Expression and Localization of C-APP and N-APP in Peripheral Neurons of Rats and Crayfish After Axotomy

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Abstract

Nerve injury induces a cascade of molecular-cellular events, leading to neuronal death or survival, where amyloid precursor protein (APP) and its proteolytic products play an important role. We studied the localization and expression of C-APP and N-APP in rat dorsal root ganglia (DRG) with transected sciatic nerve, axotomized crayfish stretch receptor neuron (SRN) and ventral nerve cord (VNC) ganglia with transected connectives. C-APP and N-APP localized predominantly in neurons, not in glial cells. Axotomy increased C-APP and N-APP expression in rat and crayfish neurons. The expression of APP in crustaceans confirms its conservative nature. In DRG, C-APP level was higher in neuronal nuclei than in cytoplasm in 24 hours post-axotomy. N-APP accumulation was not observed in DRG and crayfish neuronal nuclei. SRN axotomy resulted in C-APP and N-APP accumulation in 4–8 hours in perikaryon and its extensions, but only C-APP accumulated in nuclei. This indicates that not the whole APP, but its C-terminal product, AICD, enters the nucleus. Also, there was high level of C-APP in SRN nucleolus, suggesting possible AICD involvement in rRNA synthesis and ribosome formation. The APP accumulation in transected axons confirms its involvement in injury-induced axonal events.

Introduction

Amyloid precursor protein (APP) is a big transmembrane protein, playing an important role in various processes in different cells of living organism. It is an ancient evolutionary conservative protein^{1,2}. Its intensive studies began since 1980-th because of its central role is the pathogenesis of Alzheimer's disease (AD). However, it is involved in many other processes in the nervous system like development, differentiation, and functioning of neurons, axon growth and the formation of synaptic junctions, long-term memory formation, and in the response of nerve cells to damage^{3–5}. One of the damaging factors for the neuron is axotomy, i.e., full axonal transection, inducing a cascade of molecular-cellular events: disruption of cell homeostasis, cytoskeleton degradation, dysfunction of axonal transport and impairment of intracellular organelles, expression of genes and synthesis of important proteins⁶. As a result, many neurons die, however, in the peripheral nervous system (PNS), about 30% of damaged neurons survive the axotomy unlike those in the central nervous system (CNS), where injured neurons don't regenerate and die⁷. About 10% neurotraumas as peripheral nerve injuries. The situation is worsened by the absence of effective neuroprotector agents, which could save the damaged neurons^{8,9}.

It is suggested that in damaged neurons APP is intensively produced and transported via axon to sites where intracellular contacts are disrupted. Axotomy disrupts axonal transport, and APP accumulates in damaged axons^{10,11}.

Neurosignaling processes, developing at PNS trauma, can involve both full-size APP and products of its proteolytic degradation: sAPP α , sAPP β , A β , AICD, and other less important peptides^{12–14}. So, for example, the accumulation of C-terminal peptide AICD, binding with Fe65 protein, which stabilizes it and promotes its transition to the nucleus, was observed after axotomy. In the nucleus, this complex, together with the

Tip60 protein, promotes the transcription of a number of proteins, involved in apoptosis regulation¹⁵. However, the specific biochemical and physiological functions of APP and products of its proteolysis are yet not completely studied and are of high interest for the understanding of APP-dependent regulation pathways of survival and death of neurons after neurotrauma, for the development of effective neuroprotective agents in the future.

The purpose of this work was to study the expression and localization of APP and products of its proteolysis in neurons of vertebrates and invertebrates after axotomy. We used three models of neurotrauma: axotomy of stretch receptor neuron (SRN) (Fig. 1a) and the transection of connectives of ventral nerve cord (VNC) (Fig. 1b) of *Astacus leptodactylus* crayfish, and the sciatic nerve (SN) transection in rats (Fig. 1c).

Results

Expression and localization of APP in axotomized rat DRG

The immunofluorescent study showed that C-APP and N-APP were localized predominantly in DRG neuron, but not in glial cells, whose numerous nuclei were selectively fluorochromated in blue using Hoechst 33342²⁴ (Fig. 2,3). In the control contralateral rat ganglia, C-APP was localized in cytoplasm of neuronal somata, where its mean level was significantly higher than in nuclei (Fig. 2a,b).

In 24 hours and in seven days after the sciatic nerve transection, mean C-APP level in axotomized ipsilateral ganglia distinctly increased in neurons as compared to control contralateral ganglia, both in nuclei (threefold, $p<0.01$, and 1.8-fold, $p<0.5$, respectively) and in cytoplasm of neurons (by 33%, $p<0.05$, and by 88%, $p<0.01$, respectively) (Fig. 2b). Interesting that in 24 hours C-APP was concentrated in neuronal nuclei, where its level substantially exceeded that in the cytoplasm (Fig. 2a,b,c). The level of N-APP in the cytoplasm of control and axotomized neurons was substantially higher than in nuclei (Fig. 3a,b). In 24 hours, and, especially, in seven days after sciatic nerve transection, it increased even more by 40% ($p<0.05$) and 92% ($p<0.01$), respectively (Fig. 3b). However, unlike C-APP, N-APP accumulation was not observed in neuronal nuclei (Fig. 1a,b,c). Since the whole APP protein is not supposed to enter cell nuclei, this probably indicates that its C-terminal AICD fragment enters the nuclei and starts the transcription of both APP with some elements of its processing, and a number of proapoptotic proteins. However, in seven days this effect disappeared, and C-APP is practically not detected in nuclei, but accumulation in cytoplasm of neuronal bodies (Fig.2). The increased localization of APP in neuronal nuclei, especially in 24 hours post-axotomy, is indicated by an increased in M1 coefficient, which characterizes the colocalization of C-APP with neuronal nuclei marker NeuN (Fig. 2c). The DRG neurons are known to be unipolar. Their processes ramify in sciatic nerve into the afferent and efferent branches. Inside the ganglion, extensions of these neurons have not been detected, so there is no reason to consider APP transport along neurites.

