MECHANISM OF THE "DARK" AXONAL DEGENERATION IN THE CENTRAL NERVOUS SYSTEM

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A KÖZPONTI IDEGRENDSZERBEN LEJÁTSZÓDÓ „SÖTÉT" AXONDEGENERÁCIÓ MECHANIZMUSA
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Background and purpose – In the central nervous tissue, two types of transsection-resulted axonal degeneration are generally accepted: "watery" and "dark". The present paper deals with the assumption that the mechanism of this kind of "dark" axonal degeneration has a relationship with that of the "dark" neuronal degeneration.
Methods – A minute stab wound is inflicted in the parietal cortex of the rat brain. From 1 h to 3 months postinjury, the resulted ultrastructural events in two distant regions of the corticospinal tract (internal capsule and C3 region of the corticospinal tract) are studied.
Results – As a novel finding, the first morphological process of "dark" axonal degeneration was found to consists in a striking reduction of the distances between neighboring neurofilaments, which were readily distinguishable and apparently undamaged. This pattern (compacted ultrastructure) persisted for hours. By day 1 postinjury, the compacted axoplasmic elements aggregated into a homogenous and dense ("dark") mass in which hardly any ultrastructural elements could be distinguished. Surrounded by apparently normal or mildly abnormal myelin sheat, this mass underwent a non-isotropic shrinkage during the next three months. Morphological signs of phagocytosis were insignificant.
Conclusion – The ultrastructural events during the first day post-injury suggest a non-enzymatic mechanism as an alternative to the prevailing molecular-biological mechanism.

Keywords: *cortical stab-wound, corticospinal tract, "dark" axonal degeneration, ultrastructural compaction, non-enzymatic mechanism*

Háttér és célkitűzés – A központi idegrendszerben az átvágással vagy roncsolással előidézett axondegenerációnak két típusa van: a „sötét" (dark) és a „világos" (watery) axondegeneráció. Azt vizsgáltuk, hogy a „sötét" axondegeneráció létrejöttének mechanizmusa hasonlít-e a „sötét" idegsejtekéhez.
Módszerek – Egyetlen kis sérülést okoztunk patkányagy frontoparietalis agykérgében. Az ezt követő egy órától három hónapig tartó időintervallumban az ennek hatására bekövetkező ultrastrukturális eseményeket tanulmányoztuk a corticospinalis traktus két távoli területén (nucleus caudatus, illetve gerincvelő).
Eredmények – A „sötét" axondegeneráció – mindeddig nem vizsgált – első órájában a neurofibrillumok közti távolságok drasztikusan lecsökkentek (kompaktálódott ultrastruktúra). Az egyes neurofibrillumok jól láthatóak maradtak, és épnek tűntek az ezt követő néhány órában. A sértést követő első napra a kompaktálódott axoplazma homogén és sűrű („sötét") masszává tömörült, amelyben nem voltak megfigyelhetők individuális ultrastrukturális elemek. A sértést követő három hónap alatt – látszólag normális, vagy alig károsodott myelinburokkal körülvéve – ez a „sötét" massza anizotrop jelleggel tovább zsugorodott. Phagocytosisra utaló jel alig-alig volt található.
Következtetés – A sértést követő első órák ultrastrukturális eseményei – amelyek hasonlítanak a „sötét" idegsejtek képződésekor megfigyeltekre – arra engednek következtetni, hogy az idegátvágás okozta „sötét" axondegeneráció nem enzimatikus folyamat következménye.

Kulcsszavak: *agykéregsértés, corticospinalis traktus, „sötét" axondegeneráció, ultrastrukturális kompakció, nem enzimatikus mechanizmus*

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Differently from the commonly known ultrastructural course of the Wallerian degeneration distal to a cut or crush in the peripheral nervous system (PNS), two ultrastructural processes have been revealed in the central nervous system (CNS): "watery" and "dark" degenerations[1]. In the former, the myelin sheaths are somewhat expanded and contain either nothing or a few small multilamellar bodies self-assembled from disintegrated ultrastructural elements of the normal axoplasm. In the latter, myelin sheaths are considerably contracted and their interior is so dark and dense that hardly any ultrastructural elements can be distinguished in them.

