



Axotomy-Induced Changes of the Protein Profile in the Crayfish Ventral Cord Ganglia

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Abstract

We suggest novel experimental model of nerve injury—bilaterally axotomized ganglia of the crayfish ventral nerve cord (VNC). Using proteomic antibody microarrays, we showed upregulation of apoptosis execution proteins (Bcl-10, caspases 3, 6, and 7, SMAC/DIABLO, AIF), proapoptotic signaling proteins and transcription factors (c-Myc, p38, E2F1, p53, GADD153), and multifunctional proteins capable of initiating apoptosis in specific situations (p75, NMDAR2a) in the axotomized VNC ganglia. Simultaneously, anti-apoptotic proteins (p21WAF-1, MDM2, Bcl-x, Mcl-1, MKP1, MAKAPK2, ERK5, APP, calmodulin, estrogen receptor) were overexpressed. Some proteins associated with actin cytoskeleton (α -catenin, catenin p120CTN, cofilin, p35, myosin V α) were upregulated, whereas other actin-associated proteins (ezrin, dystrophin, tropomyosin, spectrin ($\alpha + \beta$), phosphorylated Pyk2) were downregulated. Various cytokeratins and β_{IV} -tubulin, components of intermediate filament and microtubule cytoskeletons, were also downregulated that could be the result of tissue destruction. Downregulation of proteins involved in clathrin vesicle formation (AP2 α and AP2 γ , adaptin ($\beta 1 + \beta 2$), and syntaxin) indicated impairment of vesicular transport and synaptic processes. The levels of L-DOPA decarboxylase, tyrosine, and tryptophan hydroxylases that mediate synthesis of serotonin, dopamine, norepinephrine, and epinephrine decreased. Overexpression of histone deacetylases HDAC1, HDAC2, and HDAC4 contributed to suppression of transcription and protein synthesis. So, the balance of multidirectional processes aimed either at cell death, or to repair and recovery, determines the cell fate. Present data provide integral, albeit incomplete, view on the nervous tissue response to axotomy. Some of these proteins can be probably potential markers of nerve injury and targets for neuroprotective therapy.

Keywords Nerve injury · Proteomics · Apoptosis · Cytoskeleton · Vesicular transport · Synaptic processes

Introduction

Traumatic brain or spinal cord injury is among main causes of mortality and disability of the population. The death rate of 20–50 year old people from neurotrauma is higher than death from cancer and vascular pathologies. Annually craniocerebral trauma (TBI) kills 1.5 million people in the world, and 2.5 million became disabled. Peripheral nerve injuries account for up to 10% of neurotrauma (Kobeissy 2015; Laskowitz and Grant 2016; Rabinstein 2018; Witiw and Fehlings 2015).

Neurotrauma are very diverse. Various in vitro models including cell cultures and brain sections that display different features of mechanical neuron injury were used in neuropathologic studies. They included nerve stretching, mechanical or hydrostatic compression, hydrodynamic shock, impact of falling weight, or axotomy. On the base of the obtained data, various medications for treatment of acute neurotrauma such as glutamate antagonists, antioxidants, and anti-apoptotic agents were tested, but none of them was approved. Lack of success in this direction indicates the necessity of deeper understanding the cellular and molecular mechanisms of nerve damage and subsequent neuron death, survival, or regeneration (Lichterman 2014; Kobeissy 2015; Laskowitz and Grant 2016; Rabinstein 2018; Witiw and Fehlings 2015).

Nerve injury, in particular its complete transection (axotomy), impair ionic gradients and induces influx of Na⁺ and Ca²⁺ and efflux of K⁺. The signals about the axon damage (Ca²⁺, some signaling proteins) are retrogradely transported to the neuronal body and nucleus. This stimulates gene

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expression, synthesis of necessary proteins, and their backward transport to the damaged site. Following recovery processes include restoration of the plasma membrane, organelles and cytoskeleton, nerve regeneration, and restoration of the lost nerve connections with target cells (Rishal and Fainzilber 2014). However, at severe damage, such as axotomy, these measures may be insufficient, and the neuron degenerates (Hill et al. 2016). Axotomy induces death not only neurons and glial cells in the transection site but also satellite glial cells remote from the place of injury. These processes are regulated by a complex and incompletely known signaling system (Hill et al. 2016; Rishal and Fainzilber 2014).

In order to study the signaling and epigenetic mechanisms that regulate neurodegeneration and neuroprotection after nerve damage, we introduce the novel model object—the bilaterally axotomized abdominal ganglion of the crayfish. Its ventral nerve cord (VNC) contains six ganglia (~10³ neurons each) connected by nerves. The transection of the connectives gives six bilaterally axotomized ganglia. This model is relevant for spinal nerve cord injury. Using the antibody microarrays, we studied the changes in expression of more than 200 signaling and epigenetic proteins in the bilaterally axotomized VNC ganglia at 4 h after axotomy. The results of this proteomic study identified the most reactive proteins.

Materials and Methods

Object

The crayfishes *Astacus leptodactylus* from the Don River affluents were purchased at the local market. After cutting off the crayfish tail and removal of chitin from its ventral side, the ventral nerve cord was quickly isolated and transferred to the chamber filled with van Harreveld's saline for cold-blooded animals (mM: NaCl 205; KCl 5.4; NaHCO₃ 0.24; MgCl₂ 5.4; CaCl₂ 13.5; pH 7.2–7.4). In the isolation process control VNCs were transected twice by thin ophthalmic scissors at the anterior and posterior margins before first and after sixth abdominal ganglion, respectively. The experimental VNCs were transected seven times: at the margins and between abdominal ganglia so that six bilaterally transected ganglia were obtained (Fig. 1). Control and experimental (axotomized) VNC samples obtained from 8 to 10 animals each were united and incubated in the dark at room temperature (about 22–25 °C) for 4 h.

