ and phospho-$cPLA_2$ were highly co-expressed in neurons, degenerated axons and glial cells at 24 hours post-SCI. U0126, an $ERK_{1/2}$ inhibitor decreased $ERK_{1/2}$ activation and subsequently reduced $cPLA_2$ activation [22]. RhoA is upstream of not only p38, but also $ERK_{1/2}$. Active RhoA increases $ERK_{1/2}$ and p38 activation and consequently $cPLA_2$ phosphorylation, mainly by $ERK_{1/2}$ [59]. Since Rho kinase is the main downstream substrate of

RhoA, we hypothesized that Rho kinase regulates $cPLA_2$ activation which was confirmed by the present study. Importantly, our *in vitro* and *in vivo* results demonstrated, for the first time, that inhibition of RhoA or Rho kinase remarkably down-regulated active phosphorylated $cPLA_2$. Moreover, the two isoforms of Rho kinase, $ROCK_1$ and $ROCK_2$ co-localized and directly bound with $cPLA_2$ and phospho-$cPLA_2$. Inhibition of RhoA and Rho kinase significantly reduced AA release supporting the notion that RhoA/Rho kinase regulates $cPLA_2$ activation. These data collectively indicate that RhoA and Rho kinase directly regulate $cPLA_2$ activation and subsequently cell death. However whether Rho kinase can directly phosphorylate $cPLA_2$ remains unclear and needs to be further investigated.

In summary, the current study has revealed a potential mechanism of neuronal cell death induced by TNF-α and glutamate, which is summarized in the schematic diagram of Figure 7. The combined insult of TNF-α and glutamate activates RhoA and Rho kinase and consequently activates $cPLA_2$. Active $cPLA_2$ then translocate from the cytosol to the cell and organelle membranes and hydrolyzes glycerophospholipids to produce AA and eicosanoids, and induces caspase activation and cell death. Thus, our study shows, for the first time, the mechanistic link between RhoA/Rho kinase activation, $cPLA_2$ activation, and cell death.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements:

This work was supported in part by NIH NS059622, NS073636, DOD CDMRP W81XWH-12-1-0562, Merit Review Award I01 BX002356 from the U.S. Department of Veterans Affairs, Craig H Neilsen Foundation 296749, Indiana Spinal Cord and Brain Injury Research Foundation and

Mari Hulman George Endowment Funds (XMX), and by the State of Indiana ISDH, Grant # 13679 (XW).

Author contributions

XW designed, performed experiments, analyzed data and wrote manuscript; CW designed and wrote manuscript; DE, JP performed experiments; XMX designed experiments and wrote the manuscript.

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Legend

Fig. 1 Establishment of spinal cord neuronal injury model *in vitro*. **(a)** Highly purified spinal cord neurons stained with a neurite marker βIII tubulin and neuronal marker NeuN, counterstained with a fluorescent nuclear marker DAPI. (**b**) Bright-field images show spinal cord neurons that received no treatment (control), or treatment for 24 hour with 50 ng/ml TNF-α, 3μM glutamate, or a combination of the two. Arrows show the live cells and arrowheads show the dead cells. (**c**) Neuronal death measured by the LDH assay. Values are means ± s.e.m. (n = 4). $p < 0.01$ versus control. Bar in A & B = 50 μm.

Fig. 2 TNF-α and glutamate induced activation of RhoA, Rho kinase and $cPLA_2$. **(a, b)** Western blot analysis showed that the activation of RhoA (**a**), Rho kinase and $cPLA_2$ (**b**) were significantly increased. (**c, d**) Densitometric quantification of RhoA, Rho kinase, and $cPLA_2$ activation. Data were expressed as fold change compared to the control group. Values are means ± s.e.m. (n = 4). * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$.