The data of immunoblotting confirm the results of fluorescent microscopy observations. In ipsilateral axotomized rat DRG, C-APP level in nuclear (Fig. 4a) and cytoplasmic (Fig. 4b) fractions significantly increased in 24 hours (threefold, $p<0.01$, and twofold, $p<0.05$, respectively) and in seven days (2.5-fold, $p<0.01$, and threefold, $p<0.01$, respectively) after the sciatic nerve transection as compared to the contralateral ganglia of the same animals. In four hours post-axotomy, no changes were observed, and in 24 hours, the highest increase of C-APP expression was observed in the nuclear fraction, more than two-fold higher than in the cytoplasm (Fig. 4a,b).

The level of N-APP in the nuclear fraction of DRG was low and did not change in four, 24 hours, and seven days post-axotomy (Fig. 4c). But it significantly increased in the cytoplasmic fraction about 1.5-fold ($p<0.05$) in 24 hours (but still not in four hours) and twofold in seven days ($p<0.05$) after the sciatic nerve transection (Fig. 4d). These data also indicate the appearing of C-APP, but not N-APP, in DRG cell nuclei in 24 hours post-axotomy and the accumulation of both C-APP and N-APP in the cytoplasmic fraction in 1–7 days. The first effect probably was the translocation of the transcription active fragment AICD into neuron nuclei, and the second phenomenon was an accumulation of APP in DRG neuron bodies as a result of increased synthesis and/or disrupted transport of this protein into extensions.

Expression and localization of APP in axotomized ganglia of crayfish VNC

According to the data of immunoblotting, the initial level of C- and N-APP in control ventral nerve cord ganglia was low (Fig. 5). In one hour after the bilateral axotomy of VNC ganglia, we observed a significant increase in the expression of N-APP by 34% ($p<0.05$) in the cytoplasmic fraction (Fig. 5d). In four hours, this increase became even more significant (+84%, $p<0.01$, Fig. 4d), although its level in the nuclear fraction did not significantly change (Fig. 5c). The level of C-APP in nuclear and cytoplasmic fraction of VNC ganglia significantly increased in four hours post-axotomy by 150% ($p<0.01$) and 63% ($p<0.01$), respectively (Fig. 5a,b).

Expression and localization of APP in crayfish SRN

The specifics of intracellular localization and redistribution of APP in neuron after axotomy were also studied on the CSR model. Both C-APP and N-APP, in control intact samples and in four-eight hours after axon transection, were found exclusively in SRN, but not in the surrounding glial cells, whose nuclei were imaged using Hoechst 33342 fluorochrome (Fig. 6). In intact neurons, preserving axon integrity, C-APP was localized mainly in perikaryon and less in the nucleus (Fig. 6). Interesting that C-APP level in CSR nucleolus was increased as compared to the karyoplasm, and the nucleolus was imaged distinctly (Fig. 6a). The CSR dendrites had weak fluorescence, and the axon did not fluoresce at all. The level of C-APP in the perikaryon and other parts of CSR significantly increased in four (by 50%, $p<0.05$) and especially in eight (by 150%, $p<0.001$) hours post-axotomy (Fig. 6c). In four hours after the transection, the fluorescence appeared in the axon (the fourfold increase as compared to intact neurons, $p<0.001$), which increased even more in eight hours post-axotomy (ninefold, $p<0.01$), (Fig. 6c). The level of the protein in the nucleus was increased twofold in four hours post-axotomy ($p<0.01$) and threefold in eight hours

($p < 0.001$), respectively (Fig. 6c). In eight hours, large dendrites and the areas where they are attached to the receptor muscle and the dendrite endings ramify, were clearly imaged (Fig. 6a).

N-APP, unlike C-APP, was not detected in the nucleus and the nucleolus of CSR, both in the control and post-axotomy (Fig. 6b). It was localized in SRN perikaryon, and also in the receptor muscle, in the area of its contact with dendrites (Fig. 6b). This area was noticeably wider than in case of C-APP. In four and eight hours post-axotomy, the fluorescents in these areas progressively increased and appeared in SRN axon (Fig. 6b,d). The dendritic tree was not distinguished as clear, as in case of C-APP, but the area of dendrite-muscle contact was wider and, in eight hours post-axotomy, fluoresced almost as brightly as neuronal perikaryon (Fig. 6b,d). Note that the fluorescence increase of both C-APP and N-APP in dendrites developed later than in perikaryon and axon. It significantly exceeded the control level in eight hours, but not in four hours (Fig. 6d).

Discussion

Neurotrauma is one of the leading causes of disability and death of people in the world, especially for young and middle-aged males. Injuries of peripheral nerves make a substantial contribution to this sad statistics against the background of lack of effective agents that are able to protect nerve cells^{8,9}.

Axotomy is a heavy traumatic impact for a neuron, involving full axon transection and initiating a cascade of signal and metabolic processes, leading a cell to two basic scenarios: death or regeneration with following recovery of neural connections^{27,28}. In our research, we used three models of peripheral nerve injury, which made it possible to study intracellular localization and expression of C-APP and N-APP in detail both in vertebrate neurons, like in rats, and in invertebrate neurons, like in freshwater crayfish *Astacus leptodactylus*. The anti-rabbit antibodies for mammal C-APP and N-APP, which we used in this work, recognized very well the respective epitopes in the homologous crayfish protein. This indicates the conservativity of APP and its presence in invertebrate nervous systems, not only in *C. elegans* and *Drosophila*, which was reported earlier^{1,2}, but in crustaceans as well.

APP is a universal protein, playing an important role in intracellular signaling. The products of its proteolytic degradation sAPP α , sAPP β , A β , and AICD are involved in many physiological and biochemical cell processes^{12–14}. We have demonstrated the increase in the expression of C-APP and N-APP in neurons of dorsal root ganglia of rat after the transection of sciatic nerve that innervates hindlimbs. It agrees with previous studies, which showed an axotomy-induced upregulation of APP in sensory neurons of DRG²⁹ and in motor neurons of motor nucleus of facial nerve³⁰.