Proteomic Study

The “Panorama® Antibody Microarray - Cell Signaling” kits (CSAA1), primary rabbit antibodies anti-HDAC1 (H3284) and anti-caspase 3 (C9598), anti-β-actin (A5441), and the

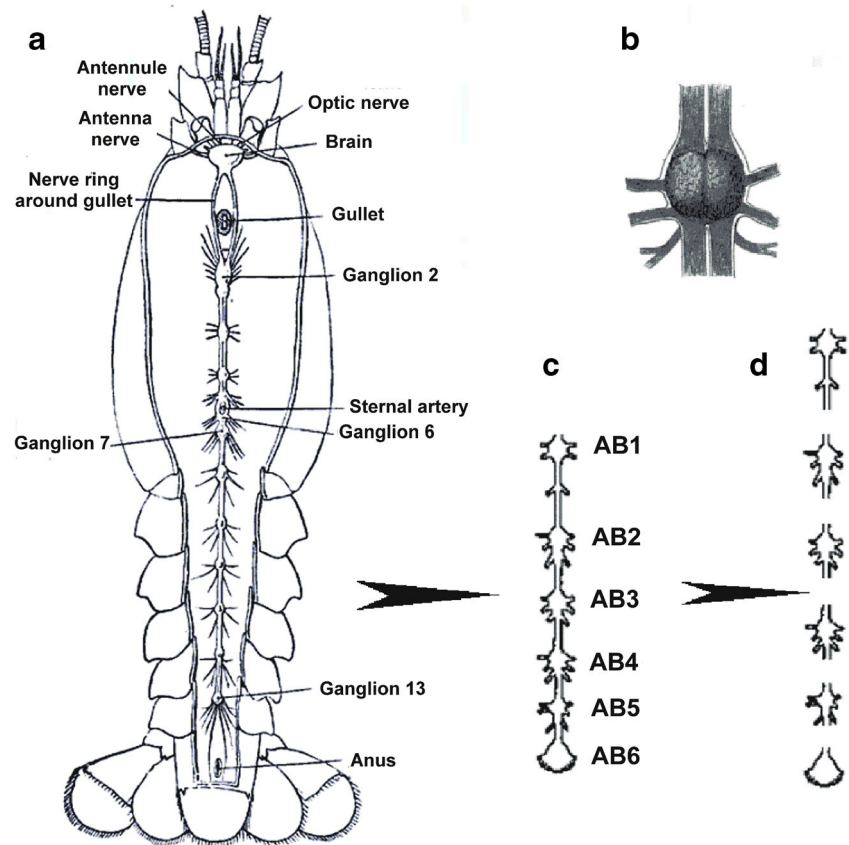
secondary anti-Rabbit IgG peroxidase antibody (A6154) were obtained from Sigma-Aldrich-Rus (Moscow).

The changes in the expression of proteins in the axotomized VNC ganglia were estimated using the “Panorama® Antibody Microarray - Cell Signaling Kit,” which consists of two identical microarrays containing microdroplets with immobilized antibodies against 224 signaling and epigenetic proteins (in duplicate). The spots with immobilized non-labeled bovine serum albumin were used as a negative control, whereas the spots with Cy3 and Cy5-conjugated BSA were used as a positive control.

At 4 h after axotomy, the control and experimental samples were weighted, homogenized by ultrasound on ice, and lysed in the Extraction/Labeling Buffer supplemented with protease and phosphatase inhibitor, and nuclease benzonase (CSAA1 components). After centrifugation (10,000 rpm, 4 min, 4 °C), protein content in experimental and control supernatants was determined using Bradford reagent. Then, the samples were diluted to 1 mg/ml protein content and incubated 30 min in the dark with fluorochromes Cy3 or Cy5, respectively. The unbound dye was removed by centrifugation (4000 rpm; 4 min) of the SigmaSpin Columns (CSAA1 components) filled with 200 μl of the labeled protein samples. One microarray was incubated (40 min, room temperature) on a rocking shaker in 5 ml of the mixture of the control and experimental samples (10 μg/ml each) labeled with Cy3 and Cy5, respectively. Another microarray was incubated with the oppositely labeled samples: Cy5 and Cy3, respectively. Such swapped staining provided verification of results and compensation of a potential bias in binding of Cy3 or Cy5 dyes to protein samples. This ensures the double test and full control of the experiment. After following triple washing in the washing buffer (CSAA1 component) and triple washing in pure water, the microarray slides were air dried overnight in the dark.

The microarrays were scanned on the GenePix 4100A Microarray Scanner (Molecular Devices, USA) at 532 and 635 nm (fluorescence maximums of Cy3 and Cy5, respectively). The fluorescence images of the antibody microarrays were analyzed and normalized (ratio-based normalization) using the software GenePix Pro 6.0. The fluorescence values in the rings around each spot served as a background. The median fluorescence value over all spot pixels was proportional to the protein content. The ratios of the experimental to control values characterized the difference in the protein level between axotomized and control VNC ganglia (Demyanenko and Uzdensky 2017). Two samples, labeled independently and reversely in duplicate, provided four experimental values for each protein. Each experiment was repeated twice, and the experiment/control ratios were averaged ($n = 8$). The Student's *t* test and 95% significance level were used. The data are presented as means ± SD. Only proteins whose expression differed more than 40% from control are shown in Table 1 and discussed.

Fig. 1 The nervous system of crayfish (a) and the scheme of axotomy of the ventral nerve cord. (b) The abdominal ganglion of the ventral nervous cord with a pair of connections. (c) The ventral nerve cord consisting of six abdominal ganglia. (d) Dissection of the ventral nerve cord