Fig. 3 Inhibition of RhoA, Rho Kinase, and $cPLA_2$ decreased neuronal death induced by TNF-α and glutamate. Spinal cord neurons were pretreated with ATK, Y27632, and CT04 at 40 minutes prior to the application of TNF-α and glutamate. After 24 hours, phase contrast images of neurons were taken (top row) and cells were stained by Propidium iodide (PI, red) and Hoechst (blue) (bottom row) in healthy neurons (**a, a1**) or neurons challenged with TNF-α and glutamate (**b-e, b1-e1**). In the latter condition, cultured were treated with ATK, a $cPLA_2$ inhibitor (**c, c1**), Y27632, a $ROCK_{1/2}$ inhibitor (**d, d1**), and CT04, a RhoA inhibitor (**e, e1**). Live neurons with clear cell bodies (top row) and blue Hoechst nuclear staining (bottom row) can be clearly seen (red and white arrows, respectively). Dead neurons with degenerating cell bodies (top row) and red PI nuclear staining (bottom row) can also be appreciated (red and white arrowhead).

Quantitative data show cell death in fold change measured with LDH releasing (**f**), and in percent change measured by the PI^+ and $Hoechst^+$ cells (**g**). Values are means ± s.e.m. (n = 4), * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$, Bars = 50 μm.

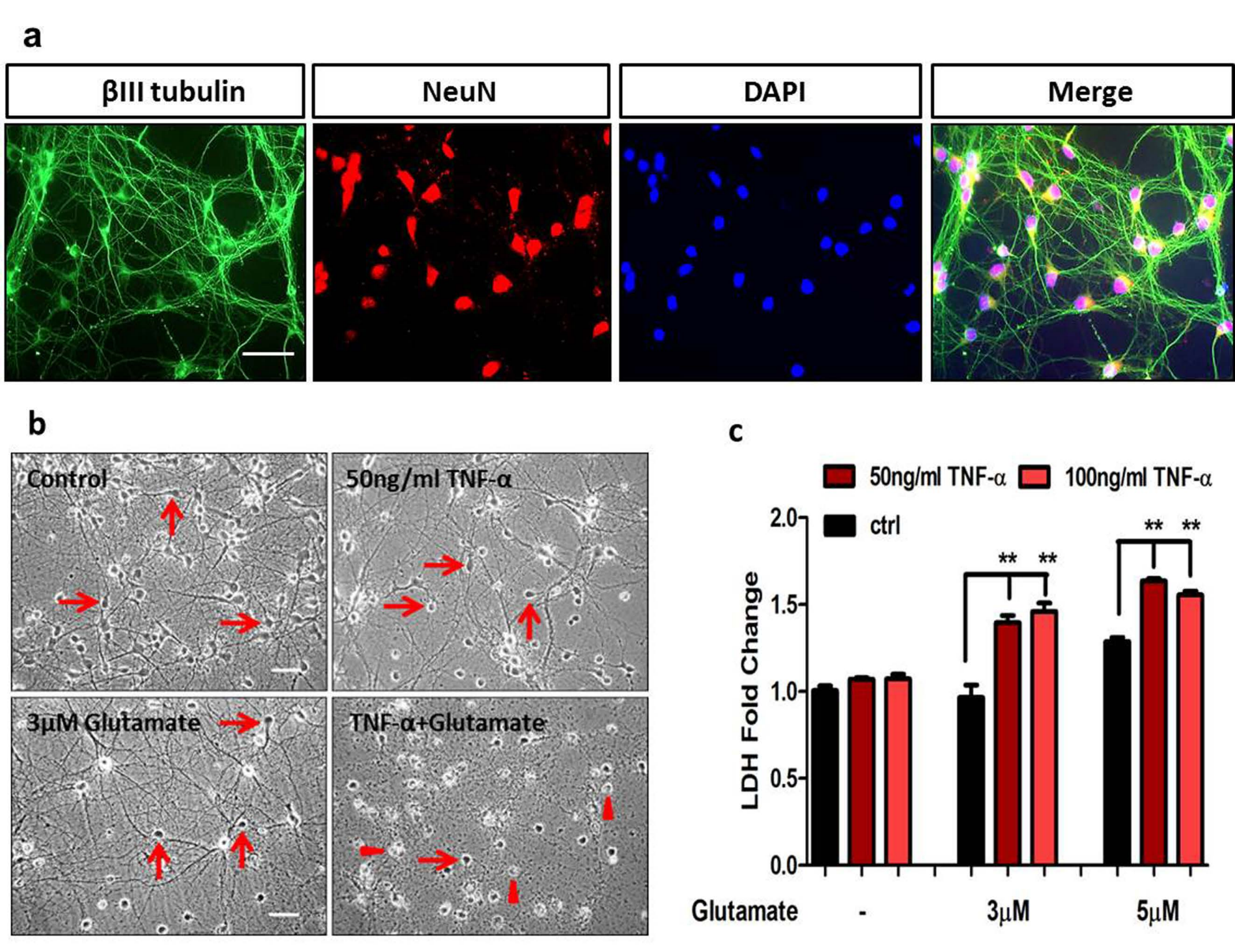
Fig. 4 RhoA/Rho kinase regulated $cPLA_2$ activation. (**a**) The inhibitors of RhoA (CT04), Rho kinase (Y27632), and $cPLA_2$ (ATK) down-regulated $cPLA_2$ activation induced by TNFα and glutamate. (**b**) The quantification data of active $cPLA_2$ and GAPDH. (**c**) The release of AA, the main downstream product of $cPLA_2$, was significantly reduced by ATK, Y27362 and CT04 (ELISA). Values are means ± s.e.m (n = 3). Data are expressed as fold change compared with the control group, **$p < 0.01$, ***$p<0.001$.

