



# The Localization of p53 in the Crayfish Mechanoreceptor Neurons and Its Role in Axotomy-Induced Death of Satellite Glial Cells Remote from the Axon Transection Site

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## Abstract

Neuron and glia death after axon transection is regulated by various signaling proteins. Protein p53 is a key regulator of diverse cell functions including stress response, DNA repair, proliferation, and apoptosis. We showed that p53 was overexpressed in crayfish ganglia after bilateral axotomy. In the isolated crayfish stretch receptor, a simple natural neuroglial preparation, which consists of a single mechanoreceptor neuron (MRN) enveloped by glial cells, p53 regulated axotomy-induced death of glial cells remote from the axon transection site. In MRN, p53 immunofluorescence was highest in the nucleolus and in the narrow cytoplasmic ring around the nucleus; its levels in the nucleus and cytoplasm were lower. After axotomy, p53 accumulated in the neuronal perikaryon. Its immunofluorescence also increased in the neuronal and glial nuclei. However, p53 immunofluorescence in the most of neuronal nucleoli disappeared. Axotomy-induced apoptosis of remote glial cells increased in the presence of p53 activators WR-1065 and nutlin-3 but reduced by pifithrin- $\alpha$  that inhibits transcriptional activity of p53. Pifithrin- $\mu$  that inhibits p53 effect on mitochondria increased axotomy-induced apoptosis of remote glial cells but reduced their necrosis. Therefore, axotomy-induced apoptosis of remote glial cells was associated with p53 effect on transcription processes, whereas glial necrosis was rather associated with transcription-independent p53 effect on mitochondria. Apparently, the fate of remote glial cells in the axotomized crayfish stretch receptor is determined by the balance between different modalities of p53 activity.

**Keywords** p53 · Neuron · Glia · Axotomy · Cell death

## Introduction

Neurotrauma, a result of head or spine injury, is one of the leading reasons of human disability and death, especially young and middle-aged men (Lichterman 2014; Kobeissy 2015; Hill et al. 2016). The emergency treatment of neurotrauma requires application of a neuroprotective medication during first hours after an accident. Unfortunately, the efficient neuroprotectors are not found yet. Therefore, deep and comprehensive studies of the molecular mechanisms of neurodegeneration are needed.

In the peripheral neurons, axon transection (axotomy) is followed either by neuron death or by axon regeneration and

restoration of connections with target cells. After nerve transection, molecular signals such as  $\text{Ca}^{2+}$  and some signaling proteins are transported retrogradely to the neuronal body and to the nucleus. They stimulate production of specific proteins, which are either transported backward to the damaged site to participate in the regeneration processes or remain in the neuronal body and regulate cell survival or death (Rishal and Fainzilber 2014).

Neuroglial interactions play a significant role in the viability and functioning of the nerve cells. They provide integrity of the nervous tissue. Neurons and glial cells support mutual survival of each other (Aldskogius and Kozlova 1998; Whiteside et al., 1998; Parpura et al. 2012). At the axon transection site, the surrounding glial cells are mechanically injured and die. However, the satellite glial cells, remote from the transection site, can die as well (Khaitin et al. 2015, 2018; Uzdensky 2018). As shown recently, in the isolated crayfish stretch receptor (CSR), a simple model object consisting of a single mechanoreceptor neuron (MRN) enveloped by satellite glial cells,  $\text{Ca}^{2+}$  and some signaling proteins (Akt, GSK-3 $\beta$ ,

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mTOR, MEK1/MEK2, MAP kinases ERK1/ERK2, and p38) regulate axotomy-induced death of remote glial cells (RGC) that surround the proximal axon segment of MRN at a distance of several millimeters from the transection site (Berezhnaya et al. 2017; Khaitin et al. 2018). The downstream proapoptotic proteins that mediate proapoptotic pathway and promote RGC apoptosis are not well-known.

Protein p53, a tumor suppressor and “genome guardian,” is known to regulate the cell cycle and stimulate apoptosis of cells with DNA damage. This eliminates malignant cells and protects the organism from tumor formation. However, p53 is involved not only in oncological processes. It is a master regulator of basic cell functions, including DNA repair, cell cycle, metabolism, and apoptosis (Nicolai et al. 2015; Aubrey et al. 2018; Marcel et al. 2018; Simabuco et al. 2018). As a transcription factor, it regulates the expression of hundreds of genes (Fisher 2017; Sullivan et al. 2018). Additionally, it participates in transcription-independent regulation of mitochondrial functions and induces apoptosis of cells with mitochondrial failure (Wang et al. 2014; Dai et al. 2016). Normally, the intracellular level of p53 is kept low, but it increases vastly under DNA breaks, oxidative damage, ionizing radiation, and other stress factors. p53 is involved in pathogenesis of various neurodegenerative diseases (Bonini et al. 2004; Culmsee and Mattson 2005; Checler and Alves da Costa 2014). These works were mainly focused on p53-mediated control of neuronal death. Its role in death of glial cells after nerve damage is less studied (Hughes et al. 1999; Jebelli et al. 2012; Ma et al. 2017). It is of interest to study the p53 role in a simple naturally integrated neuroglial system, such as CSR, subjected to axotomy.

In the present work, we showed that axotomy induced overexpression of p53 in the crayfish ganglia. The transcription-dependent p53 activity was shown to mediate axotomy-induced apoptosis of satellite glial cells remote from the transection site in the isolated crayfish stretch receptor, whereas transcription-independent p53 activity was involved in axotomy-induced necrosis of these cells. Axotomy also caused the loss of the immunoreactivity of p53 in the nucleolus and its accumulation in the cytoplasm of the crayfish mechanoreceptor neuron.

## Materials and Methods

### Chemicals

All inhibitors, antibodies, and other chemicals used in the present work were purchased from Sigma-Aldrich-Rus (Moscow, Russia).