Neurotrauma induces synthesis of important proteins, including nerve growth factor (NGF), which can increase APP expression. It was proved that NGF modulates APP expression in neurons³¹. NGF is one of the most important players in neural signal transduction, responsible for neuron survival, growth and regeneration of axons, formation of neural connections, and synthesis of neurotransmitters and

neuropeptides³². Damaged neurons can send to glial cells signals to increase the expression of neurotrophic factors, which they require for regeneration and following survival³³.

The increase in Ca^{2+} concentration and nitric oxide (NO) can be other factors of APP upregulation in axotomized neurons. Ca^{2+} is a key secondary messenger of many signal pathways, regulating cellular homeostasis. Ca^{2+} plays an important role in many neurodegenerative processes³⁴, including responses of nerve cells to nerve injury³⁵⁻³⁷. Recent studies have shown overexpression of Cav3.2 T-type Ca^{2+} channels in DRG after spinal nerve transection³⁷. Ca^{2+} can enter axon in the site of transection and induce retrograde axonal signaling³⁶. Ca^{2+} promotes NO level increase via Ca^{2+} -calmodulin system of constitutive nitric oxide synthases (NOS), including neuronal and endothelial NOS isoforms³⁸. NO is a universal neurotransmitter, involved in many physiological and pathological processes. Studies have shown that after peripheral nerve injuries, an increase in neuronal NOS (nNOS) expression in neurons is observed, and nNOS is transported to the injury site, which indicates the involvement of NO/nNOS in regeneration³⁹. Excessive production of NO induces oxidative stress, which is typical for the disturbance of intracellular homeostasis after neurotrauma¹⁶. In turn, it was shown that oxidative stress is accompanied by an increase in APP production, in particular, its proteolysis product sAPP β . At the same time, NO regulates the activity of BACE1 (beta-site APP cleaving enzyme 1) protease⁴⁰. BACE1 is actively expressed in neurons and, together with γ -secretase performs APP proteolysis to amyloid β ($\text{A}\beta$) and small C-terminal peptide AICD⁴¹.

Interesting, that a frank nuclear localization of C-APP was observed in 24 hours post-axotomy, which was not noticed in any series of experiments with N-APP. This effect was observed both in DRG neurons and in stretch receptor neurons. In the latter, it was less expressed relative to cytoplasm in four-eight hours post-axotomy. We suggest that this is C-terminal AICD fragment, because full-size APP is not expected to enter the nucleus. Recent studies have shown that full-size APP is localized in perikaryon without entering the nucleus^{42,43}. However, there are data on interaction and formation of relatively stable APP or APP fragment, exceeding AICD, with Tip60, Fe65, and Pat1a, which is further probably translocated into the nuclear area. APP can bind with GM1 ganglioside, which is widely present in nerve tissue and localized in cytoplasm, as well as in nuclear envelope⁴⁴.

Near the cell nucleus, APP couples with the Fe65 protein, which stabilizes it and facilitates its transition to the nucleus. There, together with the Tip60 protein, this complex stimulates transcription of a number of proteins, involved in the regulation of apoptosis and Alzheimer's disease. They include APP itself, β -secretase BACE1, Tip60, neprilysin, destroying $\text{A}\beta$, proapoptotic proteins p53, KAI1, GSK-3 β , CHOP, cyclins B1 and D1, regulating cell cycles, aquaporin AQP-1, cytoskeleton dynamics regulators transgelin and actin α 2, vesicular glutamate transporter VGLUT2^{41,45-48}. The absence of nuclear localization of N-APP in DRG and CSR neurons is an evidence for our suggestion. It is also confirmed by the significant decrease in C-APP level in neuron nuclei on seventh day after sciatic nerve transection, since AICD is subjected to rapid proteolysis by caspases with the formation of a fragment, named C31⁴⁸.

The high level of C-APP in nucleolus provokes a high interest. The main function of the nucleolus is the synthesis of rRNA and ribosomes, which are required to support intracellular homeostasis. C-APP localization in the nucleolus suggests its involvement in these complex processes. Earlier studies showed that APP in nucleolus can be involved in preribosomal rRNA processing and export the pre-60S ribosomal particles to the cytoplasm⁴⁴. Perhaps, in our work, we observe the AICD localization in the nucleolus being more dense than in surrounding karyoplasm. This corresponds to the recent study by Hicks et al., where the predominant localization of AICD in neuron cell nucleolus was shown^{42,43}.

The increase in the level of both C-APP and N-APP in SRN soma and its processes indicates the increased synthesis of this protein in the cytoplasm and its transport along the extensions to the periphery. Since the SRN axon is transected, APP transport is limited to the preserved part of the axon, where this protein accumulates. APP is transported along microtubules of axons and dendrites to neuron periphery. In axons, it is transported by kinesin-1, and it is unknown how is it transported in dendrites¹⁰. The increased transport of APP into SRN dendrites and their terminal parts, where they are attached to receptor muscle fibers, evokes interest. Perhaps, APP enters them from dendritic endings. Since the adhesive role of APP in the formation of synaptic contacts between the neurons is known¹⁰, the presented data can also indicate the involvement of APP in the formation of contacts between the dendrites of stretch receptor neurons with muscle fibers,

The absence of both C-APP and N-APP in axons of intact SRN, connected to the ventral nerve cord ganglion, confirms the involvement of APP in injury-induced axonal events. These data correspond to previous studies, where APP expression was not observed in healthy axons, but appeared in damaged ones⁴⁹.

However, the obtained results require further research. The study of nuclear translocation of APP C-fragments in neurons after axotomy is planned to be a subject of further studies: the role of AICD/Fe65 complex will be estimated. The inhibitor/activator analysis of C-APP and N-APP will make it possible to estimate the role of APP and products of its proteolysis in survival and death of neurons after axotomy.