Fig. 5 Rho kinase ($ROCK_1$/$ROCK_2$) was co-localized and bound directly with $cPLA_2$. (**a**) The spinal cord neurons were stained with $ROCK_1$, $ROCK_2$, and $cPLA_2$ antibodies and the images were taken at 60x magnification. Both $ROCK_1$ or $ROCK_2$ and $cPLA_2$ localized in cytoplasm. (**b**) Whole cell lysates of spinal cord neurons and Hela cells were immunoprecipitated with $ROCK_1$, $ROCK_2$ antibodies then immunobloted by phospho-$cPLA_2$ and $cPLA_2$ antibodies. The rabbit IgG antibody was used as a negative control. The data showed p-$cPLA_2$/$cPLA_2$ bound directly with $ROCK_1$ or $ROCK_2$. Bar=10 μM.

Fig. 6 Rho kinase inhibitor, Y27632, downregulated $cPLA_2$ and phospho-cPLA2 and reduced apoptosis cell death in mice T10 contusion model. (**a**) The Rho kinase inhibitor,Y27632, 1μl of 100μM was injected in mice T10 spinal cord/site around lesion area immediately after contusion injury. The spinal cords which contained lesion area were isolated post injury 24 hours and

analyzed by Western blot. (**b**) The quantitative data of densitometry. Values are means ± s.e.m. (n = 5). Data are expressed as fold change compared with the control group, *$p < 0.05$ · ** $p<0.01$.