## Objects

Two neural crayfish preparations were used as simple experimental objects. The crayfishes *Astacus leptodactylus* from Don River affluences were purchased on the local market. The crayfish abdominal stretch receptor consists of two single mechanoreceptor neurons, slowly and rapidly adapting, each enveloped by the multilayer glial sheath. These neurons are attached to the receptor muscles, which are stretched between two adjacent carapace segments. After muscle stretching, the rapidly adapting neuron generates a train of spikes, which propagate to the ventral nerve cord (VNC) ganglion. The slowly adapting neuron (MRN) permanently fires with a frequency proportional to the muscle elongation. Satellite glial cells (SGC) and their interaction with MRN have been studied in detail at the optical and ultrastructural levels (Fedorenko and Uzdensky 2009; Khaitin et al. 2015, 2018; Uzdensky et al. 2015; Berezhnaya et al. 2017).

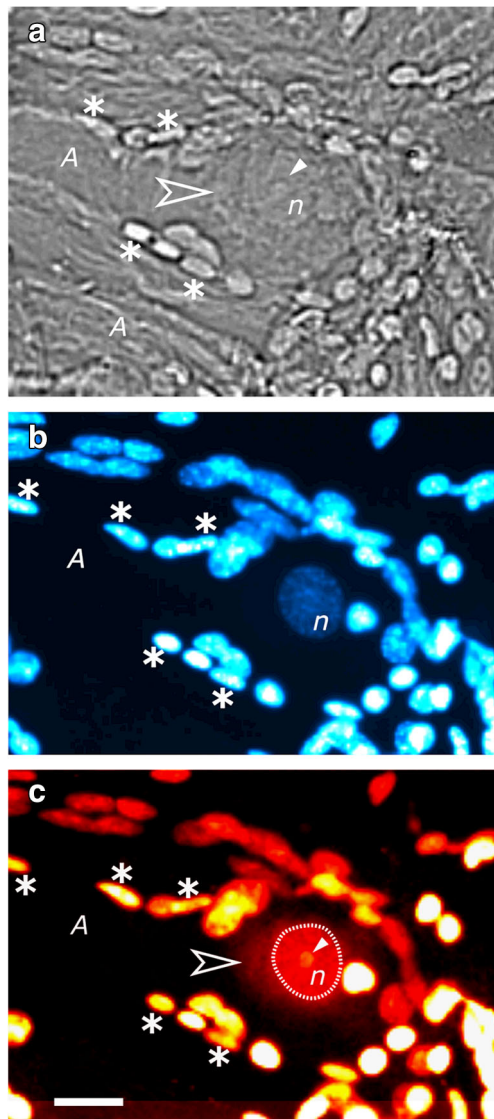
After isolation, CSR was placed into a plexiglass chamber filled with 2 ml of van Harreveld's saline (mM, NaCl 205, KCl 5.4, NaHCO<sub>3</sub> 0.2, CaCl<sub>2</sub> 13.5, MgCl<sub>2</sub> 5.4; pH 7.2–7.4) and equipped with a device for receptor muscle extension. MRN spikes were recorded extracellularly from axon by a glass pipette suction electrode, amplified, digitized, and processed on a personal computer. The initial firing level (6–10 Hz) was preset by stretching of the receptor muscle. Firing frequency was registered throughout the experiment until its complete abolition. The experiments were carried out at 23 ± 4°C.

Another object is the bilaterally axotomized crayfish VNC ganglion (Demyanenko et al. 2019). VNC consists of six ganglia connected by nerves. After cutting off the crayfish abdomen and removing the chitin carapace from the ventral side, the VNC was quickly isolated and transferred to a bath with van Harreveld's solution. Control VNC was transected twice: at the front and rear ends. In the experimental VNC, the connectives were cut in 7 sites: at the edges and between ganglia, so that 6 bilaterally axotomized ganglia were obtained.

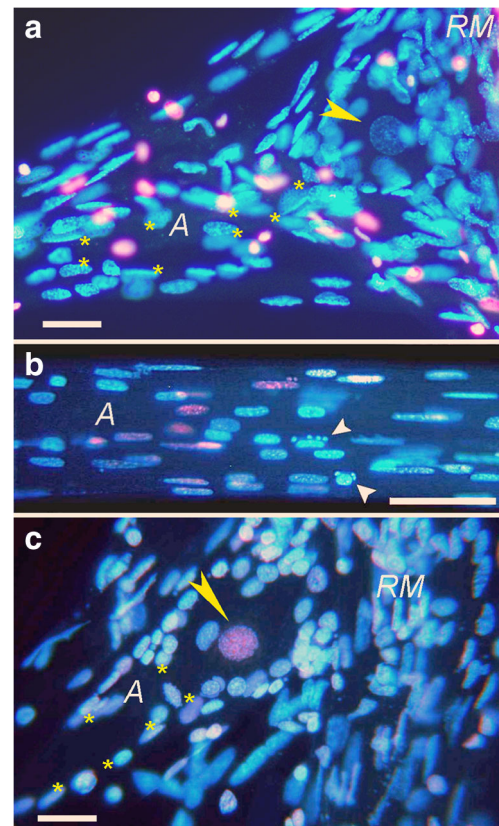
### Cell Death Assay

Dead neurons and glial cells were visualized in the crayfish stretch receptor stained with 20 μM propidium iodide and 10–20 μM Hoechst 33342 that were added into the experimental chamber at 8 h after axotomy (the interval required for apoptosis development). Then preparations were washed with van Harreveld's saline, fixed with 0.2% glutaraldehyde, repeatedly washed, and mounted in glycerol. Fluorescent images were acquired using the fluorescence microscope Olympus BX-51 (Japan) equipped with the digital camera ORCA-Flash4.0 V3 (Hamamatsu, Japan). Propidium iodide, a membrane impermeable fluorochrome, imparts red fluorescence to the nuclei of necrotic cells with the compromised plasma membrane.