Western Blot

After isolation, transection, and 4-h incubation in the dark, three experimental and three control VNCs were homogenized by ultrasound on ice in the Extraction/Labeling Buffer supplemented with nuclease benzonase and inhibitors of proteases and phosphatases (CSAA1 components). The homogenates were centrifuged 10 min at 10,000g and 4 °C. The samples containing 10–20 µg of protein were subjected to electrophoretic separation in polyacrylamide gel (7–10%) in the mini-PROTEAN Tetra cell (Bio-Rad). The protein standard was ColorBurst Electrophoresis Marker (C1992, Sigma-Aldrich). After separation, proteins were electro-transferred on the Immuno-Blot PVDF Membrane (Bio-Rad) using a Trans-Blot® Turbo Transfer System (Bio-Rad, USA). After washing by PBS, the membrane was consecutively incubated 1 h in blocking buffer (TBS 1% Casein Blocker, Bio-Rad) and incubated overnight with primary antibodies at 4 °C. The following primary rabbit antibodies, the same as in CSAA1—anti-HDAC1 (H3284, 1:1000) and anti-caspase 3 (C9598, 1:500)—were used. Anti-β-actin (A5441, 1:5000) served as a secondary antibody. The membranes were washed by TTBS (10 mM Tris-buffer, pH 8, plus 0.1% Tween-20), incubated 1 h with the secondary anti-Rabbit IgG peroxidase antibody (1:1000) at room temperature, again washed in TTBS and incubated with Clarity Western ECL. The chemiluminescence

was registered using the Gel Documentation system Fusion SL (Vilber Lourmat, France) using the software Vision Capt. Statistical analysis was performed using one-way ANOVA RM. The difference were considered to be reliable at $p < 0.05$.

Results

The changes in the protein profile in VNC ganglia at 4 h after bilateral axotomy included upregulation of 36 proteins, and downregulation of 35 proteins (Table 1). These proteins perform a variety of functions such as intracellular signaling, epigenetic processes, regulation of apoptosis, remodeling and destruction of the cytoskeleton, deterioration of vesicular transport, and synaptic processes.

The expression of various signaling proteins such as transcription factor c-Myc, protein kinases p38, PKCα, ERK1 and ERK2, MAPKAPK2 (MAPK-activated protein kinase-2), protein phosphatase MKP-1 (MAP kinase phosphatase-1), calmodulin, estrogen receptor, and APP (β-amyloid precursor protein) increased in VNC ganglia more than 1.4 times after axotomy. Simultaneously, other signaling proteins—NAK (kinase activating the transcription factor NF-κB), GAPDH (glyceraldehyde-3-phosphate dehydrogenase), EGF receptor, and protein S-100—were downregulated by 1.6–2.2-fold (Table 1).

Table 1 Changes in the expression of signaling proteins the crayfish abdominal ventral cord ganglia at 4 h after bilateral axotomy (Exp). Control samples (Ctr)-ventral nerve cords, in which nerves were transected only at margins. Mean Exp/Ctr ratios and standard deviations (SD) are shown. Ratios > 1.4 are shown

Proteins	Mean	SD	Functions
▲Exp/Ctr			
Signaling			
c-Myc	2.40	0.64	Transcription factor; controls protein synthesis proliferation, potentiates apoptosis
ERK1/2	1.77	0.23	MAP kinase. Activated by external stimuli; phosphorylates various transcription factors and cytoskeleton proteins; regulates proliferation, cell shape, and survival
ERK1	1.70	0.18	Activated by external stimuli; phosphorylates various transcription factors and cytoskeleton proteins; regulates proliferation, cell shape, and survival
p38	1.47	0.06	Stress-activated MAP kinase; stimulates brain apoptosis in stroke
PKC α	1.45	0.06	Protein kinase C α . Regulates metabolism, proliferation and apoptosis
APP	1.51	0.12	β -amyloid precursor protein. Induces oxidative stress and astrocytosis
Epigenetic processes			
HDAC 1	1.84	0.31	Histone deacetylase 1
HDAC 2	1.53	0.10	Histone deacetylase 2
HDAC 4	1.53	0.22	Histone deacetylase 4
HAT1	1.48	0.11	Histone acetyltransferase 1
PCAF	1.44	0.12	Histone acetyltransferase
Proapoptotic processes			
Bcl-10	2.10	0.27	Activates apoptosis and interacts with apoptosis regulators NF- κ B; TRAF2. IAP. Recruits TRADD and RIP in apoptosis and necroptosis
GADD153	1.94	0.16	Transcription factor. Induced by stress including ischemia and DNA damage. Inhibits proliferation, induces apoptosis
E2F1	1.82	0.21	Transcription factor; drives the cell cycle; can stimulate apoptosis if cell division is impaired
p53	1.74	0.16	Transcription factor for hundreds genes; arrests cell cycle and stimulates apoptosis
NGFR p75	1.64	0.14	NGF receptor. Proapoptotic. Inhibits growth of regenerating axons and induces their degeneration
NMDAR 2a	1.57	0.06	Glutamate receptor: Ca ²⁺ -channel, excitotoxicity, apoptosis
SMAC/DIABLO	1.70	0.24	Apoptosis; activates caspases 9, 3, 6 and 7
AIF	1.66	0.11	Induces apoptotic DNA fragmentation and chromatin condensation
Caspase 3	1.83	0.17	Execution of apoptosis
Caspase 3 active	1.48	0.05	Execution of apoptosis
Caspase 6	1.76	0.11	Execution of apoptosis
Caspase 7	1.67	0.12	Execution of apoptosis
PSR	1.63	0.06	Phosphoserine receptor. Recognition and removal of apoptotic cells
Anti-apoptotic proteins			
MKP-1	1.66	0.21	Protein phosphatase. Anti-apoptotic neuroprotector activated under oxidative stress in Ca ²⁺ -dependent manner
MAPKAPK2	1.61	0.11	Activated by p38 and phosphorylates chaperons hsp25/hsp27 under stress
Calmodulin	1.59	0.14	Anti-apoptotic neuroprotector; stops p38 and JNK signaling, Ca ²⁺ -binding protein. Activates numerous cellular proteins
Bcl-x	1.85	0.47	Antiapoptotic Bcl-2 family protein
p21Waf-1	1.68	0.16	p53-dependent inhibitor of proliferation and apoptosis
Mcl-1	1.49	0.29	Antiapoptotic Bcl-2 family protein
Estrogen receptor	1.59	0.12	Anti-inflammatory and antioxidants action. Modulates protein synthesis and inhibits apoptosis. Protects brain against ischemia
ERK5	1.52	0.06	Anti-apoptotic neuroprotector; activated under oxidative stress in a Ca ²⁺ -dependent manner
Cytoskeleton			
p120CTN	1.79	0.19	Binds E- and N-cadherins to actin cytoskeleton and signaling proteins. Participates in cytoskeleton remodeling during cell movements and shape changes. Involved in neuroprotection during brain ischemia
α -catenin	1.59	0.13	Binds cadherins to actin cytoskeleton. Neuroprotector activity at brain ischemia.
Cofilin	1.73	0.18	Actin depolymerization. Involved in cytoskeleton remodeling, cell motility, endocytosis