While numerous morphological and molecular-biological reports have dealt with the consequences of experimentally transected or crushed axons in the PNS and in the optic nerve, there is a paucity of relevant studies in the brain. The present paper involves a qualitative ultrastructural analysis of the "dark" axonal degeneration in the corticospinal tract caused by a minute stab-wound in the motor area of the frontoparietal cortex of the rat brain. Since each of the morphological patterns of axon degeneration is severely influenced by the causing event and the pathological circumstances in and around the injured region[2], we studied two distant areas, the internal capsule and the C3 region of the dorsal corticospinal tract.

Materials and methods

ANIMAL EXPERIMENTS

Animal care and handling were carried out in accordance with order 243/1998 of the Hungarian Government, which is an adaptation of directive 86/609/EGK of the European Committee Council. Before surgical procedures including transcardial perfusion fixation, each rat was anesthetized by the intraperitoneal administration of 2 ml/kg of a 1:1 mixture of 25 mg/ml Thiopental (Biochemie GmbH, Austria) and 5 mg/ml Seduxen (Richter Gedeon Rt, Hungary).

The scalp of each of 33 Wistar rats weighing between 200 and 250 g was incised longitudinally, the edges of the wound were stretched apart, and the soft tissue was removed from the calvaria. A round hole, 2 mm in diameter, centered 0.5 mm rostrally from the bregma and 5 mm laterally from the midline was then carefully drilled into the skull on the right side without damaging the dura, which was then pricked in 30 rats. Thereafter, each skull of these rats was fixed in the head holder of a stereotaxic apparatus. Thereafter a sharp-pointed rod 1 mm in diameter was firmly attached to its horizontal arm, the position of which could be adjusted in three perpendicular directions by micrometer screws. By moving forward the rod, a 1-mm-wide, 4-mm-long stab-wound was made in the brain cortex **(Figure 1A)** at the coronal plane, 1 mm rostral to the bregma, and the horizontal plane, 1.5 mm below the plane passing through the interaural line[3]. These rats were divided into 10 groups, with three rats in each. Immediately after creation of the stab wound, the heart of each of the rats in group 1 was exposed, the left chamber was catheterized, and the right atrium was opened. After a 1-min. transcardial washing with physiological saline, 500 ml of an electronmicroscopic fixative was perfused through the heart, 100 ml at the highest possible rate, then 400 ml in drops, within 30 min. The fixative was prepared by mixing 250 ml of 0.2 M sodium cacodylate, 50 ml of 20% paraformaldehyde, 50 ml of 25% glutaraldehyde, 25 ml of 0.1 M calcium chloride and 125 ml of 10% polyvinylpyrrolidone K25, followed by adjustment of the mixture to pH 7.5 with a few drops of 0.1 M hydrochloric acid.

After suturing the scalp, the 27 non-perfused rats in groups 2-10 were placed onto a warm plate until waking up, and were perfusion-fixed transcardially (see above) 1 h, 3 h, 7 h, 17h, 1 day, three days, 1 week, 1 month or three months later, and the 3 non-stabbed (control) rats 1 week later. Thereafter the rats were placed in a refrigerator (4 °C) until brain autopsy, which was performed 1 day later (in order to avoid the artifactous *post-mortem* formation of "dark" neurons[4].