Hoechst 33342 imparts blue fluorescence to the nuclear chromatin. It visualizes intact nuclei of living cells and fragmented nuclei of apoptotic cells (Figs. 1 and 2). Nucleus fragmentation is the final apoptosis stage when the no-return point has passed. The percentage of red nuclei of necrotic glial cells stained by propidium iodide and the number of the fragmented nuclei of apoptotic glial cells stained by Hoechst 33342 were counted around the proximal 2-mm axon segment, where apoptosis of glial cells was higher. These glial cells were located at a distance of 5–8 mm from the axon transection site (Berezhnaya et al. 2017; Khaitin et al. 2018). One-way



**Fig. 1** The crayfish stretch receptor neuron and surrounding glial cells. **a** Brightfield image; **b** Hoechst 33342 staining that imparts blue fluorescence to the cell nuclei; **c** p53 immunofluorescence. Large arrowhead, the p53-immunopositive perikaryon region; n, the neuron nucleus; small arrowhead, the nucleolus; dashed line, the perinuclear ring with increased p53 immunofluorescence; asterisks, glial nuclei. Scale bar 20  $\mu$ m



**Fig. 2** Isolated crayfish stretch receptor fluorochromed by Hoechst 33342, which imparts blue fluorescence to the nuclear chromatin and visualizes nuclei of living cells and fragmented nuclei of apoptotic cells (small arrowheads on **b**), and propidium iodide, which imparts red fluorescence to the nuclei of necrotic cells with damaged plasma membrane. **a** and **c** Stretch receptors with living and necrotic sensory neurons (blue and red nuclei, respectively; big arrowheads). **A** axon; **RM** receptor muscle; yellow asterisks, glial nuclei. Scale bars on **a** and **c** 20  $\mu$ m and on **b** 40  $\mu$ m

ANOVA and Student's t-test for independent samples were used.  $M \pm SEM$ ;  $n = 10$ .

### Application of p53 Inhibitors or Activators

After 30 min control recording of neuronal activity, the inhibitor or activator of p53 was added into the chamber. The following p53 activators were used: nutlin-3 (1  $\mu$ M), WR-1065 (100  $\mu$ M), or RITA (10  $\mu$ M). p53 inhibitors are pifithrin- $\alpha$  (PFT- $\alpha$ , 5  $\mu$ M) and pifithrin- $\mu$  (PFT- $\mu$ , 5  $\mu$ M). They were present in the chamber during 8 h after axotomy. Their concentrations were chosen to be approximately twofold lower than the concentration, which abolishes MRN firing for 3–4 h.

### Immunofluorescence Microscopy

To determine the localization of p53 in MRN and glial cells before and 4 or 8 h after axotomy, we used the method of CSR isolation that preserved its connection to the VNC ganglion



(Khaitin et al. 2015). These CSRs were incubated in the van Harreveld's saline during different time intervals after axon transection and then were labeled by the primary monoclonal anti-p53 antibody produced in mouse (P5813, Sigma-Aldrich). This antibody was diluted 1:100 in PBST (10 mM PBS with 2.7 mM KCl, 137 mM NaCl, 0.1% Tween 20, pH 7.4) and used at 25 °C. Anti-Mouse IgG1 ( $\gamma$ 1) labeled with CF™ 555 (SAB4600302; Sigma-Aldrich) was used as a secondary antibody (dilution 1:500 in PBS). The CSR preparations were fixed for 24 h by 4% paraformaldehyde. After following 24-h washing in PBS containing 1% bovine serum albumin, 0.2% NaN<sub>3</sub>, and 1% TritonX-100, they were incubated 24 h with the primary antibody, washed, incubated 24 h with the secondary antibody, washed again during 24 h at + 4 °C, and mounted in glycerol.

The CSR preparations were fluorochromed 10 min with Hoechst 33342 (40  $\mu$ M) at + 25 °C, washed, mounted in glycerol, and studied using Olympus BX-51 microscope equipper with the digital camera ORCA-Flash4.0 V3 (Hamamatsu, Japan) at the excitation wavelengths around 535 nm for the Anti-Mouse IgG1 ( $\gamma$ 1) labeled with CF555 and at 365 nm for Hoechst 33342. Fluorescence was recorded at  $\lambda > 580$  nm and  $\lambda > 460$  nm, respectively (Figs. 1 and 2).

The intensities of p53 immunofluorescence in various CSR elements, the MRN nucleus, the nucleolus, the perikaryon area with visually distinguishable p53 location, the bright cytoplasm ring around the nucleus, and the nuclei of glial cells that surround the 2-mm long proximal axon segment, were measured in different points (10 points in the nucleus and in the p53-containing cytoplasm regions and 3 points in the nucleolus and glial nuclei) using the ImageJ software (Fig. 1d). The background fluorescence was measured outside the CSR. The fluorescence intensity in the each location was averaged. The data were normalized by the background fluorescence:

$$I = \frac{I_m - I_b}{I_b},$$

where  $I_m$  is the mean intensity in the studied area (nucleolus, nucleus, perikaryon, or glia) and  $I_b$  is the average background fluorescence outside CSR. One-way ANOVA was used for statistical evaluation of differences between various experimental groups: Intact (Int) and 4 or 8 h after axotomy.  $M \pm$  SEM;  $n = 8$ .