Fig. 7 Schematic drawing illustrates the proposed mechanism of RhoA/Rho kinase and $cPLA_2$ activation following spinal cord injury. The combined TNF-α and glutamate insults activate RhoA/Rho kinase and $cPLA_2$. The phospho-$cPLA_2$ re-localizes to cell and organelle membrane to hydrolyze phospholipids to arachidonic acid (AA) and eicosanoids to induce neuronal death. Inhibition of RhoA, $ROCK_{1/2}$, and $cPLA_2$ with CT04, Y27632, and ATK reduces $cPLA_2$ activation and neuronal death.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3μM Glutamate
TNF-α+Glutamate
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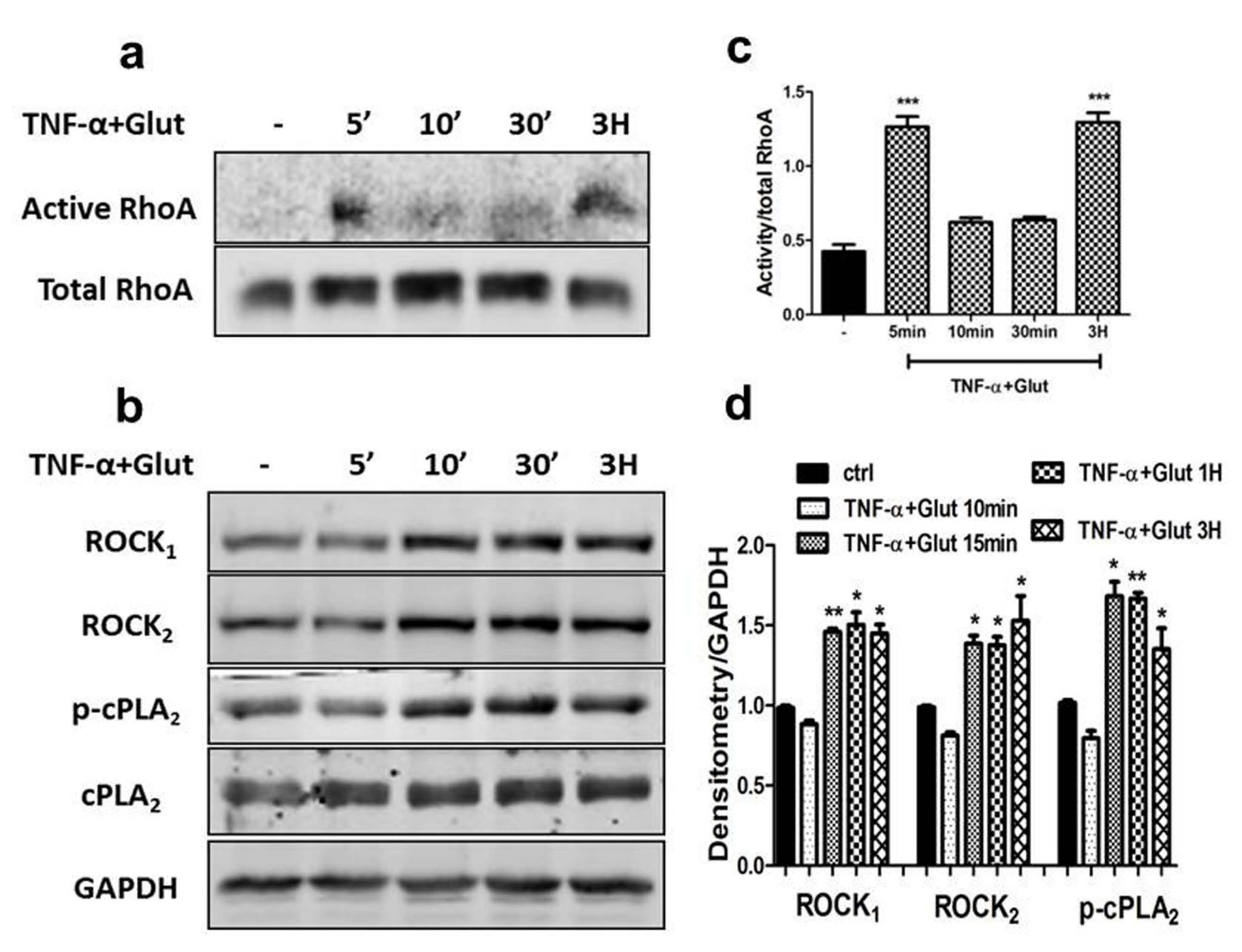