TISSUE PROCESSING AND STAINING

The brain and segment C3-C5 of the spinal cord of each rat were vibratome-sectioned coronally at 150 µm. Every tenth vibratome section was stained with a silver method[5], which selectively stains the compacted ultrastructure[6] mentioned later. Specifically, the sections were dehydrated with graded 1-propanol, treated at 56 °C for 16 h with 1-propanol containing 0.6% sulfuric acid and 2% water, rehydrated with graded 1-propanol, washed with 1% acetic acid for 5 minutes, placed into a special physical developer until the sections turned light brown, dehydrated with 1-propanol, cleared with clove oil and covered with Canada balsam. From the other vibratome sections, those 2×2-mm^2 parts were dissected for electron microscopy that contained many "dark" axons in the adjacent silver-stained sections. These specimens were post-fixed with a 1:1 mixture of 2% osmium tetroxide and 3% potassium fer-

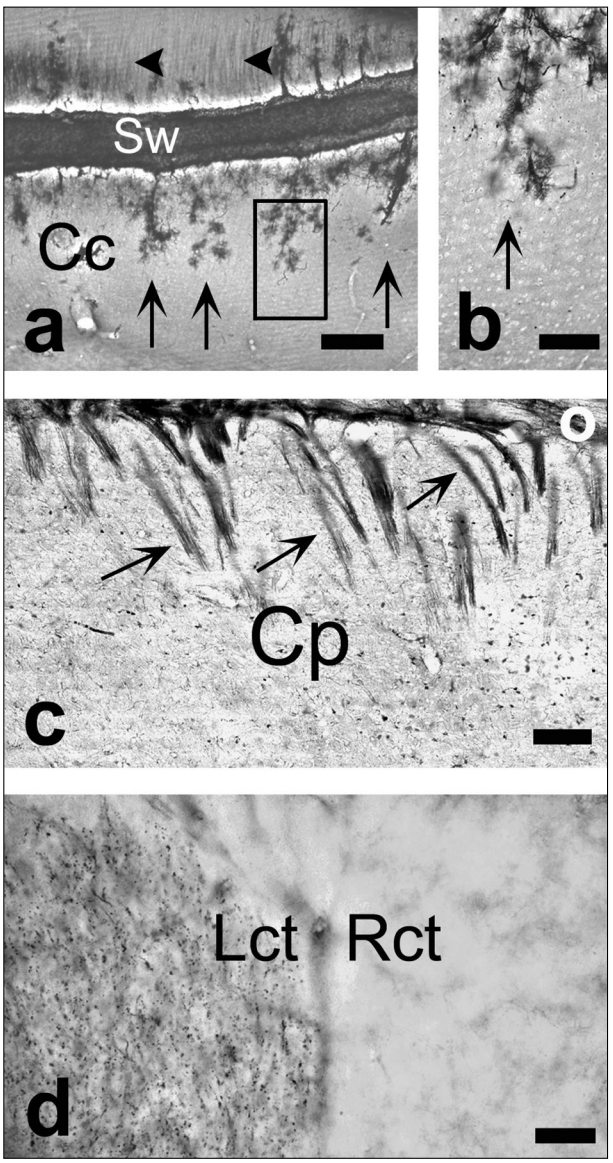

Figure 1. *Silver-stained areas in 150-μm-thick coronal CNS sections cut from rats perfusion-fixed immediately after creation of the stab-wound* **(A** *and* **B)**, *17 h later* **(C)** *and three days later* **(D)**. **A** *and* **B**: *Right cerebral cortex (Cc) at the plain of the stab-wound (Sw); arrowheads point to neuronal dendrites, and arrows to groups of compacted perivascular astrocytes.* **B**: *Magnification of the area boxed in* **A**. **C**: *Right internal capsule (Cp) at the plain of the stab-wound; arrows point to longitudinal bundles of damaged axons.* **D**: *C3 regions of the corticospinal tract; tiny black dots in the left tract (Lct) correspond to cross-sections of "dark" axons; Rct marks the right tract. Scale bars: a=450 μm, b=150 μm, c=300 μm, d=50 μm*

rocyanide for 1 h at room temperature, and then flat-embedded in Durcupan ACM. Semithin sections were cut at 1.0 μm, air-dried onto microscopic slides previously coated with Vectabond adhesive, and stained for 1 min. at 90 °C in a solution containing 0.05% toluidine blue, 0.05% sodium tetraborate and 0.1% saccharose (pH 9.5). Following examination of the light-microscopic picture, the most suitable 1×1-mm^2 was trimmed out from each embedded specimen. Thin sections were cut from these at 40 nm and stained with uranyl acetate and lead citrate in the usual manner. Ultrastructural investigations were carried out with a Jeol JEM 1200EX transmission electronmicroscope.