Table 1 (continued)

Proteins	Mean	SD	Functions
p35	1.63	0.14	Associated with the adhesion complex β -catenin/N-cadherin. Participates in growth and navigation of axons and neuron migration during cortex neurogenesis and neuromediator release.
Myosin V α (LE-16)	1.55	0.06	Mediates transport of vesicles, organelles and mRNA along actin filaments in neurons and glial cells
▼Exp/Ctr			
Signaling			
NAK	1.68	0.54	Kinase activating NF- κ B. Activated by growth factors and protein kinase C
S-100	2.16	0.25	Ca ²⁺ -binding protein; regulates activity of various enzymes and transcription factors, Ca ²⁺ homeostasis
GAPDH	1.78	0.18	Glyceraldehyde-3-phosphate dehydrogenase involved in expression of antiapoptotic genes
Receptor EGF	1.61	0.21	Stimulates proliferation, differentiation and DNA reparation upon EGF binding.
Epigenetic processes, proliferation			
Cdc7 kinase	1.59	0.13	Initiates and regulates proliferation
Topoisomerase – 1	1.64	0.17	Involved in cell division and transcription
Trf-1	1.55	0.21	Negatively regulates length of telomeres
Histone H3 (Ac-Lys9)	4.13	1.01	Acetylated histone H3
Histone H3 (Ac-Lys9, pS10)	4.62	0.73	Acetylated and phosphorylated histone H3
NTF2	1.69	0.41	Transfers proteins from cytoplasm into the nucleus
Apoptosis			
FADD	1.64	0.41	Fas-associated protein with death domain. Participates in apoptosis initiation
p14 arf	1.38	0.25	Initiates p53-dependent cell cycle arrest and apoptosis
Cell protection			
AOP-1	1.51	0.17	Mitochondrial antioxidant protein (peroxiredoxin-3)
Cytoskeleton			
Ezrine	1.75	0.47	Adapter linking actin filaments to the plasma membrane; regulates adhesion, cytoskeleton remodeling, cell shape, migration, apoptosis
Spectrin ($\alpha + \beta$)	3.10	0.38	Maintains the plasma membrane integrity and cytoskeleton structure. Spectrin cleavage causes membrane blebbing and apoptosis
Dystrophin	3.63	1.78	Component of the scaffold, which binds actin filaments to the plasma membrane
Tropomyosin	3.34	2.06	Associated with actin cytoskeleton
Phospho-Pyk2 (pY579/580)	2.35	0.72	Focal adhesion kinase specific for CNS; phosphorylated Pyk2 influences ERK, JNK and p38 to modulate cytoskeleton, cell morphology, ion channels, cell division and death, neuronal activity
Plakoglobin	1.51	0.10	Homolog of β -catenin. Component of desmosomes and adhesive contacts
β_{1V} -Tubulin	6.00	2.04	Microtubular component. Regulates assembly of microtubules and their interaction with other proteins
β -Tubulin polyglutamated	3.56	0.63	Microtubular component. Regulates assembly of microtubules and their interaction with tau and other proteins
Tau, phosphorylated (pS199/202)	2.00	1.25	Phosphorylated protein tau, regulates assembly and stabilization of microtubules
MAP1	1.42	0.20	Binds to microtubules and regulates their assembly
Cytokeratin 7	5.45	3.50	Intermediate element, cytoskeleton
Cytokeratin 8,60	1.45	0.19	Intermediate element, cytoskeleton
Cytokeratin 19	2.70	0.89	Intermediate element, cytoskeleton
Vesicular transport, synaptic processes			
Adaptin ($\beta 1 + \beta 2$)	2.03	0.40	Vesicular transport, clatrin vesicles
AP2 α	2.43	0.22	Vesicular transport, clatrin vesicles
AP2 γ	3.68	2.24	Vesicular transport, clatrin vesicles
ARNO (Cytohesin 2)	1.69	0.57	Regulates protein sorting and membrane trafficking; controls Golgi structure and function
ARNO3 (GRP1; Cytohesin-3)	1.86	0.60	Regulates protein sorting and membrane trafficking; controls Golgi structure and function
Syntaxin	6.21	3.63	Docking of synaptic vesicles and neuromediator secretion
DOPA decarboxylase	6.51	4.08	Dopamine synthesis

Table 1 (continued)

Proteins	Mean	SD	Functions
Tryptophan hydroxylase	1.72	0.51	Serotonin synthesis
Tyrosine hydroxylase	1.72	0.45	Synthesis of catecholamines (L-DOPA, dopamine, epinephrine, norepinephrine) from tyrosine

Axotomy caused significant upregulation of a number of proapoptotic proteins in the VNC ganglia (Table 1) that include (a) apoptosis execution proteins: Bcl-10, SMAC/DIABLO, AIF, caspases 6, 7, 3, and the active form of caspase 3, and phosphoserine receptor PSR, which marks the apoptotic cells; (b) transcription factors that regulate expression of various apoptotic proteins: E2F1, p53, and GADD153 (growth arrest- and DNA damage-inducible gene 153); and (c) proteins that perform various cellular functions, but can initiate apoptosis in specific conditions: NGF receptor p75 and glutamate receptor NMDAR2a. The upregulation of caspase 3 at 4 but not 1 h after axotomy was confirmed by western blot (Fig. 2).

At the same time, different anti-apoptotic and prosurvival proteins—Bcl-x, Mcl-1, p21WAF-1, ERK5, calmodulin, and estrogen receptor—were upregulated. The anti-apoptotic tendency also included downregulation of mitochondrial antioxidant AOP-1 and proapoptotic proteins FADD (Fas-associated protein with death domain) and p14arf (Table 1).