## Western Blotting

After the bilateral axotomy, VNC ganglia were incubated during 4 h in van Harreveld's saline at 22–24 °C. Control and experimental samples from 3 animals were united to obtain the necessary tissue mass. They were homogenized 2 min on ice using the ultrasonic homogenizer in the extraction/labeling

buffer (E0655, Sigma Aldrich) supplemented with protease and phosphatase inhibitors and nuclease benzonase. The homogenates were centrifuged (10 min, 10,000 g, 4 °C), and the supernatant was collected. The samples containing 10–20  $\mu$ g of protein were subjected to electrophoretic separation in the polyacrylamide gel in the presence of sodium dodecyl sulfate using a Mini-PROTEAN Tetra cell (Bio-Rad). After the separation, the antibodies were electrotransferred to the PVDF Immuno-Blot membrane (Bio-Rad) using the Trans-Blot® Turbo Transfer System (Bio-Rad, USA). After washing, the membrane was incubated 1 h in the blocking buffer (TBS, 1% casein blocker, Bio-Rad) and incubated overnight at 4 °C with monoclonal anti-p53 antibody produced in mouse (P5813, SigmaAldrich, 1:400) and anti- $\beta$ -actin (A5441, SigmaAldrich; 1:5000). The membranes were then washed with TTBS (10 mM Tris buffer with 0.1% Tween 20, pH 8), incubated 1 h at room temperature with the secondary antibody anti-rabbit IgG-peroxidase (A6154, SigmaAldrich; 1:1000), washed in TTBS again, and incubated with Clarity Western ECL. Chemiluminescence was recorded using the Fusion SL gel documentation system (Vilber Lourmat, France) and the Vision-Capt software. One-way Anova RM was used for statistical analysis.  $M \pm$  SEM;  $n = 5$ . The significance level,  $p < 0.05$ .

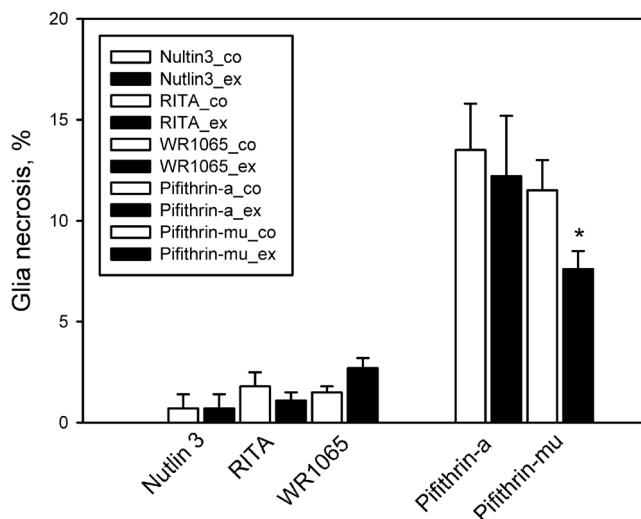
## Results

### The Involvement of p53 Death of Mechanoreceptor Neuron and Remote Glial Cells Induced by Axotomy

After axotomy, MRN fired regularly 6–8 h or more till irreversible abolition of neuronal activity. The level of necrosis of the mechanoreceptor neuron was low. Necrosis dependence on modulation of p53 by different inhibitors or activators was not registered. Nuclear fragmentation typical for the terminal apoptosis stages was not observed in these neurons.

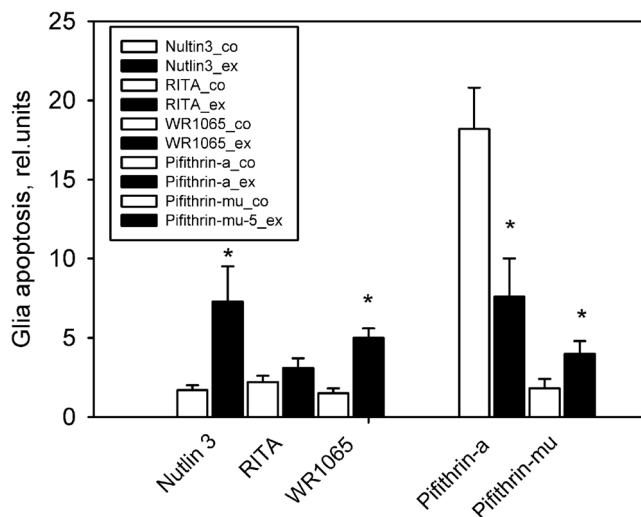
The level of necrosis of remote glial cells at 8 h after axotomy was also insignificant, < 3–4%, and was not increased by p53 activators nutlin-3, RITA, or WR-1065 (Fig. 3). In the experiments with pifithrins- $\alpha$  and pifithrin- $\mu$ , when the post-axotomy incubation was increased up to 9 h, glial necrosis in the control series without p53 modulators increased to 11–13%. In these experiments, inhibition of p53 by pifithrin- $\mu$ , but not pifithrin- $\alpha$ , reduced necrosis of RGC by 35% ( $p < 0.05$ ; Fig. 3). Since pifithrin- $\mu$  is known to inhibit the transcription-independent effect of p53 on mitochondria, one can suggest that p53-mediated injury of mitochondria was involved in necrosis of remote glial cells in the axotomized CSR.

Axotomy induced apoptosis in some satellite glial cells remote from the axon transection site. This effect was higher



**Fig. 3** Effect of p53 activators: RITA (10  $\mu$ M), WR-1065 (100  $\mu$ M), and nutlin-3 (1  $\mu$ M), as well as p53 inhibitors: pifithrin- $\alpha$  (PFT- $\alpha$ , 5  $\mu$ M) and pifithrin- $\mu$  (PFT- $\mu$ , 5  $\mu$ M) on necrosis of glial cells in the isolated crayfish stretch receptor after axotomy.  $M \pm SEM$ ;  $n = 10$ . \* $p < 0.05$ .