Conclusion

Thus, we studied the expression of C-APP and N-APP on three models of neurotrauma from vertebrates and invertebrates. It was demonstrated that APP is present not only in the nervous systems of invertebrates like *C. elegans* or *Drosophila*, but in crustaceans as well, confirming the conservative nature of this protein. C-APP and N-APP were localized predominantly in neurons, but not glial cells in DRG. Axotomy induced the increase in the level of C-APP and N-APP in DRG and crayfish neurons. The increase in the level of C-APP, but not N-APP in SRN and DRG nuclei in four, eight, 24 hours, and seven days post-axotomy, accordingly, indicates that soluble AICD fragment, but not the whole APP, enters the nucleus. The high level of APP in SRN nucleus can indicate the role of AICD in rRNA synthesis and ribosome formation. The accumulation of C- and N-APP in damaged axons confirms the role of APP in injury-induced axonal events.

Materials And Methods

Reagents

Xyla (2% xylazine hydrochloride solution) was purchased from Interchemie Werken "de Adelaar" BV (Netherlands). Telazol (a combination of tiletamine and zolazepam hydrochlorides) was purchased from Zoetis (United States). All antibodies, used in this work, were purchased from "Sigma-Aldrich-Rus" company (Moscow, Russia)

Objects and procedure of axotomy

The study was performed on three model objects: isolated abdominal crayfish stretch receptor (CSR) (Fig. 1a), bilaterally axotomized ventral nerve cord ganglia of crayfish (Fig. 1b), dorsal root ganglia of rats with transected sciatic nerve (Fig. 1c).

Isolated abdominal CSR. CSR is a convenient for study neuroglial object, consisting of two stretch receptor neurons, surrounded by the envelope, consisting of satellited glial cells, and attached to the two receptor muscles (Fig. 1a). There are two CSRs in each ventral segment, located on the inner surface of dorsal carapace. The mechanoreceptor neurons have several big dendrites, ramifying on the receptor muscle into smaller branches, and an axon. When the muscle is stretched, the dendrite membrane depolarizes, and the receptor potential is generated. The slowly adapting neuron constantly generates electric spikes with frequency, proportional to the extension of the receptor muscle, while the other neurons has phasic activity in the form of a series of spikes in response to the extra stretching of the muscle. The spikes propagate to the ventral nerve cord ganglion^{16–18}. The ultrastructure of this neuron was studied in detail in many works^{19–21}. It proved itself a convenient model object for studying the response of neurons and glial cells to various damaging impacts including axotomy¹⁷.

The CSRs were isolated via the technique, developed in our laboratory, which preserves an intact receptor with neurons keeping their connection to the corresponding ventral nerve cord ganglion¹⁸. The receptors were isolated with small fragments of chitin carapace which there were attached to, and placed into a plexiglas chamber, filled with 2 ml van Harreveld's solution for crustaceans (mM: NaCl - 205; KCl -5.4; NaHCO₃ - 0.24; MgCl₂ - 5.4; CaCl₂ - 13.5; pH 7.2-7.4). The chamber was equipped with one fixed hook and one mobile hook to adjust the spiking frequency via the stretching of receptor muscles. We established the spiking frequency about 6-10 Hz. The cessation of bioelectrical activity was interpreted as a "functional death" of the neuron. Such SRN were removed from the study. The experiments were performed at the temperature 25±4 °C. The action potential on the axon was recorded extracellularly via suction glass microelectrode, filled with van Harreveld's solution. The spikes were amplified on the bioelectrical activity amplifier. The axon was transected with ophthalmic scissors at 8-10 mm from the neuronal body. Changes in the spiking frequency were recorded continuously before the fixation in paraformaldehyde.

Bilaterally axotomized crayfish ventral nerve cord ganglia. VNC consists of six ganglia, connected via nerve tracts (Fig. 1b)^{17,22}. After cutting off the crayfish abdomen and removal of chitin, the ventral nerve cord was quickly excised and placed into a chamber, filled with the saline. During the isolation of control samples, VNC was transected with ophthalmic scissors before the first and after the sixth ganglions. The experimental VNC were transected 7 times: at the ends (like in the controls) and between ganglia, so that six bilaterally axotomized ganglia were obtained (Fig. 1b). Control or experimental (axotomized) samples of VNC ganglia were taken from five crayfishes and placed together and incubated for one or four hours in the saline at room temperature (22-25 °C).

Dorsal root ganglia of rats with transected sciatic nerves. Dorsal roots ganglia (DRG) of rats contain somata of sensory neurons. Axons of these neurons are included into sciatic nerve responsible for the innervation of hind limbs (Fig. 1c). The rats were anesthetized via intramuscular administration of 0.75 ml mixture of Xyla and telazol in 2:1 ratio. The surgery for the sciatic nerve transection and isolation of DRS was performed according to the protocol, described by Savastano et al.²³. The intact contralateral DRG were used as controls. The anesthetized rats were decapitated using guillotine in 24 hours or seven days after the unilateral sciatic nerve transection.

Animal keeping. The experiments involving sciatic nerve transection and the extraction of dorsal root ganglia and were carried out on adult Wistar male rats (200–250 g), kept in standard cages in groups of 4–5 animals with free access to food and water. The animal holding room was maintained in standard conditions: 12 light/12 dark cycle, 22–25 °C, an air exchange rate of 18 changes per hour. The experiments involving the isolation of crayfish stretch receptor and ventral nerve cord were varied out on crayfishes, kept in special containers, filled with fresh water at 3-6 °C.