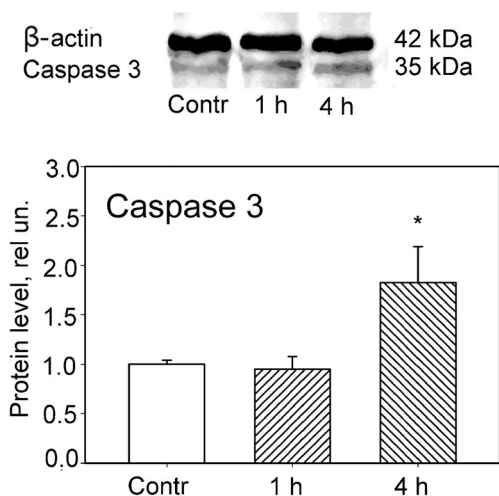
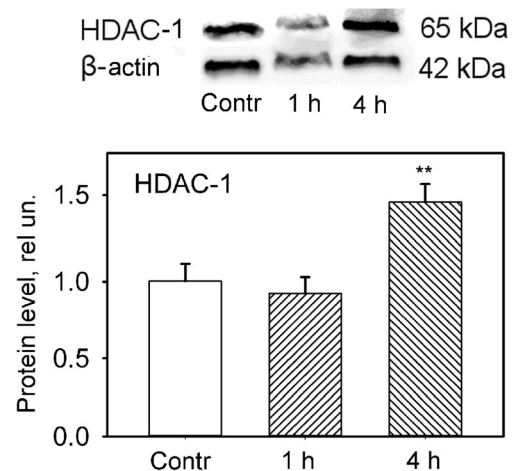
The increase in the protein levels may be partially associated with 44–48% overexpression of histone acetyltransferases HAT1 and PCAF, because histone acetylation facilitates protein synthesis. However, the acetylation of histone H3 decreased more than four times. Probably, it was associated with 53–84% upregulation of histone deacetylases HDAC1, HDAC2, and HDAC4 (Table 1). The upregulation of HDAC1 in the VNC ganglia at 4 but not 1 h after axotomy

was confirmed by western blot (Fig. 3). Possibly, certain signaling cascades and transcription proteins but not epigenetic processes could be responsible for the upregulation of specific proteins in the VNC ganglia after axotomy.

Downregulation of cdc7 kinase, topoisomerase 1, Trf-1 (telomere-binding factor-1), and NTF-2 (nuclear transport factor 2) indicated the deteriorations of the cell cycle.

The expression of various cytoskeleton elements differently changed in the axotomized ganglia. The downregulation of cytokeratins 7, 8.60, and 19 indicated the impairment of intermediate filaments. The microtubule cytoskeleton was also deteriorated. Actually, tubulin β_{IV} and its polyglutamated form were downregulated. This could be associated with the downregulation of MAP1 and phosphorylated tau protein, which bind to the microtubules and regulate their assembly. More complicated changes of the actin cytoskeleton were observed in the axotomized VNC ganglia. Cofilin, protein p35, and catenins alpha and p120CTN and myosin V α (LE-16) were overexpressed (Table 1). However, a number of proteins such as ezrine, spectrin ($\alpha + \beta$), and dystrophin that bind actin cytoskeleton to the plasma membrane, tropomyosin, which binds to the actin cytoskeleton, phosphorylated form of focal adhesion kinase Pyk2, and placoglobin that participates in adhesive contacts, were downregulated.

Vesicular transport and synaptic processes were also impaired. The expression of proteins AP2 α , AP2 γ , and adaptins ($\beta 1 + \beta 2$), which mediate formation of clathrin vesicles,

**Fig. 2** The data of western blot analysis of caspase 3 expression in the crayfish ventral cord after bilateral axotomy. * $p < 0.05$ **Fig. 3** The data of western blot analysis of HDAC-1 expression in the crayfish ventral cord after bilateral axotomy. ** $p < 0.01$

decreased. Additionally, syntaxin involved in docking of synaptic vesicles to the synaptic membrane, and cytohesins 2 and 3 (ARNO and ARNO3), which control protein sorting and membrane traffic in Golgi complexes, were downregulated. The synthesis of neurotransmitters catecholamines, dopamine, and serotonin was also disrupted, as indicated by the downregulation of DOPA decarboxylase, tyrosine, and tryptophan hydroxylases (Table 1).

Discussion

In the present work, we introduced the novel experimental model for study of the axotomy consequences—the bilaterally axotomized ganglia from the crayfish ventral cord. This object is rather simple, inexpensive, and easily prepared. Control VNCs were also axotomized at their edges, but much less. They consisted of four non-axotomized and two edge ganglia, which were transected unilaterally.

Axotomy disrupts the neuronal communications between the VNC ganglia. Main injurious factors at the axon transection site include impairment of ionic homeostasis with Ca^{2+} and Na^+ penetration and loss of K^+ that leads to depolarization, edema, disruption of energy metabolism, and formation of the signaling complex that is transported retrogradely to the neuronal body (Rishal and Fainzilber 2014). The present study demonstrated that axotomy induces up- or downregulation of diverse proteins involved in cell signaling, epigenetic processes, apoptosis regulation, cytoskeleton remodeling, vesicular transport, and synaptic processes. Protein downregulation was apparently the result of tissue destruction and proteolysis, whereas upregulation could be the result of additional synthesis. However, the signaling pathways and transcription factors that regulate expression of specific genes in the injured nervous tissue are still incompletely known.

Protein biosynthesis in the cell is globally regulated by epigenetic processes such as acetylation/deacetylation of histones that increase or decrease the transcriptional activity of the genome. Acetylation of histone H3 loosens DNA packing in the chromatin and facilitates binding of transcription factors and RNA polymerase II to gene promoters. Deacetylation of histone H3, oppositely, leads to chromatin condensation and suppression of protein synthesis. The observed upregulation of histone acetyltransferases HAT1 and PCAF could stimulate protein biosynthesis (Contestabile and Sintoni 2013; Kukucka et al. 2013). However, fourfold decrease in the acetylation of histone H3 that could be performed by histone deacetylases HDAC1, HDAC2, and HDAC4, which were upregulated, suggests that epigenetic processes did not play a significant role in protein upregulation in the VNC ganglia after axotomy. Probably, activation of some signaling cascades and transcription factors was more important.