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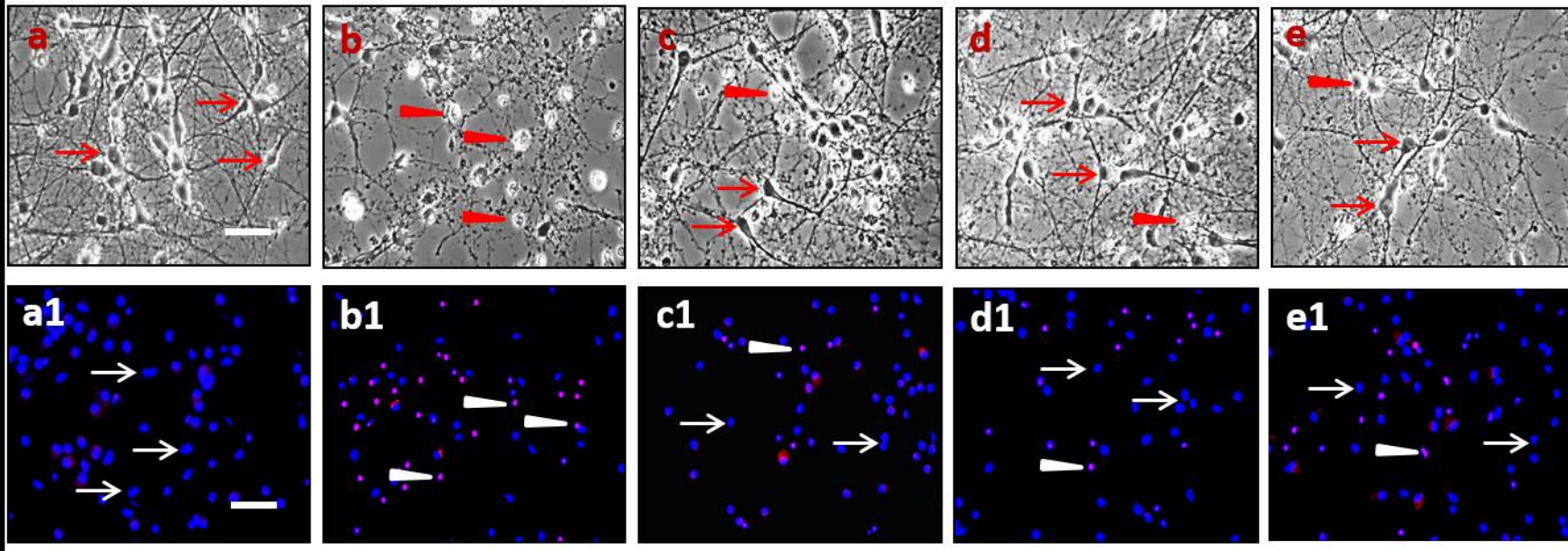
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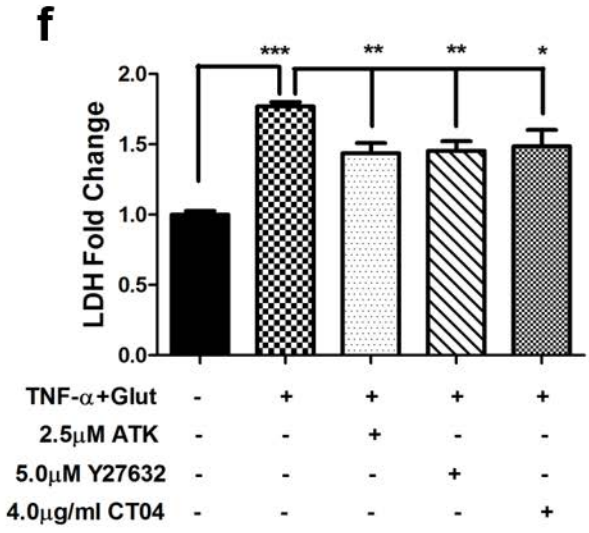
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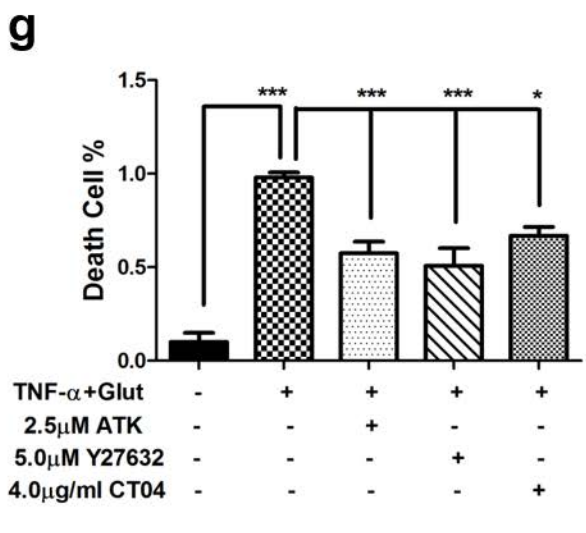