in the experiment with pifithrin- $\alpha$  where, unlike 8-h incubation in other experiments, glial apoptosis was detected at 9 h post-axotomy. The incubation time in this experimental group was increased because we anticipated that p53 inhibitor can suppress apoptosis. Our experiments confirmed that axotomy-induced apoptosis of RGC was mediated by p53. In fact, RGC apoptosis was higher in the presence of p53 activators nutlin-3 and WR-1065, whereas p53 inhibitor pifithrin- $\alpha$  suppressed apoptosis. Another p53 inhibitor, pifithrin- $\mu$ , oppositely, enhanced glial apoptosis ( $p < 0.05$ ; Fig. 4). One can suggest that the transcription-associated activity of p53, which is inhibited by pifithrin- $\alpha$ , was involved in axotomy-induced apoptosis of

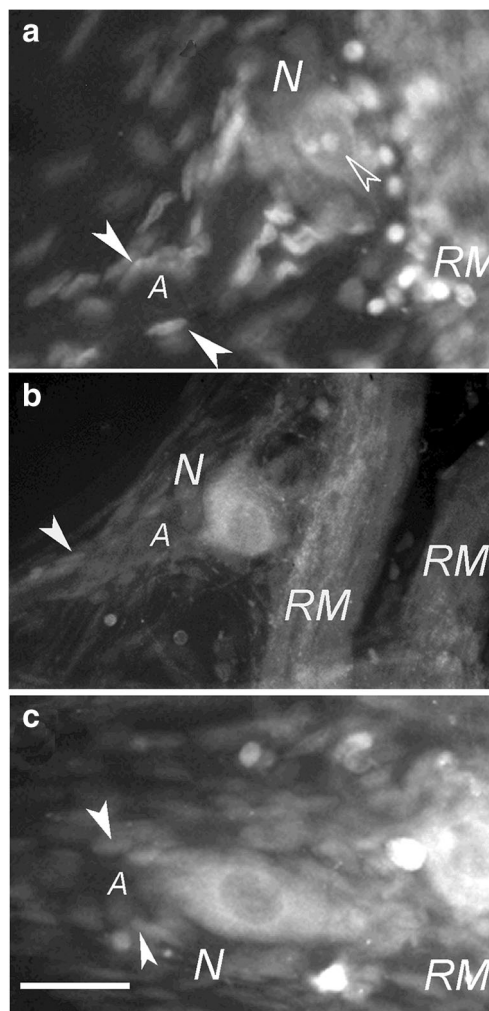


**Fig. 4** Effect of p53 activators: RITA (10  $\mu$ M), WR-1065 (100  $\mu$ M), and nutlin-3 (1  $\mu$ M), as well as p53 inhibitors: pifithrin- $\alpha$  (PFT- $\alpha$ , 5  $\mu$ M) and pifithrin- $\mu$  (PFT- $\mu$ , 5  $\mu$ M) on apoptosis of glial cells in the isolated crayfish stretch receptor after axotomy.  $M \pm SEM$ ;  $n = 10$ . \* $p < 0.05$ ; \*\* $p < 0.01$

glial cells. On the other hand, some p53-controlled mitochondrial processes might exert the anti-apoptotic effect on remote glial cells.

### Localization and Expression of p53 in the Crayfish Mechanoreceptor Neuron

Immunofluorescence microscopy showed that in the control CSRs, in which MRN axons were not damaged and transferred spikes to the VNC ganglion, p53 immunofluorescence was highest in the nucleolus and in the narrow, 2–4  $\mu$ m widths, cytoplasmic ring around the nucleus. The p53 immunofluorescence in the neuronal karyoplasm and in the glial nuclei, where nucleoli were not observed, was noticeably lower. The p53 immunofluorescence in the perikaryon beyond the bright ring was low; it was almost

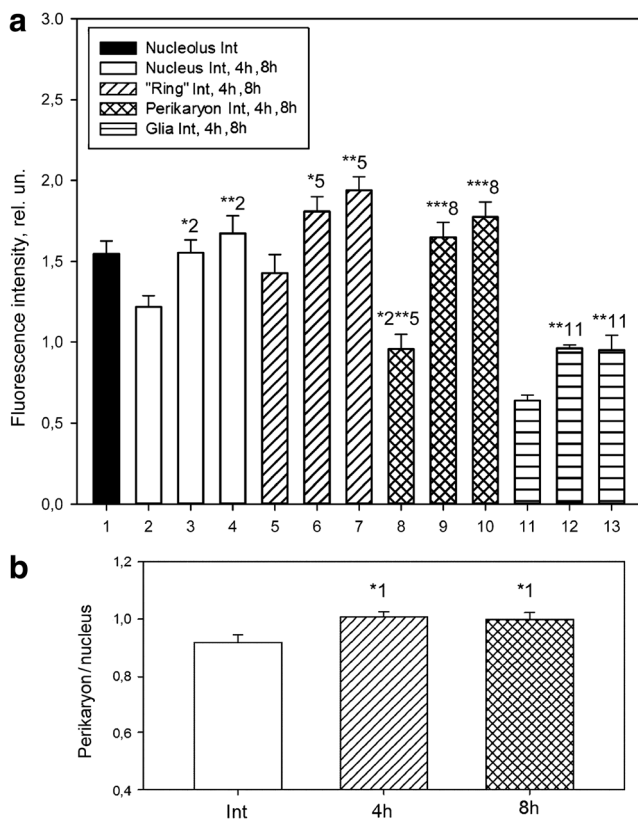


**Fig. 5** Localization of p53 immunofluorescence in the isolated crayfish stretch receptor. **a** Intact non-axotomized stretch receptor; **b–c** Axotomized stretch receptors 4 and 8 h after axon transection. **A** axon; **N** nucleus of the mechanoreceptor neuron; **RM** receptor muscle; white-stroked arrowheads indicate neuronal nucleoli; white-filled arrowheads indicate nuclei of glial cells. Scale bar 20  $\mu$ m