Immunofluorescent microscopy

For the detection of APP and, partially, products of its proteolysis, we used antibodies, specifically recognizing N- or C-terminal fragments of APP molecule (N-APP and C-APP). According to the manufacturer's data (SigmaAldrich), the anti-rabbit N-APP (SAB4200536) antibody recognized the N-terminal extracellular domain of human, rat, or murine APP and the products of its proteolysis, sAPP α and sAPP β . The anti-rabbit C-APP (A8717) antibody is selective for the 676-695 amino acid sequence at the C-terminal of APP, and also for AICD peptide.

To estimate the localization of N-APP and C-APP in stretch receptor neurons after axotomy, isolated SRN-ganglion complexes were placed into the chambers, axotomized and incubated in van Harreveld's solution for four or eight hours. Intact (not axotomized) complexes were used for the control. After that, the axotomized samples, as well as the intact control samples, were fixed in 4% paraformaldehyde for 24 hours, then washed for 24 hours in phosphate-buffered saline (PBS), containing 1% bovine serum albumin (BSA), 0.2% NaN₃, and 1% TritonX-100. After that, the samples were incubated with primary antibodies for C-APP or N-APP, which were diluted in the 1:100 ratio in PBST (10 mM PBS with 2.7 mM KCl, 137 mM NaCl, 0.1% Tween 20, pH 7.4). Then the samples were again washed in PBS for 24 hours

and in incubated for 24 hours with anti-rabbit IgG (H+L), labeled with CF™ 488A (SAB4600045; 1:500), used as a secondary antibody, and again washed in PBS for 24 hours. All 24-hour incubations were performed at 4 °C in refrigerator. Then we added fluorochrome Hoechst 33342 (40 µM), which selectively stains nuclear chromatin in blue²⁴, incubated for ten minutes, three times washed in PBS and placed into 60% glycerol for microscopy.

To detect N-APP and C-APP in DRG in 24 hours or seven days after sciatic nerve transection, we quickly extracted the fourth and the fifth ganglia, fixed them for six hours in 4% paraformaldehyde and incubated for 48 hours in 20% sucrose at 4 °C. Then the ganglia were placed into 4% agarose gel (Lowmelt agarose, SigmaAldrich). Sliced of agarose blocks with 20 µm thickness were obtained in the Leica VT 1000 S vibratome (Germany). After washing in PBS, they were incubated for one hour at room temperature with 5% BSA and 0.3 Triton X-100 to block nonspecific binding sites. Then the slices were incubated with primary rabbit antibodies for C-APP or N-APP (1:100) and murine antibody for the neurospecific nuclear protein NeuN (MAB377, Sigma, 1:100) overnight at 4 °C. After three-times washing in PBS, the slices were incubated with the secondary antibodies anti-rabbit IgG (H+L), CF™ 488A (SAB4600045; 1:500) and anti-mouse IgG1 (γ1), CF™ 555 (SAB4600302; 1:500). The absence of primary antibodies was the negative control. Neuronal and glial nuclei were fluorochromed with Hoechst 33342 (40 µM, 10 min in PBS), then were washed three times for five minutes. Then the slices placed into 60% glycerol for microscopy.

The fluorescent microscopy study was performed on the Olympus BX-51 microscope (Japan), equipped with digital camera OrcaFlash 4.0 V3 (Hamamatsu, Japan).

The intensity of APP fluorescence in the nucleus, perikaryon, axon, and dendrites of SRN was measured using the free ImageJ software (NIH, United States) and averaged. The data were normalized after the subtraction of the background:

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

where I_m is a mean fluorescence intensity in a given area and I_b is a mean background fluorescence outside the object. In the experiments on rat DRG, where the background areas were not present within the limits of the ganglia, the normalization was not performed. The colocalization of C-APP and N-APP with neuron marker NeuN was estimated using ImageJ software with JACoP plugin²⁵. The colocalization coefficient M1 indicates the fraction of pixels in the green channel (APP) relative to the overall signal, recorded in the red channel (a neuron marker NeuN)²⁶. We used at least 100 cells in the calculations. For the quantitative estimation of mean fluorescence level of APP in experimental or control DRG samples, we used ten control and ten experimental images for each of six rats. We estimated the area mean fluorescence of cytoplasm and nucleus for each cell and averaged the obtained values. The analysis of mean fluorescence level in crayfish SRN was performed for one image for each of 10 experimental or 10 control samples.

Immunoblotting

The expression of N-APP and C-APP in nuclear and cytoplasmic fractions of axotomized crayfish ventral nerve cord ganglia or rat dorsal root ganglia was studied using the western blot technique. To obtain the sufficient amount of material for each post-axotomy time period, we combined fourth and fifth lumbar DRG ganglia, taken from three rats, or five experimental or control VNC.

Cytoplasmic and nuclear fractions were extracted using the nuclear fraction extraction kit CellLytic NuCLEAR (SigmaAldrich). The sediment contained cell nuclei, and the supernatant, where the nuclear histone H3 protein was not detected, was used as the cytoplasmic fraction.

The samples, containing 10-20 mkg of protein per 15 mkl, were subjected to electrophoretic separation in polyacrylamide gel (7-10%) in the presence of sodium disulfate in mini-PROTEAN Tetra cell (Bio-Rad, United States). For standard protein marker, we used ColorBurst Electrophoresis Marker (C1992, Sigma-Aldrich). After the separation, the proteins were subjected to electrotransfer to PVDF membrane (polyvinyl difluoride membrane 162-0177, Bio-Rad), using the Trans-Blot® Turbo Transfer System (Bio-Rad). After washing in PBS, the membrane was sequentially incubated for one hour in the blocking buffer (TBS 1% Casein Blocker, Bio-Rad), and overnight at 4 °C with primary rabbit antibodies for C-APP (A8717, SigmaAldrich, 1:500) or N-APP (SAB4200536, SigmaAldrich, 1:500), and β -actin (A5441, 1:5000). After the incubation, the membranes were washed in Tris-buffer with addition of 0.1% Tween-20 (TTVS, 10 mM, pH 8), and incubated for one hour and room temperature with secondary antibody for rabbit IgC peroxidase (A6154, SigmaAldrich, 1:1000). Protein detection was performed on the Clarity Western ECL Substrate (Bio-Rad). Chemiluminescence was analyzed using the Fusion SL gel documentation system (Vilber Lourmat, France). The obtained images were processed using the VisionCapt software kit (Vilber Lourmat).