5.0μM Y27632 - - - + -
4.0μg/ml CT04 - - - - +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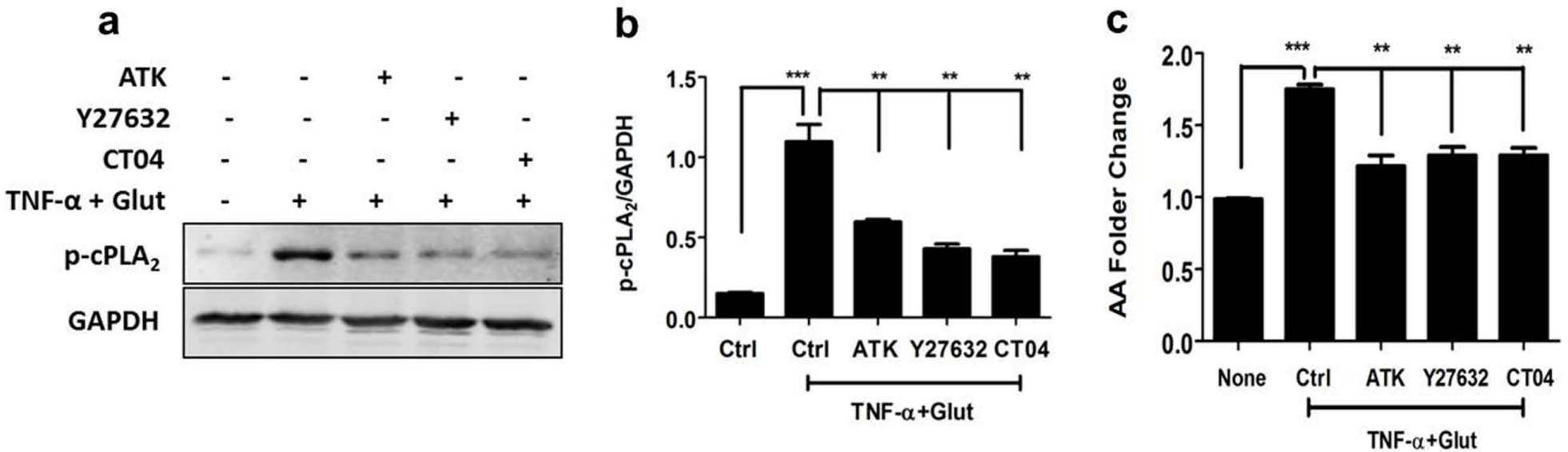
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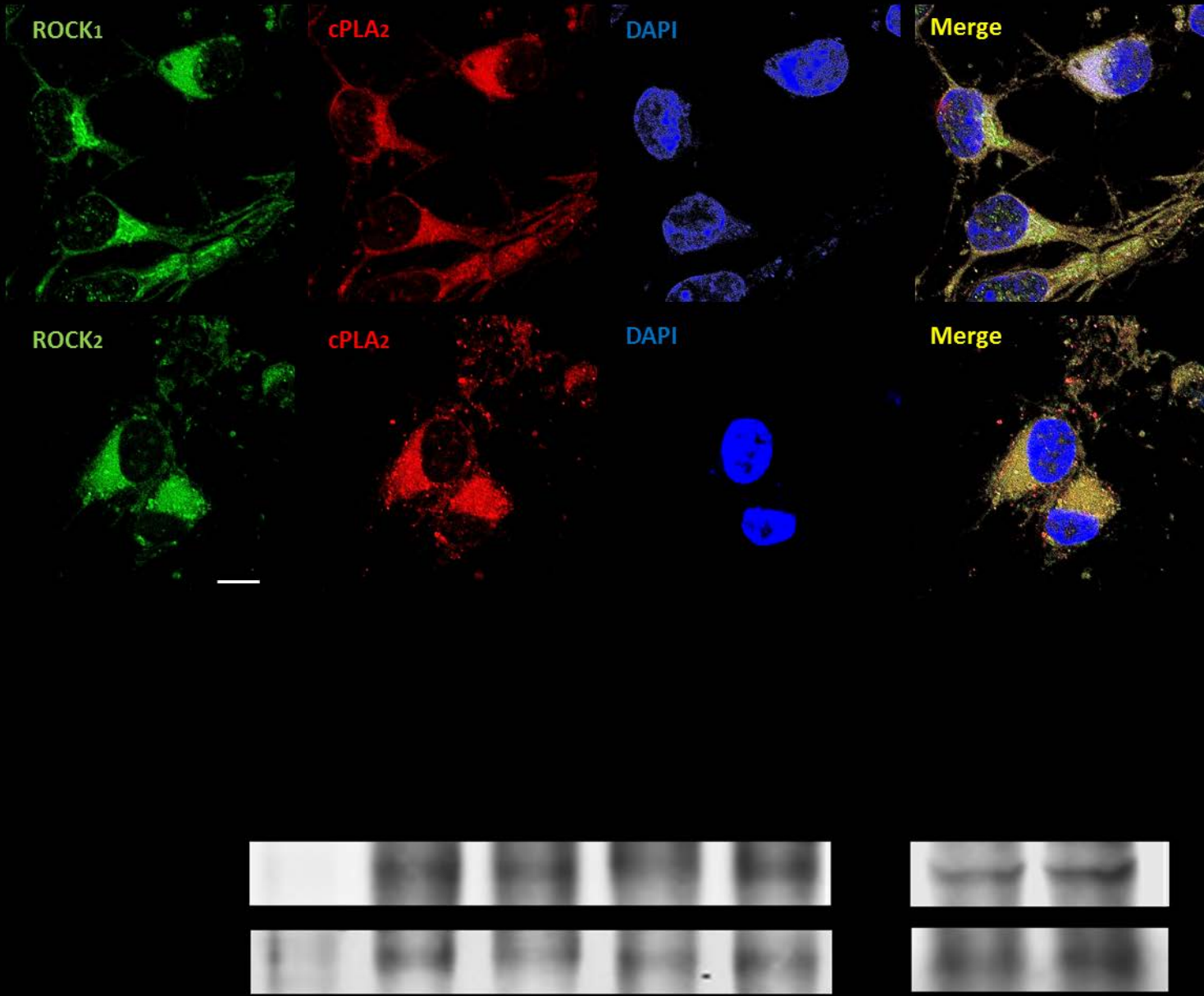
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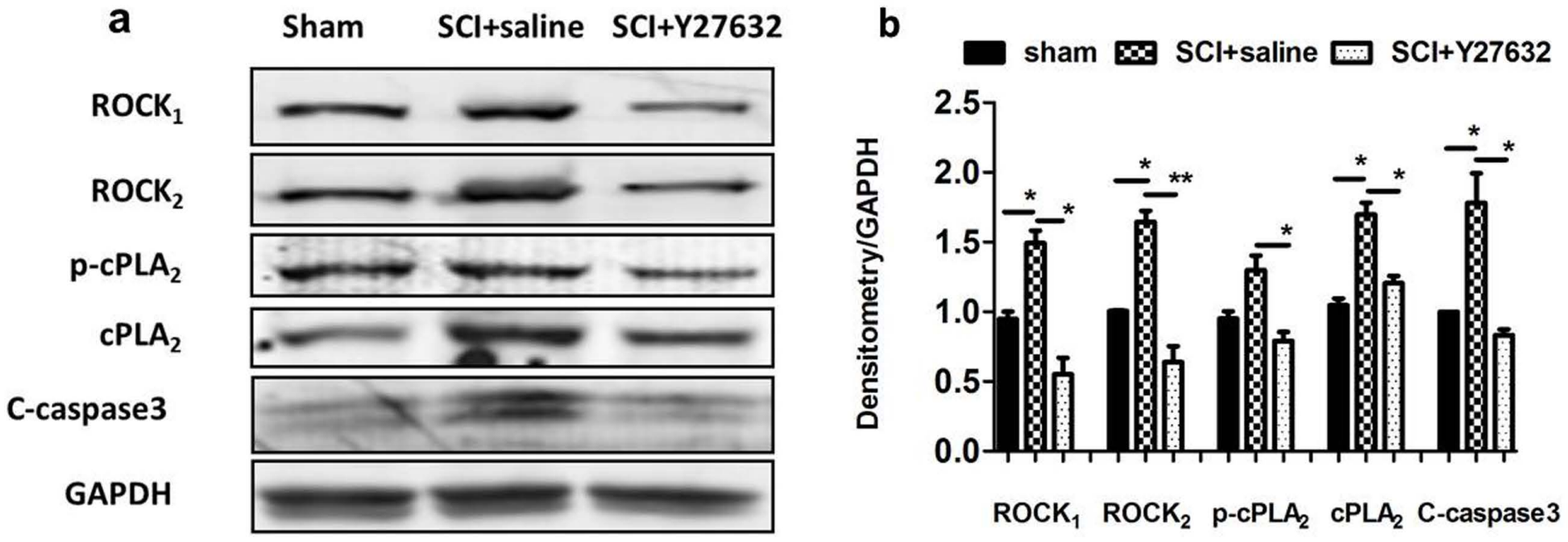