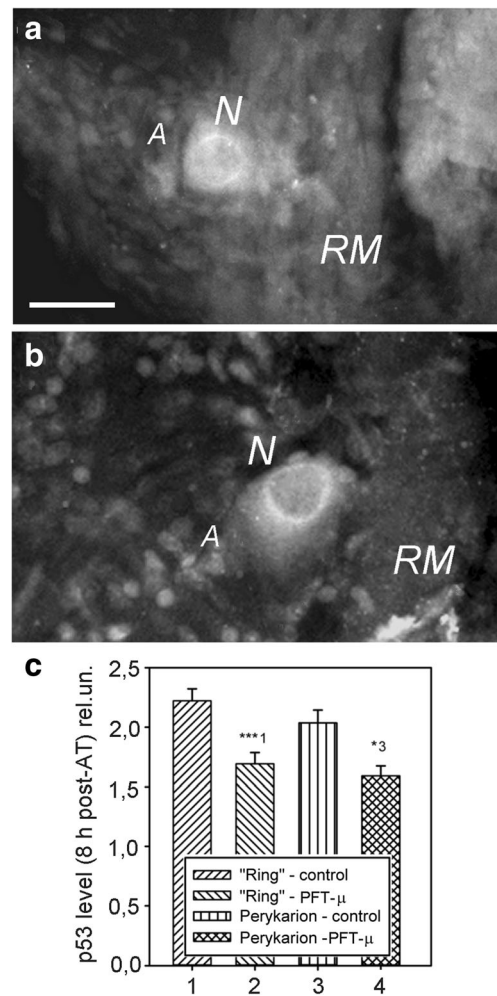
absent in 2/3 of neurons. Axons did not fluoresce (Fig. 5a).

At 4–8 h after axotomy, the p53 immunofluorescence increased in the neuronal and glial karyoplasm, in the MRN perikaryon, and in the perinuclear ring as compared with the intact CSRs (Fig. 6a). In the MRN soma, a p53-positive fluorescing area was observed mainly in the perikaryon, which is rich in mitochondria and other intracellular organelles (Fedorenko and Uzdensky 2009). The p53 immunofluorescence of the MRN nucleolus was less intensive and observed seldom, only in 1/3 of CSRs (Fig. 5b–c). The p53 immunofluorescence in the perikaryon of the control intact preparations was initially lower than in the nucleus and in the ring (Fig. 5a). However, at 4–8 h after axotomy, it became significantly higher ( $p < 0.05$ ; Fig. 6b) which was shown using the paired statistical comparison for each CSR.

Since pifithrin- $\mu$  can inhibit p53 binding to mitochondria (Strom et al. 2006; Nijboer et al. 2011), we studied its effect on location of p53 in the MRN at 4 or 8 h after axotomy (Fig. 7a–b). The distribution of p53 was similar to that without pifithrin- $\mu$ : p53 immunofluorescence was



**Fig. 6** **a** The dynamics of p53 immunofluorescence in different parts of the axotomized crayfish stretch receptor: neuronal nucleus, perinuclear ring, perikaryon cytoplasm, and nuclei of glial cells surrounding the proximal axon segment before and at 4 or 8 h after axotomy. **b** Perikaryon/nucleus ratio. Int intact control CSR.  $M \pm SEM$ ;  $n = 8$ . \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$

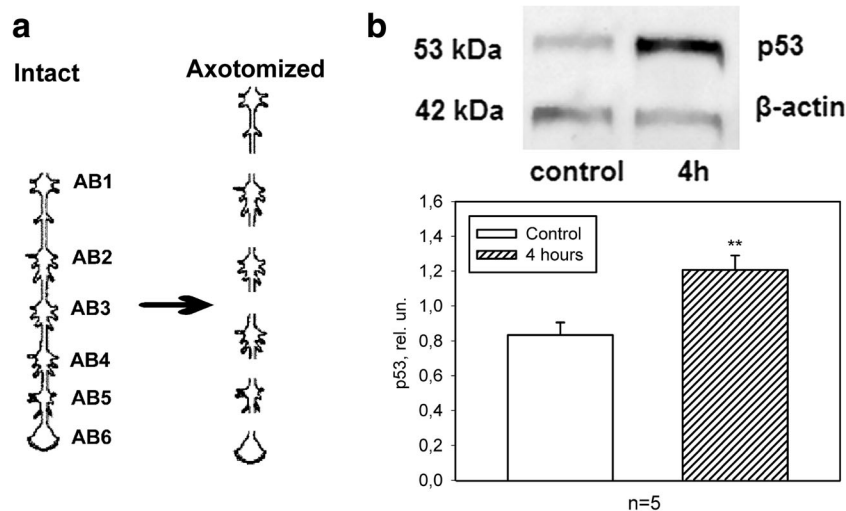


**Fig. 7** Localization of p53 immunofluorescence in the isolated crayfish stretch receptor at **a** 4 or **b** 8 h after axotomy in the presence of pifithrin- $\mu$  (PFT- $\mu$ , 15  $\mu$ M) and **c** the effect of pifithrin- $\mu$  on the averaged level of p53 in the perikaryon cytoplasm and cytoplasmic perinuclear ring; control - axotomy without pifithrin- $\mu$ .  $M \pm SEM$ ;  $n = 8-10$ . \* $p < 0.05$ ; \*\*\* $p < 0.001$ . A axon; N nucleus of the mechanoreceptor neuron; RM receptor muscle. Scale bar 20  $\mu$ m

observed in the extranuclear ring and in the perikaryon. The neuronal nucleus did not fluoresce. However, the area occupied by p53 in the perikaryon and its overall p53 immunofluorescence in this region were lower than in the absence of pifithrin- $\mu$  (Fig. 7). This showed that pifithrin- $\mu$  partly inhibited the binding of p53 with neuronal mitochondria.