Results

LIGHT-MICROSCOPIC FINDINGS

In the neocortex of the rats perfusion-fixed immediately after the injury, the silver method stained red blood cells within the stab-wound, neuronal dendrites and perivascular astrocytes above it, and perivascular astrocytes but no axons under it **(Figures 1A, B)**. In the internal capsule, silver-stained axons appeared first in the 7-h rats and were numerous in the rats that survived for 17-h **(Figure 1C)** or longer. In the C3 region of the dorsal corticospinal tract, silver-stained axons appeared first in the 3-day rats **(Figure 1D)**.

ULTRASTRUCTURAL FINDINGS IN THE AXOPLASM

In the internal capsule, no abnormality was seen either in the rats sacrificed immediately after the cortical injury or in the 1-h and 3 h rats. In several axons of the internal capsule of the 7-h rats, the distances between the ultrastructural components of the axoplasm (neurofilaments, microtubules, mitochondria, endoplasmic reticulum cisternae and axolemma) were strikingly reduced, the electron density of neurofilaments and neurofilament sidearms, and that of the spaces between them were increased (compacted ultrastructure; **Figures 2A, B**). In a very few cases, a fluid filled space was observed between the compacted axoplasm and the surrounding myelin sheath **(Figure 2C)**. In the 17-h rat, besides numerous compacted axon profiles (427 in 500 damaged profiles), several axon profiles (73 in 500 damaged profiles) displayed homogenous and dense interior in which hardly any ultrastructural elements could be distinguished **(Figure 3A)**. In a few cases, axons with a normal, a compacted and a homogenous and dense ultrastructure were seen next to each other **(Figure 3B)**. In the rats that survived for 1 day or longer, no compacted axon profiles were seen in 500 homogenous and dense