Statistical analysis

The statistical analysis was performed using OneWay ANOVA with Dunnett's a posteriori test. The result are presented as Mean $\pm$ SEM.

Declarations

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Authors' contributions. Study concept, design, and methodology: Stanislav V. Rodkin, Andrey M. Khaitin, Valentina A. Dzreyan, Svetlana V. Demyanenko; Material preparation – isolation of CSR: Stanislav V. Rodkin, Andrey M. Khaitin, Moez A. Eid; Material preparation – isolation of VNC: Moez A. Eid; Material preparation – sciatic nerve transection, isolation of DRG: Maria A. Pitinova; Data collection from CSR (immunofluorescence study): Stanislav V. Rodkin, Andrey A. Khaitin; Data collection from VNC ganglia (western blot): Svetlana V. Demyanenko; Data collection from DRG (immunofluorescence study, western blot) – Stanislav V. Rodkin, Valentina A. Dzreyan; Data analysis and investigation – Stanislav V. Rodkin, Andrey A. Khaitin, Valentina A. Dzreyan, Svetlana V. Demyanenko; Writing - original draft preparation,

figures: Stanislav V. Rodkin; Writing – review, translation, and editing: Andrey M. Khaitin; Funding acquisition and supervision: Svetlana V. Demyanenko.

Competing Interests Statement. The authors declare no competing interests.

Ethics declaration. The study did not involve human participants. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All experimental procedures were carried out in accordance with the European Union guidelines 86/609/EEC for the use of experimental animals and local legislation for ethics of experiments on animals. The animal protocols were evaluated and approved by the Animal Care and Use Committee of the Southern Federal University (Approval No 08/2016). No special randomization was performed to allocate subjects in the study. All the authors complied with the ARRIVE guidelines.

References

1. Jacobsen, K. T. & Iverfeldt, K. Amyloid precursor protein and its homologues: a family of proteolysis-dependent receptors. *Cell. Mol. Life Sci.* **66**, 2299–2318 (2009).
2. Guo, Q., Wang, Z., Li, H., Wiese, M. & Zheng, H. APP physiological and pathophysiological functions: insights from animal models. *Cell Res.* **22**, 78–89 (2012).
3. Dawkins, E. & Small, D. H. Insights into the physiological function of the β -amyloid precursor protein: beyond Alzheimer's disease. *J. Neurochem.* **129**, 756–769 (2014).
4. Coronel, R. *et al.* Neuronal and Glial Differentiation of Human Neural Stem Cells Is Regulated by Amyloid Precursor Protein (APP) Levels. *Mol. Neurobiol.* **56**, 1248–1261 (2019).
5. Müller, U. C., Deller, T. & Korte, M. Not just amyloid: physiological functions of the amyloid precursor protein family. *Nat. Rev. Neurosci.* **18**, 281–298 (2017).
6. Dzreynan, V., Rodkin, S., Nikul, V., Pitinova, M. & Uzdensky, A. The Expression of E2F1, p53, and Caspase 3 in the Rat Dorsal Root Ganglia After Sciatic Nerve Transection. *J. Mol. Neurosci.* **71**, 826–835 (2021).
7. Dzreynan, V. A., Rodkin, S. V., Pitinova, M. A. & Uzdensky, A. B. HDAC1 Expression, Histone Deacetylation, and Protective Role of Sodium Valproate in the Rat Dorsal Root Ganglia After Sciatic Nerve Transection. *Mol. Neurobiol.* **58**, 217–228 (2021).
8. Hill, C. S., Coleman, M. P. & Menon, D. K. Traumatic Axonal Injury: Mechanisms and Translational Opportunities. *Trends Neurosci.* **39**, 311–324 (2016).
9. Laskowitz, D. & Grant, G. *Translational Research in Traumatic Brain Injury. Translational Research in Traumatic Brain Injury* (CRC Press, 2016). doi:10.1201/b18959.
10. Muresan, V. & Ladescu Muresan, Z. Amyloid- β precursor protein: Multiple fragments, numerous transport routes and mechanisms. *Exp. Cell Res.* **334**, 45–53 (2015).
11. Wang, E. *et al.* Molecular Mechanisms Underlying Effects of Neural Stem Cells against Traumatic Axonal Injury. *J. Neurotrauma* **29**, 295–312 (2012).