### Expression of p53 in the Crayfish VNC Ganglia After Bilateral Axotomy

Immunoblotting showed 1.5-fold overexpression of p53 in the cytoplasmic fraction of the crayfish ventral nerve cord ganglia at 4 h after bilateral axotomy as compared with the non-transected control VNC ( $p < 0.01$ ; Fig. 8).



**Fig. 8** Expression of p53 in the crayfish ventral nerve cord ganglia at 4 h after bilateral axotomy. **a** Transection of connectives between crayfish VNC ganglia provides 6 bilaterally axotomized ganglia; **b** Western

blotting results: increase in the p53 level in VNC ganglia at 4 h after axotomy.  $M \pm SEM$ ;  $n = 5$ . \*\*  $p < 0.01$

## Discussion

The multifunctional protein p53 plays diverse roles in cells. As a transcription factor, it controls expression of hundreds and perhaps thousands genes involved in the regulation of metabolism, stress reactions, proliferation, apoptosis, and other cellular processes (Morrison et al. 2003; Bonini et al. 2004; Culmsee and Mattson 2005; Nicolai et al. 2015; Fisher 2017; Simabuco et al. 2018; Sullivan et al. 2018). Its proapoptotic activity is associated with stimulation of biosynthesis of caspase 6, Puma, Noxa, Apaf-1, and other proteins involved in different apoptosis stages (Jebelli et al. 2012; Akhter et al. 2014; Wan et al. 2014; Wang et al. 2014). It can also function in the transcription-independent mode. Being translocated to mitochondria, it impairs bioenergetic processes and stimulates release of cytochrome c and AIF that induce apoptosis (Wang et al. 2014; Dai et al. 2016; Aubrey et al. 2018). In normal cells, the level of p53 is low due to rapid MDM2-dependent ubiquitination and proteasomal degradation (Gottifredi and Prives 2001). However, extensive DNA damage and stress caused by diverse harmful factors such as hypoxia, excitotoxicity, oxidative stress, ionizing radiation, ribonucleotide depletion, or disruption of the nucleolar function induce overexpression of p53 (Bonini et al. 2004; Culmsee and Mattson 2005; Checler and Alves da Costa 2014; Wan et al. 2014; Wang et al. 2014). In the nervous system, not only neuronal but also astrocytic p53 coordinates the pathogenesis of various neurodegenerative diseases (Jebelli et al. 2012).

Our experiments showed the upregulation of p53 in the various invertebrate model objects: in the bilaterally axotomized crayfish VNC ganglia, in the nuclei and cytoplasm of the mechanoreceptor neuron, and in the nuclei of glial cells remote from the axon transection site at 4–8 h after axotomy. p53 is synthesized in the cytoplasm, and then it is

transported into the nucleus. The unbound p53 forms a complex with MDM2 that transports it back to the cytoplasm where it rapidly degrades in proteasomes. Its accumulation in the nucleus may be associated with intense synthesis, rapid posttranslational translocation into the nucleus, and impeded nuclear-cytoplasmic transport. Accumulation of p53 in the cytoplasm may occur if the rate of its synthesis is higher than the rates of degradation and nuclear transport. The observed accumulation of p53 in both nuclear and cytoplasmic compartments could be due to both factors. Phosphorylation prevents interaction of p53 with MDM2, impedes degradation, and stabilizes the p53 pool. A variety of protein kinases such as MAP kinases JNK, p38, ERK, and others phosphorylate p53 (Culmsee and Mattson 2005). Since axotomy does not induce DNA breaks, the potential signals for p53 synthesis include transcription factors such as E2F1, c-Myc, p38, etc. The overexpression of these signaling proteins in the axotomized crayfish VNC ganglia has been demonstrated in our recent proteomic study (Demyanenko et al. 2019).

The fragmentation of the neuronal nuclei that can indicate cell apoptosis was not observed in MRN at 4–8 h after axotomy as well as after photooxidative stress and action of various pharmacological agents in our previous studies (Uzdensky et al. 2002, 2015). As suggested earlier, in MRN, apoptosis is intrinsically blocked, probably because this unique single neuron is vitally important for movement control and animal survival. Nevertheless, axotomy induced upregulation and accumulation of the apoptosis promoter p53 in crayfish neurons. Possibly, in MRN, apoptosis may develop later, beyond the 8-h incubation interval that was studied in the present work. During this interval, the majority of MRNs continued firing and evidently lived longer than the satellite glial cells. It is also possible that in MRN, apoptosis can occur differently than in glial cells, without nucleus fragmentation.



Apoptosis of CSR glial cell was observed not only near the axon transection site where these cells may be mechanically injured but also around the axon regions remote from this place for several millimeters (Berezhnaya et al. 2017; Khaitin et al. 2018). The effect of p53 on cell survival or death is determined by the balance between the transcription-dependent nuclear and transcription-independent mitochondrial apoptosis pathways. The application of various activators and inhibitors showed p53 involvement in axotomy-induced apoptosis of remote glial cells. Among these modulators, WR-1065 is known as an activator and reactivator of p53 (Wiman 2006). RITA and nutlin-3 prevent MDM2-dependent p53 degradation (Chipuk and Green 2006; Wiman 2006). Suppression of glial apoptosis in the presence of pifithrin- $\alpha$  indicated the proapoptotic role of p53 in these cells and suggested that p53-mediated apoptosis was associated with its transcriptional activity. Pifithrin- $\alpha$  is known to block the translocation of p53 into the nucleus where it inhibits the p53-mediated transcription and expression of various apoptotic genes (Gudkov and Komarova 2005; Chipuk and Green 2006). On the other hand, the present data showed that pifithrin- $\mu$  did not influence apoptosis of MRN in the axotomized CSR but enhanced apoptosis of remote glial cells (Fig. 4). This effect was not in agreement with the data on its antiapoptotic activity. Pifithrin- $\mu$  is known to block the p53 binding to mitochondria and following inhibition of the transcription-independent mitochondrial apoptosis pathway, which is associated with inhibition of the anti-apoptotic Bcl-2 family proteins, permeabilization of the outer mitochondrial membrane, decrease in mitochondrial potential, and bioenergetics failure (Strom et al. 2006; Nijboer et al. 2011; Dong et al. 2012; Zhang et al. 2013; Wan et al. 2014; Wang et al. 2014; Maj et al. 2017; Glas et al. 2018). In the case of CSR axotomy, the transcription-dependent pathway of glial apoptosis apparently prevailed over the mitochondrial pathway, which was inhibited by pifithrin- $\mu$ . However, the mitochondrial effects of p53 rather than regulation of transcriptional activity were possibly involved in the axotomy-induced necrosis of glial cells that was reduced in the presence of pifithrin- $\mu$ , but not pifithrin- $\alpha$ .