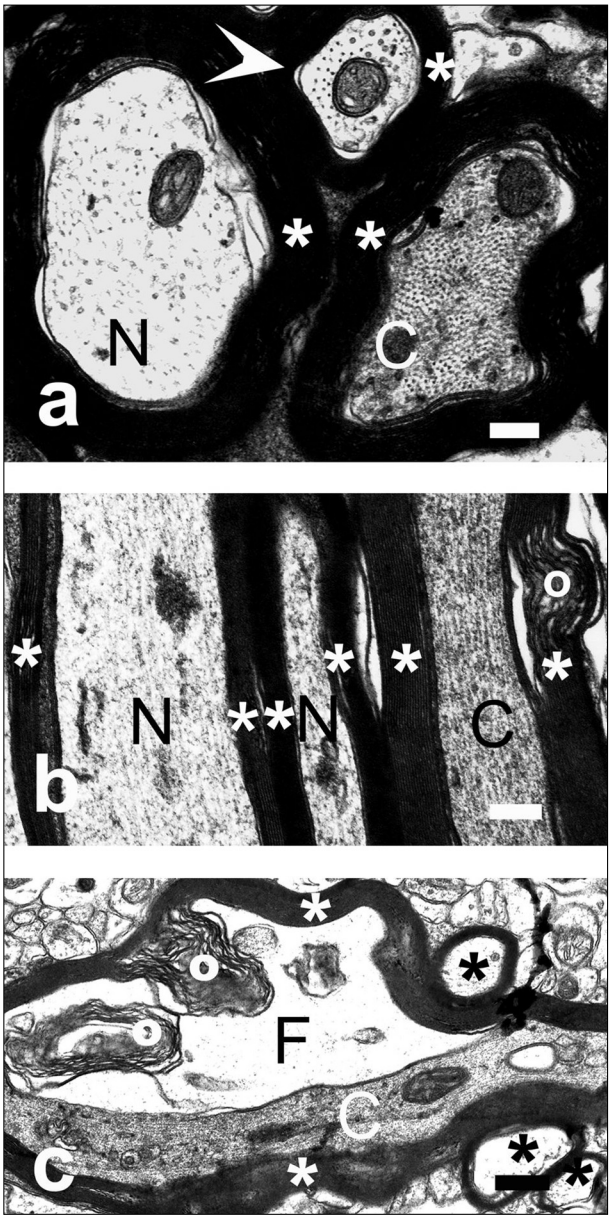

Figure 2. *Cross-sections* **(A)** *and longitudinal sections* **(B** *and* **C)** *of axons in the internal capsule of a rat perfusion-fixed 7 h after creation of the stab-wound. N marks normal axons, C compacted axons, F a fluid-filled space between the axolemma and myelin sheath, and white asterisks myelin sheaths. In* **A**, *a white arrowhead points to a normal axon cut exactly perpendicular to its long axis. In* **B** *and* **C**, *white open circles mark sites of multilamellar formations protruding from myelin sheaths, and black stars normal axons. Scale bars:* **A** *and* **B**=*200 nm,* **C**=*500 nm*

axon profiles **(Figure 4A)**. During the 3-month period investigated, the homogenous and dense axoplasm underwent a slow deformation due to anisotropic shrinkage **(Figures 4B, C)**. In the areas and time points investigated, no "watery" profiles in myelinated axons were seen. In non-myelinated axons, neither compacted nor homogenous and dense profiles were encountered.

In the C3 region of the dorsal corticospinal tract, similar ultrastructural sequences of "dark" axonal degeneration were seen as in the internal capsule, but with a 2-day delay. Specifically, neither compacted nor homogenous and dense axon profiles were observed in the rats that survived for 7h, 17 h and 1 day. At day 3 post injury, scattered among homogenous and dense axon profiles (331 in 500 damaged profiles), several compacted axon profiles (169 in 500 damaged profiles) were present. In the rats that survived for 1 week or longer, axons with a compacted ultrastructure were no longer seen, while numerous axons displayed homogenous and dense interior. Several of these underwent some deformation due to anisotropic shrinkage by three months post injury.

ULTRASTRUCTURAL FINDINGS IN MYELIN SHEATS

In the areas and time points investigated, neither axonal spheroids (striking focal dilatations of the myelin sheath filled with piled-up components of the axoplasmic ultrastructure or dens bodies transformed from them) nor any morphological signs pointing to "watery" degeneration (swollen myelin profiles exhibiting either an empty interior or an interior containing a few small areas of lamellar material) were observed. The structure of the myelin sheath around the affected axoplasms remained normal or mildly abnormal during the first week postinjury; only a low proportion of them displayed lamellar separation **(Figure 3B)** or multilamellar protrusion **(Figure 2B, C)**. Proliferated, redundant or apparently purposeless myelin sheaths were scarce. In the 3-months rats, we saw several "dark" axons surrounded by myelin sheaths that displayed multiple lamellar separations **(Figure 4A, C)**. However, neither complete nor serious disintegration of myelin sheath was seen.

ULTRASTRUCTURAL FINDINGS IN PHAGOCYTOTIC CELLS

In the areas and times investigated, macrophage infiltration or phagocytosis by microglial cells was not observed. Oligodendrocytes did not display any morphological alteration (proliferation, apoptosis, necrosis or phagocytosis). In an insignificant number (five cases in an area where 500 "dark"-degenerating axons were monitored), dark bodies of non-cleared origin were observed in astrocytes.