12. Kobayashi, S. *et al.* Temporal–spatial expression of presenilin 1 and the production of amyloid- β after acute spinal cord injury in adult rat. *Neurochem. Int.* **56**, 387–393 (2010).
13. Chow, V. W., Mattson, M. P., Wong, P. C. & Gleichmann, M. An Overview of APP Processing Enzymes and Products. *NeuroMolecular Med.* **12**, 1–12 (2010).
14. Brunholz, S. *et al.* Axonal transport of APP and the spatial regulation of APP cleavage and function in neuronal cells. *Exp. Brain Res.* **217**, 353–364 (2012).
15. Liu, C. *et al.* APP upregulation contributes to retinal ganglion cell degeneration via JNK3. *Cell Death Differ.* **25**, 663–678 (2018).
16. Rodkin, S. V., Kovaleva, V. D., Berezhnaya, E. V., Neginskaya, M. A. & Uzdensky, A. B. Ca^{2+} - and NF- κB -dependent generation of NO in the photosensitized neurons and satellite glial cells. *J. Photochem. Photobiol. B Biol.* **199**, 111603 (2019).
17. Rodkin, S. *et al.* 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. *J. Mol. Neurosci.* **70**, 532–541 (2020).
18. Khaitin, A. M., Rudkovskii, M. V. & Uzdensky, A. B. The method of isolation of the crayfish abdominal stretch receptor maintaining a connection of the sensory neuron to the ventral nerve cord ganglion. *Invertebr. Neurosci.* **15**, 176 (2015).
19. Fedorenko, G. M. & Uzdensky, A. B. Ultrastructure of neuroglial contacts in crayfish stretch receptor. *Cell Tissue Res.* **337**, 477–490 (2009).
20. Rudkovskii, M. V., Fedorenko, A. G., Khaitin, A. M., Pitinova, M. A. & Uzdensky, A. B. The effect of axotomy on firing and ultrastructure of the crayfish mechanoreceptor neurons and satellite glial cells. *Mol. Cell. Neurosci.* **107**, 103534 (2020).
21. Fedorenko, G., Neginskaya, M., Fedorenko, A. & Uzdensky, A. The paired neuroglial and interglial membranes in the crayfish stretch receptor and their local disorganization. *J. Neurosci. Res.* **93**, 707–713 (2015).
22. Demyanenko, S., Dzreyan, V. & Uzdensky, A. Axotomy-Induced Changes of the Protein Profile in the Crayfish Ventral Cord Ganglia. *J. Mol. Neurosci.* **68**, 667–678 (2019).
23. Savastano, L. E. *et al.* Sciatic nerve injury: A simple and subtle model for investigating many aspects of nervous system damage and recovery. *J. Neurosci. Methods* **227**, 166–180 (2014).
24. Crowley, L. C., Marfell, B. J. & Waterhouse, N. J. Analyzing Cell Death by Nuclear Staining with Hoechst 33342. *Cold Spring Harb. Protoc.* **2016**, pdb.prot087205 (2016).
25. Bolte, S. & Cordelières, F. P. A guided tour into subcellular colocalization analysis in light microscopy. *J. Microsc.* **224**, 213–232 (2006).
26. Fletcher, P. A., Scriven, D. R. L., Schulson, M. N. & Moore, E. D. W. Multi-Image Colocalization and Its Statistical Significance. *Biophys. J.* **99**, 1996–2005 (2010).
27. Khaitin, A., Rudkovskii, M. & Uzdensky, A. Ca^{2+} mediates axotomy-induced necrosis and apoptosis of satellite glial cells remote from the transection site in the isolated crayfish mechanoreceptor. *Mol.*

Cell. Neurosci. **88**, 7–15 (2018).

28. Duan, X. *et al.* Subtype-Specific Regeneration of Retinal Ganglion Cells following Axotomy: Effects of Osteopontin and mTOR Signaling. *Neuron* **85**, 1244–1256 (2015).
29. Scott, J. N., Parhad, I. M. & Clark, A. W. β -Amyloid precursor protein gene is differentially expressed in axotomized sensory and motor systems. *Mol. Brain Res.* **10**, 315–325 (1991).
30. Palacios, G., Palacios, J. M., Mengod, G. & Frey, P. β -Amyloid precursor protein localization in the Golgi apparatus in neuron and oligodendrocytes. An immunocytochemical structural and ultrastructural study in normal and axotomized neurons. *Mol. Brain Res.* **15**, 195–206 (1992).
31. Nishimura, I., Takazaki, R., Kuwako, K., Enokido, Y. & Yoshikawa, K. Upregulation and antiapoptotic role of endogenous Alzheimer amyloid precursor protein in dorsal root ganglion neurons. *Exp. Cell Res.* **286**, 241–251 (2003).
32. Minnone, G., De Benedetti, F. & Bracci-Laudiero, L. NGF and Its Receptors in the Regulation of Inflammatory Response. *Int. J. Mol. Sci.* **18**, 1028 (2017).
33. Lobanov, A. V. & Uzdensky, A. B. Protection of Crayfish Glial Cells but not Neurons from Photodynamic Injury by Nerve Growth Factor. *J. Mol. Neurosci.* **39**, 308–319 (2009).
34. Gemes, G. *et al.* Depletion of Calcium Stores in Injured Sensory Neurons-Anatomic and Functional Correlates. *Anesthesiology* **111**, 393–405 (2009).
35. Kobeissy, F. H. *Brain neurotrauma: Molecular, neuropsychological, and rehabilitation aspects. Brain Neurotrauma: Molecular, Neuropsychological, and Rehabilitation Aspects* (2015). doi:10.1201/b18126.
36. Rishal, I. & Fainzilber, M. Axon–soma communication in neuronal injury. *Nat. Rev. Neurosci.* **15**, 32–42 (2014).
37. Tomita, S. *et al.* Cav3.2 overexpression in L4 dorsal root ganglion neurons after L5 spinal nerve cutting involves Egr-1, USP5 and HMGB1 in rats: An emerging signaling pathway for neuropathic pain. *Eur. J. Pharmacol.* **888**, 173587 (2020).
38. Eroglu, E. *et al.* Real-time visualization of distinct nitric oxide generation of nitric oxide synthase isoforms in single cells. *Nitric Oxide* **70**, 59–67 (2017).
39. Cristino, L., Pica, A., Della Corte, F. & Bentivoglio, M. Co-induction of nitric oxide synthase, Bcl-2 and growth-associated protein-43 in spinal motoneurons during axon regeneration in the lizard tail. *Neuroscience* **101**, 451–458 (2000).
40. Muche, A., Arendt, T. & Schliebs, R. Oxidative stress affects processing of amyloid precursor protein in vascular endothelial cells. *PLoS One* **12**, e0178127 (2017).
41. Bukhari, H. *et al.* Small things matter: Implications of APP intracellular domain AICD nuclear signaling in the progression and pathogenesis of Alzheimer’s disease. *Prog. Neurobiol.* **156**, 189–213 (2017).
42. Hicks, D. TDP-43 and amyloid precursor protein processing: implications for Alzheimer’s disease. *Neural Regen. Res.* **16**, 1402 (2021).