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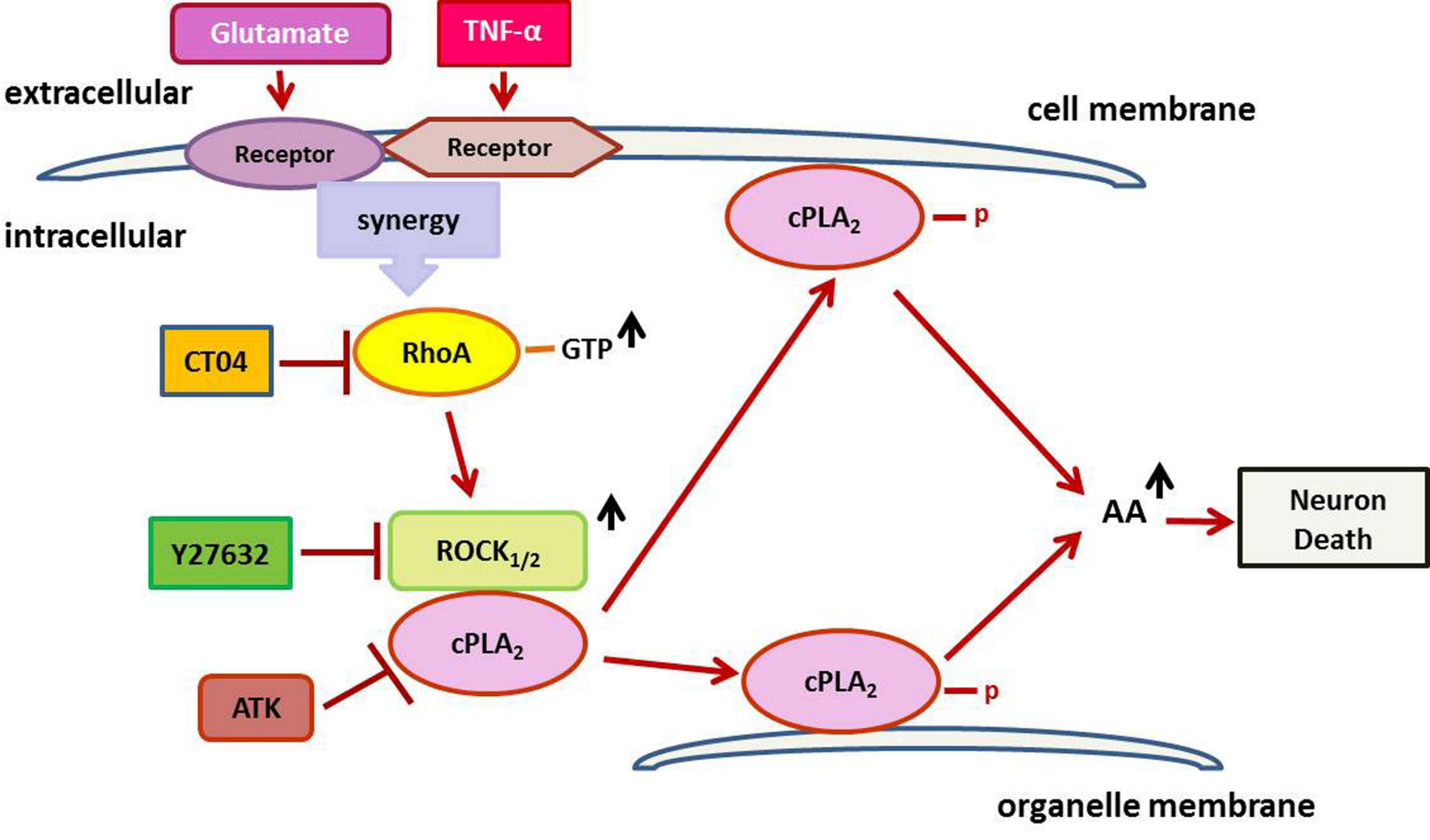
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Neuron Death
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cPLA2
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organelle membrane