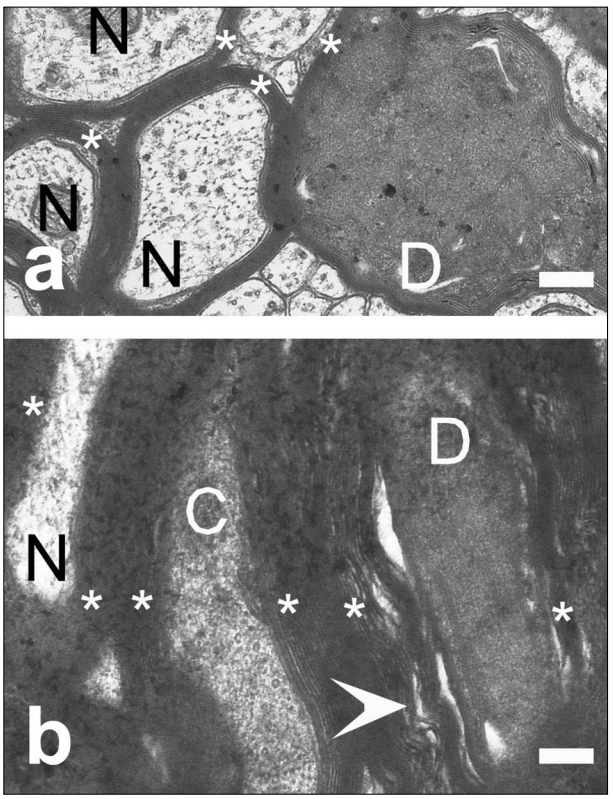

Figure 3. *Cross-sections* **(A)** *and longitudinal sections* **(B)** *of axons in the internal capsule of a rat perfusion-fixed 17 hours after creation of the stab-wound. N marks normal axons, C compacted axons, D homogenous and dense ("dark") axons, and white asterisks myelin sheaths. In* **B**, *a white arrowhead points to a site of lamellar separation of myelin. Scale bars:* **A**=*500 nm,* **B**=*200 nm*

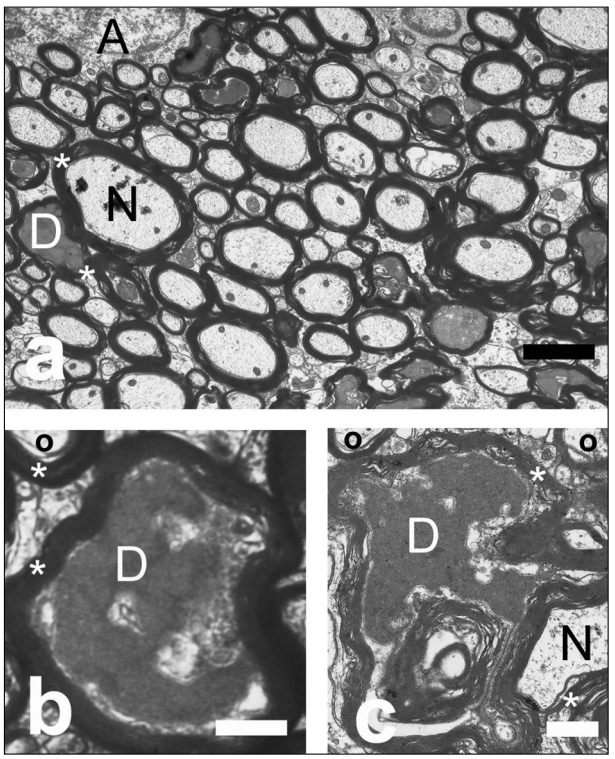

Figure 4. *Normal (N) and "dark" (D) axons in the internal capsule of a rat perfusion-fixed 3 months after creation of the stab-wound. White asterisks mark myelin sheaths. In* **B** *and* **C**, *open circles mark normal axons. Scale bars:* **A**=*2 μm,* **B** *and* **C**=*500 nm*

Discussion

ULTRASTRUCTURAL SEQUENCE OF "DARK" AXONAL DEGENERATION

Findings of other authors

Previous transmission electronmicroscopic studies on the "dark" axonal degeneration in the CNS stated that, in its first morphological stage, the myelin sheath appears unchanged, while the axoplasm is transformed into a homogenous and dense ("dark") mass in which hardly any elements of the normal axoplasm can be distinguished, even at high magnifications[1, 7].