The proteomic experiments revealed the downregulation of some proteins that regulate the cell cycle in the axotomized ganglia: Cdc7 kinase that initiates and regulates the cell division, topoisomerase-1 that is involved in DNA replication, Trf-1 that regulates the telomere length, and NTF2 that transfers proteins from the cytoplasm into the nucleus.

Pro-apoptotic Proteins

Axotomy induced overexpression of various proteins that execute the apoptotic program in the VNC ganglia: SMAC/DIABLO, AIF, and caspases 3, 6, and 7. In the axotomized VNC ganglia, apoptosis could be initiated by different pathways. Overexpression of glutamate receptors NMDAR2a induces massive Ca^{2+} influx and excitotoxic cell death (Quillinan et al. 2016). The presence of glutamate NMDA receptors in the crayfish nervous system and their role in neuroglial relationships has been earlier reported (Gafurov et al. 2002). The receptor of neurotrophic factors p75 induces neuronal apoptosis upon binding of neurotrophins (Roux and Barker 2002). Its presence in the crayfish nervous system has been recently reported (Kolosov et al. 2016). Bcl-10, whose level increased twofold after axotomy, interacts with different apoptosis regulators such as NF- κ B, TRAF2, IAP, and recruits TRADD and RIP proteins that mediate apoptosis and necroptosis (Thome 2004).

Transcription factor GADD153 is usually weakly expressed in normal cells. However, DNA damage, or inhibition of proliferation, or other damage induce its upregulation and following expression of the downstream genes. Its activation leads to ROS production, glutathione depletion, downregulation of Bcl-2, and finally to apoptosis (Onoue et al. 2005; Oyadomari and Mori 2004). Probably, overexpression of GADD153 in the axotomized VNC ganglia could induce apoptosis.

More than twofold overexpression of the ubiquitous transcription factor c-Myc in the axotomized VNC ganglia was the greatest among studied proteins. c-Myc is a multifunctional master regulator of diverse cellular processes such as anabolic and energy metabolism, cell growth, proliferation, and apoptosis depending on the cell context (Dang 2012). The default function of c-Myc is suggested to be at the head of cascades that drive apoptosis rather than cell cycle progression (McMahon 2014). C-Myc controls the expression of 10–15% of all genes due to regulation of global chromatin structure. In the complex with proteins Max and TRRAP, it attracts histone acetyltransferases GCN5 and TIP60, which maintain euchromatin in an open, accessible state thereby facilitating protein synthesis (Eilersm and Eisenman 2008; Knoepfler 2007). Belin et al. (2015) reported that c-Myc mRNA was overexpressed in the mouse dorsal root ganglion at 1 day after sciatic nerve injury. However, its level decreased 3 days later. This could lead to survival and regeneration of peripheral

sensory neurons. The expression of c-Myc at shorter intervals after nerve injury was less studied. One can suggest that the upregulation of c-Myc in VNC ganglia at 4 h after axotomy was proapoptotic.

Overexpression of stress-activated MAP kinase p38 played apparently a proapoptotic role in the axotomized VNC ganglia. Similarly, the damage to the optic nerve induced NMDA receptor-mediated glutamate neurotoxicity, in which p38 mediated apoptosis of retinal ganglion cells (Kikuchi et al. 2000). Oppositely, inhibition of p38 reduced loss of primary sensory neurons in the rat dorsal root ganglia after sciatic nerve transection (Agthong et al. 2012).

Another master regulator in the cell is the proapoptotic protein p53 (Culmsee and Mattson 2005; Morrison et al. 2003). It is a universal transcription factor for hundreds or even thousands genes (Fischer 2017; Sullivan et al. 2018). It stimulates the expression of various pro- and anti-apoptotic proteins such as caspase 6 and p21Waf-1, which were also overexpressed in the axotomized VNC. The early induction of p53 and pro-apoptotic Bcl-2 family proteins, which mediate mitochondrial dysfunction and the release of proapoptotic proteins, was suggested to be the leading mechanism of apoptosis induction in the ischemic brain (Culmsee and Landshamer 2006; Culmsee and Mattson 2005). Perhaps, axotomy induced the similar proapoptotic mechanism in the VNC ganglia.

The pro-apoptotic activity of p53 is associated with activation of E2F1 that transcriptionally controls the expression of this protein (Hou et al. 2002). E2F1 was also upregulated in the crayfish VNC ganglia after axotomy. It is a master regulator of a variety of cellular processes including the cell cycle, DNA repair, and apoptosis (Raimundo et al. 2012). It controls the expression of a variety of proapoptotic proteins such as caspases 3, 7, 8, and 9, p53, SMAC/DIABLO, Apaf-1, and Bcl-2 family pro-apoptotic proteins (Engelmann and Pützer 2010; Pelengaris et al. 2002). In turn, its expression is controlled by p38 and c-Myc (Bretones et al. 2015; de Olano et al. 2012).

The decrease of the viability of VNC ganglia cells after axotomy was also associated with downregulation of some prosurvival proteins. The decrease in the level of mitochondrial antioxidant AOP-1 could lead to oxidative cell damage. Axotomy also decreased the level of NAK, a protein kinase that activates the prosurvival transcription factor NF- κ B (Häcker and Karin 2006). Another downregulated protein in the axotomized ganglia is S-100. It is a Ca^{2+} sensor in the cell. After Ca^{2+} binding, it regulates Ca^{2+} homeostasis and activity of various enzymes and transcription factors. S-100 is the biochemical marker of brain injury (Korfias et al. 2006). EGF receptor stimulates DNA reparation, proliferation, and differentiation upon EGF binding (Scafidi et al. 2014). Its downregulation in the axotomized VNC ganglia also decreased the prosurvival capability of neuronal and glial cells.