At 4 and especially 8 h after axon transection, p53 was accumulated in the MRN perikaryon area (Fig. 5) abundant with mitochondria (Fedorenko and Uzdensky 2009). The Western blot data also showed the overexpression of p53 in the cytoplasmic fraction of the crayfish VNC ganglia after bilateral axotomy (Fig. 8). Pifithrin- $\mu$ , which inhibits the p53 binding to mitochondria, reduced the axotomy-induced area of the cytoplasmic location of p53 (Fig. 7). This effect was incomplete, possibly due to lesser inhibitory effect of pifithrin- $\mu$  as compared with the rate of p53 accumulation in the cytoplasm and its binding to mitochondria.

It is of interest that in the intact, non-axotomized MRN, p53 was concentrated in the narrow cytoplasmic ring around the nucleus. The p53 immunofluorescence in this ring did not

disappear, but became brighter after axotomy (Figs. 5, 6, and 7). The mechanism of its formation is not clear. Our previous electron-microscopic data did not show any specific structures in this area that differ from other cytoplasm regions in the MRN perikaryon (Fedorenko and Uzdensky 2009). Other authors also did not observe a similar ring in the neuronal perikaryon. One can suggest that after synthesis in the cytoplasm, p53 may retain near the nuclear membrane before its import through the nuclear pores. Possibly, the rate of p53 synthesis was higher than the rate of its degradation and the rate of its nuclear transport.

The accumulation of p53 in the neuronal nucleoli is also of interest. Nucleolus is known to be the ribosome factory (McLeod et al. 2014). Ribosome biogenesis is necessary for the neuron life. Under stress conditions, the nucleolus participates in sensing the cell damage. Its integrity and activity are critical for regulation of p53 level and activity in the cell (Woods et al. 2015). Nucleolus impairment leads to activation of p53 and transfers the injury signal to the systems that control cell metabolism, homeostasis, and survival (Erickson and Bazan 2013; Parlato and Kreiner 2013). At 4–8 h after MRN axotomy, the averaged nuclear level of p53 increased, but fluorescing nucleoli were observed only in rare experiments. The lack of excessive p53 in the nucleoli might be associated with the disassembly of the multiprotein complex that controls p53 binding to the nucleolar structures (Erickson and Bazan 2013). In glial cells, p53 was distributed rather uniformly throughout the nucleoplasm, and nucleoli were not observed.

In conclusion, p53 was upregulated in the crayfish VNC ganglia and mechanoreceptor neurons. In the isolated crayfish stretch receptor, p53 participated in axotomy-induced apoptosis and necrosis of glial cells that envelop the proximal axon region remote from the transection site. Axotomy-induced apoptosis of glial cells was due to p53-regulated transcriptional processes, whereas glial necrosis was rather associated with p53 effect on mitochondria. In the crayfish mechanoreceptor neuron, p53 was accumulated in the nucleolus and in the narrow cytoplasmic ring around the nucleus. Its level in the karyoplasm and cytoplasm was much lower. After MRN, axotomy p53 accumulated in the cytoplasm; its nucleolar p53 immunofluorescence disappeared in the majority of neurons.

Some unresolved problems remain. It is unclear how axon transection induces apoptosis of glial cells remote for several millimeters from the transection site? What molecules serve as proapoptotic neuroglial signals, and how they stimulate p53 to regulate death of these cells? One can suggest the involvement of  $\text{Ca}^{2+}$  or NO as small-molecular-weight messengers in glial apoptosis. Just after axotomy,  $\text{Ca}^{2+}$  can penetrate into the damaged glial cells at the transection site and propagate along the glial syncytium. Alternatively, it can enter into the transected axon and induce retrograde signaling cascades, which transfer some proapoptotic molecules to glial cells (Khaitin et al.



2018). Nitric oxide is easily transported between cells and also promotes apoptosis.  $\text{Ca}^{2+}$  activates neuronal NO synthase that produces NO. In astrocytes, NO induces p53-mediated translocation of Bax to mitochondria that causes apoptosis (Yung et al. 2004). Further investigations should check these hypotheses.

**Authors' Contributions** Stanislav Rodkin: Immunofluorescence study of p53 localization in neuron and glial cells

Andrey Khaitin: Immunofluorescence study of p53 localization in neuron and glial cells and study of effects of pifithrin- $\alpha$  and pifithrin- $\mu$  on neurons and glial cells

Maria Pitinova: Study of effects of pifithrin- $\alpha$  and pifithrin- $\mu$  on neurons and glial cells

Valentina Dzreyan: Immunoblotting of p53 expression in VNC ganglia

Valeria Guzenko: Immunoblotting of p53 expression in VNC ganglia

Mikhail Rudkovskii: Study of effects of RITA on neurons and glial cells

Svetlana Sharifulina: Study of effects of nutlin-3 and WR-1065 on neurons and glial cells

Anatoly Uzdensky: Work management; analysis of results, and writing the paper

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**Ethical Approval** All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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