Marques et al.[7] reported that the neurofilaments are aggregated but not decomposed in this stage, since each of their subunits can be detected for several weeks postinjury by immuno-fluorescence or immuno-electronmicroscopic methods. As regards the fate of the affected axons, the dense axoplasm was stated to undergo a months-long anisotropic shrinkage (deformation), while the lamellar structure of the surrounding myelin sheath slowly loosens, but remains easily recognizable. *Ludwin*[8] found that this morphology can persist for at least several months, depending on the nature and location of the initiating insult. In comparison, the peripheral nerves which undergo Wallerian degeneration are disintegrated in a few hours and phagocytosed within two weeks[9].

Our present findings

As a novel finding, we demonstrated that the homogenous and dense ultrastructural stage of the "dark" axonal degeneration is preceded by a striking decrease in the distances between readily distinguishable ultrastructural components of the axoplasm (compacted ultrastructure). The process of its development (ultrastructural compaction) is accompanied by a considerable shrinkage of the axoplasm. We assume that the surplus fluid accumulates transiently between the axolemma and myelin sheath

and then leaves the axon probably through the nodes of Ranvier. Concerning the fate of the compacted axons, our observations were similar to those described in the previous section. The cause why relevant papers by other authors did not report on the compacted ultrastructural stage consists probably in the fact that these did not deal with the period preceding postinjury day 1.

The spreading nature of "dark" axonal degeneration

Marques et al.[7] suggested that the processes of "dark" and "watery" degenerations are independent. They posited that focal aggregation of neurofilaments progresses into „dark" degeneration, while their focal disintegration progresses into "watery" degeneration, both types beginning at the injury site and spreading downstream along the axon. This suggestion explicitly implies that the whole length of an axon displays the same type of degeneration at variable time-dependent distances from the injured area.

Our observations that no "watery" axon profiles were present among more than one thousand of compacted or homogenous and dens profiles, and the "dark" degeneration did not begin simultaneously at numerous points throughout the whole length of the affected axon support the relevant suggestion of Marques et al.[7], and is compatible with the spreading nature[10, 11] of ultrastructural compaction.

CLEARANCE OF THE "DARK" AXONS

Findings of other authors

There is no agreement on the nature of the cells that remove the degenerated axons from the tissue, even when a single CNS area, the optic nerve, is concerned. *Bignami* and *Ralston*[12] reported that this task is carried out by macrophages that emerge around capillaries, and later assume the light-microscopic morphological characteristics of microglial cells. By contrast, *Cook* and *Wisniewski*[2] posited that the entry of hematogenous cells into the CNS is restricted to the site of the injury, while at some distance from it endogenous cells are involved in the phagocytosis. In areas remote from the injury site, oligodendrocytes[13], microglial cells and astrocytes, but not oligodendrocytes[14], or microglial cells, astrocytes and oligodendrocytes[1] were variably reported to be responsible for the phagocytosis. Weeks after quasi-total aspiration of the sensorimotor cortex, which results in huge numbers of closely oriented "dark"-degenerating axons in the corticospinal tract, *Basiri* and *Doucette*[15] found that primarily microglial cells are involved in the phagocytosis. The discrepancies between these observations may result from differences between the animal species involved and the injury modes applied.

Our present findings

In the present study, the insignificant number of morphological signs indicating phagocytosis suggests that thinly scattered "dark"-degenerating axons cannot activate the resting microglial cells even in months. This suggestion is supported by a finding in a quantitative study of age-related changes in the CNS[16] stating that the resting microglial cells do not multiply or transform into the phagocytotic form in the vicinity of solitary "dark" axons running through an otherwise undamaged environment.

MECHANISM OF THE FORMATION OF "DARK" AXONS

Head-injury-resulted ultrastructural compaction in studies of other authors

Pettus et al.[17], discovered that an impact-acceleration (a momentaneous and non-contusing) head injury led, among others, to a special ultrastructural consequence in axons: a striking reduction in the distances between readily distinguishable and seemingly intact neurofilaments, accompanied by the disappearance of most neurofilament side-arms and slight damages in other ultrastructural components. This state, which was named neurofilament compaction, did not change considerably from 5 min to 6 h postinjury. Shorter or longer survival periods were not investigated. A quantitative study by the same team[18] established that the density of the neurofilaments increased to approximately twice the normal value, resulting in an about 50% volume reduction in the axoplasm. The affected axons were scattered diffusely among uninjured neighbors.