Exposure to the outside of membrane phosphatidylserine is a signal “eat me,” which allows to recognize apoptotic cells and their fragments and stimulate their phagocytic removal (Li et al. 2003). Upregulation of the phosphatidylserine receptor (PSR) indicated the apoptotic process in the axotomized VNC ganglia.

Anti-apoptotic Processes

The overexpression of diverse anti-apoptotic Bcl-2 family proteins such as Bcl-x and Mcl-1 occurred along with upregulation of pro-apoptotic proteins in the axotomized VNC ganglia. Axotomy also induced the upregulation of p21Waf that suppresses p53-mediated apoptosis. Similarly, the upregulation of p21 after axotomy was involved in proliferation of microglia in the transected rat facial nucleus (Yamamoto et al. 2012).

MAP kinases ERK1 and ERK2 phosphorylate and activate various transcription factors and cytoskeleton proteins, and thus regulate cell proliferation, mobility, and survival. In our experiments, the augment of ERK1 was approximately the same as that of ERK1 and ERK2—about 70%, i.e., ERK1 almost entirely determined the reaction of the crayfish neural tissue to axotomy. The upregulation of MAPK-activated protein kinase-2 (MAPKAPK2) after axotomy could be also neuroprotective. Under stress, MAPKAPK2 is phosphorylated by p38, and then it phosphorylates chaperones HSP25 and HSP27, which stabilize protein structure and prevent apoptosis (Piao et al. 2005).

The overexpression of estrogen receptors, which exert a potent neuroprotective and neurotrophic effect, could play a neuroprotective role in the axotomized ganglia. Similarly, estrogens attenuated cell death after cerebrovascular stroke or neurotrauma (Wise et al. 2001). The presence of estrogen receptors in the freshwater crayfish was reported by Paolucci et al. (2002).

Protein phosphatase MKP-1 dephosphorylates proteins and stops their activation. It is known to abolish the proapoptotic activity of MAP kinases JNK and p38 in the ischemic brain, thus participating in the endogenous neuroprotective mechanism (Liu et al. 2014). So, overexpression of MKP-1 in the axotomized VNC ganglia could be neuroprotective.

Calmodulin is a major Ca^{2+} -binding protein in the brain. Ca^{2+} /calmodulin-dependent signaling pathways regulate gene expression, cell survival, production and secretion of neurotransmitters, cytoskeleton remodeling, axonal transport, and other physiological processes. Calmodulin and calmodulin-dependent kinases CaMKII α and CaMKIV are abundant in the brain (Solà et al. 1999). Despite abundance, the calmodulin level increased even more (+ 59%) in the axotomized VNC ganglia that could activate physiological and protective processes in the injured nervous tissue.

Upregulation of protein kinase C α (PKC α) and ERK5, which are activated by Ca²⁺, could be also associated with physiological and protective processes in the axotomized VNC ganglia (Su et al. 2014). Likewise, upregulation of PKC- α after axon injury in the retinal ganglion cells participated in neurite outgrowth in mice (Wu et al. 2003).

Interesting, amyloid precursor protein (APP) was overexpressed in the axotomized crayfish ganglia. APP is involved in the pathogenesis of Alzheimer's disease. It also accumulates in injured nerves (Ahlgren et al. 1996). APP is involved in neuronal differentiation, neuritic outgrowth, synapse formation, long-term memory, and maintaining brain integrity. It is present in the nervous system of various invertebrates (Ewald and Li 2012; Guo et al. 2012; Poeck et al. 2012). APP overexpression in the ischemic brain was considered to play the neuroprotective role (Nalivaeva and Turner 2013). Possibly, it participated in the protection processes in the axotomized VNC ganglia.

The glycolysis enzyme GAPDH could be involved in apoptosis in the axotomized ganglia. The expression of the GAPDH gene remains stable under changing cellular conditions. However, it was shown to contribute to diverse cellular functions unrelated to glycolysis including DNA replication, DNA repair, nuclear RNA export, membrane fusion, and cytoskeletal organization. Pathologically, GAPDH was involved in regulation of apoptosis and neurodegenerative disorders (Berry and Boulton 2000; Tatton et al. 2000).

The downregulation of FADD, which initiates apoptosis upon binding of the extracellular Fas ligand to Fas receptor, and p14arf, which initiates p53-dependent cell cycle arrest and apoptosis (Fadeel and Orrenius 2005), also contributed to anti-apoptotic tendencies in the axotomized VNC ganglia.

Cytoskeleton

An important result of traumatic nerve damage is the changes in expression of cytoskeleton proteins and proteins involved in intercellular interactions. Actin cytoskeleton is responsible for maintaining the cell shape, its remodeling, cell migration, and intracellular movements. In the axotomized VNC ganglia, α -catenin and catenin p120CTN, which bind the intracellular domains of N-cadherin, a component of the cell-cell adhesion complex, to the actin cytoskeletons (Ishiyama and Ikura 2012), were over-expressed. These catenins regulate the cytoskeleton architecture and dynamics. On the contrary, cofilin depolymerizes fibrillar actin that is necessary for cytoskeleton rearrangement and cell motility. Under stress conditions, cofilin forms rods that impede axonal transport and contribute to synaptic deficit (Maloney and Bamberg 2007). The functional role of its overexpression in the axotomized VNC ganglia is not clear. Protein p35, a component of almost all neurons, forms a complex with Cdk5 kinase that mediates binding

of the β -catenin-associated adhesion complex to the actin cytoskeleton. Upregulation of p35 in the VNC ganglia after axotomy may be associated with the cytoskeleton depolymerization and neuronal death, as in the case of cerebral ischemia (Hayashi et al. 1999).

Myosin V α is highly expressed in the brain. It participates in the transport of vesicles, organelles, and mRNA along actin fibers, in exocytosis of secretory vesicles and recycling endosomes in neurons and glial cells. Myosin V α is upregulated in the proximal stump of severed sciatic nerve. It also transfers mRNA from Schwann cells to axons of injured neurons in both vertebrates and invertebrates (Calliari et al. 2002; Sotelo et al. 2013). Its upregulation in the VNC ganglia after axotomy was apparently associated with recovery processes.