43. Hicks, D. A., Jones, A. C., Pickering-Brown, S. M. & Hooper, N. M. The cellular expression and proteolytic processing of the amyloid precursor protein is independent of TDP-43. *Biosci. Rep.* **40**, (2020).
44. Okamoto, M. Immunoreactivity of the amino-terminal portion of the amyloid-beta precursor protein in the nucleolus. *Neurosci. Lett.* **521**, 82–87 (2012).
45. Beckett, C., Nalivaeva, N. N., Belyaev, N. D. & Turner, A. J. Nuclear signalling by membrane protein intracellular domains: The AICD enigma. *Cell. Signal.* **24**, 402–409 (2012).
46. Pardossi-Piquard, R. & Checler, F. The physiology of the β -amyloid precursor protein intracellular domain AICD. *J. Neurochem.* **120**, 109–124 (2012).
47. Koistinen, N. A. *et al.* Nuclear localization of amyloid- β precursor protein-binding protein Fe65 is dependent on regulated intramembrane proteolysis. *PLoS One* **12**, e0173888 (2017).
48. Multhaup, G., Huber, O., Buée, L. & Galas, M.-C. Amyloid Precursor Protein (APP) Metabolites APP Intracellular Fragment (AICD), A β 42, and Tau in Nuclear Roles. *J. Biol. Chem.* **290**, 23515–23522 (2015).
49. Truong, P. H. *et al.* Amyloid precursor protein and amyloid precursor-like protein 2 have distinct roles in modulating myelination, demyelination, and remyelination of axons. *Glia* **67**, 525–538 (2019).

Figures

Figure 1

Experimental models of axotomy. (a) – stretch receptor neuron, stained with Hoechst 33342, which imparts blue fluorescence to nuclear chromatin and images all cell nuclei, and propidium iodide, staining

necrotic cell nuclei in red, and the schematic picture of SRN morphology: N - nucleus, P – perikaryon, D – dendrites, g – glial cells, a – axon. (b) – axotomized ventral nerve cord ganglia. (c) – dorsal ganglia of rat

Figure 2

Fluorescence microscopy: (a) expression of C-APP (green fluorescence) in rat DRG neurons in 24 hours and seven days after sciatic nerve transection. Scale bar 50 mkm. (b) mean fluorescence of anti-C-APP antibody in nuclei and cytoplasm of neurons in axotomized ipsilateral (n=6) and control

contralateral(n=6) DRG in 24 hours and seven days after sciatic nerve transection. (c) colocalization coefficient M1 of C-APP and neuronal nuclear marker in axotomized ipsilateral (n=6) and control contralateral (n=6) DRG in 24 hours and seven days after sciatic nerve transection. Denotation: Ipsi – axotomized ipsilateral ganglion, contra – contralateral control ganglion. NeuN – neuronal nuclear marker; C-APP+NeuN – the overlapping of C-APP antibody fluorescence and NeuN fluorescence. Hoechst – Hoechst 33342 fluorescence, imaging all neuronal and glial cell nuclei. *p < 0.05; **p < 0.01

Figure 3

Fluorescence microscopy: (a) expression of N-APP (green fluorescence) in rat DRG neurons in 24 hours and seven days after sciatic nerve transection. Scale bar 50 μ m. (b) mean fluorescence of anti-N-APP antibody in nuclei and cytoplasm of neurons in axotomized ipsilateral (n=6) and control contralateral (n=6) DRG in 24 hours and seven days after sciatic nerve transection. (c) colocalization coefficient M1 of N-APP and neuronal nuclear marker in axotomized ipsilateral (n=6) and control contralateral (n=6) DRG in 24 hours and seven days after sciatic nerve transection. Denotation: ipsi – axotomized ipsilateral ganglion, contra – contralateral control ganglion. NeuN – neuronal nuclear marker; N-APP+NeuN – the overlapping of N-APP antibody fluorescence and NeuN fluorescence. Hoechst – Hoechst 33342 fluorescence, imaging all neuronal and glial cell nuclei. *p < 0.05; **p < 0.01; ***p<0.001

Figure 4

Immunoblotting. Changes in C-APP and N-APP level in nuclear and cytoplasmic fractions of axotomized ipsilateral DRG in 24 hours and seven days after sciatic nerve transection in rats in comparison with contralateral ganglia of the same animals. (a) C-APP, nuclear fraction. (b) C-APP, cytoplasmic fraction. (c) N-APP, nuclear fraction. (d) N-APP, cytoplasmic fraction. Denotation: ipsi – axotomized ipsilateral ganglion, contra – contralateral control ganglion. OneWay ANOVA. $M \pm SEM$. $n=6$. * $p < 0,05$; ** $p < 0,01$

Figure 5

Immunoblotting. Changes in C-APP and N-APP level in nuclear and cytoplasmic fractions of bilaterally axotomized ventral nerve cord ganglia of crayfish in one and four hours after the transection of interganglionic connectives: (a) C-APP, nuclear fraction, four hours. (b) C-APP cytoplasmic fraction, four hours post-axotomy. (c) N-APP, nuclear fraction, one and four hours post-axotomy. (d) N-APP cytoplasmic

fraction, one and four hours post-axotomy. Denotation: contr – intact control. OneWay ANOVA. $M \pm SEM$. $n=6$. * $p < 0,05$; ** $p < 0,01$

Figure 6

Fluorescence microscopy: (a, b) Intracellular distribution of C-APP (a) and N-APP (b) in crayfish stretch receptor in four and eight hours post-axotomy and its colocalization with nuclear marker Hoechst33342. Scale bar 100 μm . (c, d) Fluorescence of anti-C-APP (c) and anti-N-APP (d) antibody in different parts of stretch receptor neuron: nucleus, perikaryon, axons, and dendrite of intact neurons (Int), connected with ventral nerve cord ganglia ($n=10$) and in axotomized neurons ($n=10$) in four and eight hours after axon transection. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$