The ultrastructural compaction in axons resulted by impact-acceleration head injury is still widely considered to be a delayed morphological manifestation of calpain-mediated spectrin proteolysis, initiated by an uncontrolled influx of Ca^{2+} into the axoplasm through the axolemma perturbed focally by some head injury-generated intracerebral shearing force[19]. *Narciso* et al.[1] believed that the molecular-biological process mentioned above governs also the axon degeneration resulting from transection of the optic nerve. *Saggu* et al.[14] put forward the view

that an increased axoplasmic Ca^{2+} level induces calpain-mediated spectrin proteolysis either in most axoplasmic ultrastructural components leading to their disintegration ("watery" axonal degeneration), or in the neurofilament side-arms only, resulting in neurofilament aggregation ("dark" axonal degeneration).

Head-injury-resulted ultrastructural compaction in our earlier studies

Gallyas et al.[10, 11] observed that the compaction of axonal neurofilaments is completed even in rats perfusion-fixed immediately after a weight-drop (a momentaneous and non-contusing) head injury. Not only the neurofilaments but also the other ultrastructural elements appeared normal. Quantitative analysis[10, 11] demonstrated that the degree of compaction (volume reduction) was commensurate with that found by *Pettus* and *Povlishock*[18]. The compaction began simultaneously in each affected neuron and axon at the moment of injury and was completed in neurons within seconds[20].

Any enzyme-mediated mechanism of head-injury caused ultrastructural (neurofilament) compaction in axons is contradicted by a previous observation of our team[10] that this process can take place even in rats head-injured *post-mortem* under conditions that are extremely unfavorable for enzyme-mediated processes. Quantitative comparisons[10] showed that the degrees of *in-vivo* and *post-mortem* compactions were nearly equal. It was suggested that any molecular-biological event detectable after the ultrastructural compaction must be a consequence but not the cause of it.

The ultrastructural sequence of head-injury resulted and transection resulted "dark" axons are similar.

Using a highly-controlled weight-drop head-injury device[21] that is capable of producing compacted axons scattered in an otherwise undamaged environment, Gallyas et al.[11] found that the fraction of compacted axons, which did not recover in one hour, persisted for several hours, thereafter underwent homogenization and thickening, and then months-long anisotropic shrinkage. This morphological sequence is similar to that of transection-resulted "dark" axonal degeneration found in the present study. This fact suggests that the mechanisms of their formation and fate may also be the same.

Ultrastructural compaction is a common cell-biologic phenomenon in mammalian cells

Rapid dramatic reduction in the distances between seemingly intact ultrastructural elements was observed not only in axons, but also throughout the whole soma-dendrite domain of diffusely distributed neurons, even in intracellular areas where neurofilaments are scarce or absent[10]. In addition to momentaneous and non-contusing head injuries, a condenser-discharge electric shock[22] and some pathobiochemical processes taking place in neurological diseases such as hypoglycemia[23], ischemia[24], and epilepsy[25] can also initiate ultrastructural compaction. Furthermore, this phenomenon was observed not only axons and the whole the soma-dendrite domain of neurons, but also in astrocytes[26], oligodendrocytes, pericytes and endothelial cells[27], and in many cell types of non-nervous mammalian tissues (referred by[28]). Consequently, ultrastructural compaction should be regarded as a common response of mammalian cells and their protrusions to various insults of both physical and chemical nature[29].

Gel-to-gel phase transition may propel the ultrastructural compaction in transected axons

As suggested in several earlier papers of our team[27, 29, 30], the ultrastructural compaction in head-injury produced neuronal soma-dendrite domains and axons proceeds with gel-to-gel phase transition, which is an established physicochemical (non-enzymatic) process[31–33]. A comparison of the arguments mentioned in the previous subheadings suggests that the ultrastructural compaction in the transection-resulted "dark" axonal degeneration may go forward also with this non-enzymatic mechanism.

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