Some proteins associated with actin cytoskeleton such as ezrin, dystrophin, placoglobin, spectrin ($\alpha + \beta$), and tropomyosin were downregulated in the axotomized VNC ganglia. Ezrin mediates linking the actin cytoskeleton to the plasma membrane. It regulates adhesion, cytoskeleton rearrangements, shape, and cell migration, and participates in intracellular signaling and apoptosis. Dystrophin is a component of the scaffold, which binds actin filaments to the plasma membrane. Placoglobin is a homolog of β -catenin. It is a component of desmosomes and adhesive contacts. Spectrin ($\alpha + \beta$) that is located on the intracellular side of the plasma membrane maintains its integrity and forms a framework for the actin cytoskeleton. Tropomyosin stabilizes fibrillar actin and mediates the formation of a complex actin network in cells. It controls the dynamics of actin filaments and the cell shape during migration and cytokinesis. The downregulation of these proteins was apparently the result of the nervous tissue destruction after axotomy.

After formation of adhesion contacts, the focal adhesion kinase Pyk2, specific for the nervous system, is phosphorylated and stimulates MAP kinases ERK, JNK, and p38. This modulates the architecture of the cytoskeleton, cell morphology, neuronal activity, cell division, and death. The reduced phosphorylation of Pyk2 after axotomy could be associated with a destruction of the ganglion tissue.

The downregulation of elements of the microtubular cytoskeleton — β_{IV} -tubulin and its polyglutamated form, proteins MAP1 (microtubule associated protein 1) and tau linked to microtubules and regulating their assembly, stability, and connections to other proteins — was also the result of destructive processes in the axotomized ganglia. Likewise, downregulation of these proteins was observed in the isolated chick cortical neurons in the presence of NaCN and calcium ionophore ionomycin (Hutter-Paier et al. 2000). Similarly, the decrease in the levels of cytokeratins 7, 19, and 8.60, elements of the intermediate filaments that form the third cytoskeleton in the cell, could be associated with destruction of the nervous tissue.

Vesicular Transport and Synaptic Processes

Vesicular transport plays an important role in protein processing and sorting after synthesis, in intracellular traffic between ER, Golgi apparatus and plasma membrane, in endo- and exocytosis, and in synaptic transmission. Our experiments showed that axotomy reduced the levels of some proteins involved in vesicular transport and synaptic processes in the VNC ganglia: AP2 α and AP2 γ that form the envelope of clathrin vesicles, and adaptin ($\beta 1 + \beta 2$) that is involved in vesicle formation, and cytohesins 2 and 3 (ARNO) that regulate protein sorting and membrane trafficking, and control Golgi structure and function.

Vesicular transport plays a central role in synaptic transmission. The downregulation of synaptic proteins such as syntaxin involved in docking of synaptic vesicles, fusing with the synaptic membrane, and release of neurotransmitters (Jahn and Fasshauer 2012), led apparently to deterioration of synaptic processes in the axotomized VNC ganglia.

The expression of proteins involved in the metabolism of neurotransmitters was also decreased. In particular, tyrosine hydroxylase that produces L-DOPA from L-tyrosine during biosynthesis of dopamine, norepinephrine, and epinephrine, L-DOPA decarboxylase, which transforms L-DOPA into dopamine, and tryptophan hydroxylase, which synthesizes serotonin, were downregulated.

Conclusion

In the present work, the novel experimental model of nerve injury consequences, the bilaterally axotomized ganglia of the crayfish ventral cord, was suggested. Proteomic antibody microarray study showed the upregulation of 36 proteins involved in signal transduction and epigenetic regulation, pro- and antiapoptotic processes, impairment of actin, microtubule and intermediate filament cytoskeletons, vesicular transport, and synaptic transmission after bilateral transection of the inter-ganglion connectives. Simultaneously 35 proteins were downregulated. Among upregulated proteins were apoptosis execution proteins (Bcl-10, caspases 3, 6, and 7, SMAC/DIABLO, AIF), proapoptotic signaling proteins and transcription factors (E2F1, p53, c-Myc, p38, GADD153), and multifunctional proteins capable of initiating apoptosis in specific situations (p75, NMDAR2a). Simultaneously, overexpression of some anti-apoptotic proteins (Bcl-x, Mcl-1, p21WAF-1, MDM2, MKP1, MAKAPK2, ERK5, APP, calmodulin, estrogen receptor) was observed. Some proteins associated with the actin cytoskeleton and responsible for its stability and rearrangement of the cell shape, migration, intracellular movements, and intercellular interactions (α -catenin, catenin p120CTN, cofilin, p35, myosin V α) were upregulated, whereas other proteins associated with actin cytoskeleton

(ezrin, dystrophin, tropomyosin, spectrin ($\alpha + \beta$), phosphorylated Pyk2) were downregulated. The components of microtubule and intermediate filament cytoskeletons (β_{IV} -tubulin and various cytokeratins) were downregulated that probably was the result of tissue destruction. Vesicular transport and synaptic processes were impaired. The levels of adaptin ($\beta 1 + \beta 2$), clathrin vesicles AP2 α and AP2 γ proteins, and syntaxin decreased. L-DOPA decarboxylase, tyrosine and tryptophan hydroxylases that mediate synthesis of serotonin, dopamine, norepinephrine, and epinephrine were downregulated. Overexpression of histone deacetylases HDAC1, HDAC2, and HDAC4 contributed to suppression of transcription and protein synthesis. The balance of multidirectional processes aimed either at cell death, or repair and recovery, determines the fate of the cells and tissue.

The present data provide the integral, albeit incomplete, view on the nervous tissue response to axotomy. A comparison of the obtained data on axotomy-induced changes in protein composition in the crayfish VNC ganglia with that induced by photothrombotic stroke in the rat brain cortex, which were studied using the same microarrays (Demyanenko and Uzdensky 2017), showed that despite significant differences in the object of study and the method of the nervous tissue damage, many changes in protein profile were similar, both qualitatively and quantitatively. Such similarity suggests some common mechanisms in responses of the nervous system of different animals to different damage modalities. Probably, some of these proteins can be potential markers of nerve injury and targets for neuroprotective therapy.

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