

Diazoxide blocks or reduces microgliosis when applied prior or subsequent to motor neuron injury in mice

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HIGHLIGHTS

- Diazoxide blocks axotomy induced microgliosis when applied prior to the injury.
- Diazoxide reduces microgliosis when applied after the axotomy.
- Oculomotor nucleus exhibits reduced microgliosis compared to other motor nuclei.
- Diazoxide restores altered mitochondrial morphology in motoneurons after axotomy.

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ABSTRACT

Diazoxide (DZX), an anti-hypertonic and anti-hypoglycemic drug, was shown to have anti-inflammatory effects in several injured cell types outside the central nervous system. In the brain, the neuroprotective potential of DZX is well described, however, its anticipated anti-inflammatory effect after acute injury has not been systematically analyzed. To disclose the anti-inflammatory effect of DZX in the central nervous system, an injury was induced in the hypoglossal and facial nuclei and in the oculomotor nucleus by unilateral axonal transection and unilateral target deprivation (enucleation), respectively. On the fourth day after surgery, microglial analysis was performed on tissue in which microglia were DAB-labeled and motoneurons were labeled with immunofluorescence. DZX treatment was given either prophylactically, starting 7 days prior to the injury and continuing until the animals were sacrificed, or postoperatively only, with daily intraperitoneal injections (1.25 mg/kg; in 10 mg/ml dimethyl sulfoxide in distilled water). Prophylactically + postoperatively applied DZX completely eliminated the microglial reaction in each motor nuclei. If DZX was applied only postoperatively, some microglial activation could be detected, but its magnitude was still significantly smaller than the non-DZX-treated controls. The effect of DZX could also be demonstrated through an extended period, as tested in the hypoglossal nucleus on day 7 after the operation. Neuronal counts, determined at day 4 after the operation in the hypoglossal nucleus, demonstrated no loss of motor neurons, however, an increased Feret's diameter of mitochondria could be measured, suggesting increased oxidative stress in the injured cells. The increase of mitochondrial Feret's diameter could also be prevented with DZX treatment.

1. Introduction

Diazoxide (7-chloro-3-methyl-2H-1,2,4-benzothiadiazine 1,1-

dioxide; DZX), which was originally introduced to lower blood pressure and treat hypoglycemia, is nowadays considered as a drug with multiple targets (Coetzee, 2013). Among other effects, it has been shown to

Abbreviations: A β ₁₋₄₂, amyloid- β ; a.u., arbitrary units; K_{ATP}, adenosine-5'-triphosphate dependent potassium; ATP, adenosine-5'-triphosphate; CCD, charge-coupled device; CH, convex hull; ChAT, choline acetyltransferase; CNS, central nervous system; DAB, diaminobenzidine tetrahydrochloride; DMSO, dimethyl sulfoxide; DZX, diazoxide; Iba1, ionized calcium-binding adaptor molecule 1; mK_{ATP}, mitochondrial adenosine-5'-triphosphate dependent potassium; PBS, phosphate buffered saline; RTV, real-time viewing; sCMOS, scientific complementary metal-oxide semiconductor; s.e.m., standard error of the mean; TPBS, phosphate buffered saline with 0.2% triton x-100; VEH, vehicle (dimethyl sulfoxide (DMSO))

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induce anti-inflammatory responses in several cell types that were injured in different ways, such as in *in vitro* cardiac cells during glucose-induced injury (Liang et al., 2017), in rat liver injury caused by ischemia/reperfusion (Nogueira et al., 2014), or in rat small intestine damage induced by indomethacin (Menozzi et al., 2011). It has also been shown to suppress the inflammatory response to coronary artery bypass grafts in humans (Wang et al., 2004). In the central nervous system (CNS), DZX has been shown to have neuroprotective properties in hypoxic/ischemic injury (Lei et al., 2018; Zhang et al., 2014), and in acute experimental subdural hematoma (Nakagawa et al., 2013). However, along with the neuroprotective action of DZX, its presumable anti-inflammatory effect, represented by a decreased microglial activity in the CNS, has not been systematically analyzed after acute neuronal lesion. In the present study, to test the anti-inflammatory effect of DZX, motor neurons in anatomically well-defined brainstem nuclei were surgically injured, either with axonal transection (hypoglossal, and facial motor neurons) or target deprivation (oculomotor neurons), then microglial activation was characterized in the appropriate nuclei. Under pathological conditions, such as after acute lesion, microglial cells change their phenotype (Ransohoff and Perry, 2009), migrate to the site of injury, reduce the complexity of their shape by retracting and thickening their branches (Kettenmann et al., 2011), which results in a more compact appearance of the immunostaining for microglial cells in the proximity of the injured neurons. Our immunostaining quantification procedure, by taking into account the change in the density and volume of microglial cells, arrives at a single robust parameter characterizing the activation of microglial cells in the entire volume of the motor nuclei affected by the injury. These data indicate that prophylactically + postoperatively applied DZX completely eliminated the microglial response in each of the examined motor nuclei. If DZX was applied only postoperatively, reduced microglial activation of a significantly smaller magnitude could be detected in each nucleus when compared to the non-DZX-treated controls. The anti-inflammatory effect of DZX, if applied only postoperatively, was most prominent in the oculomotor nucleus, in which the injury-induced microglial activation was relatively moderate compared to the hypoglossal and facial nuclei.

2. Results

Qualitatively, in each of the injured motor nuclei, a marked elevation of microglial staining could be observed 4 days after the operation. The increase of microglial staining was more obvious in the facial nucleus (Fig. 1A) than in the hypoglossal nucleus (Fig. 2A). In the oculomotor nucleus a moderate, but clearly recognizable rise of microglial reaction could be observed (Fig. 3A). Dimethyl sulfoxide (DMSO) + DZX, in the pretreatment + postoperative treatment paradigm, as estimated visually, completely blocked the microglial activity in the facial- (Fig. 1C versus Fig. 1A), in the hypoglossal- (Fig. 2C versus Fig. 2A), and in the oculomotor nucleus (Fig. 3C versus Fig. 3A). The organic solvent (DMSO), used as a vehicle (VEH), seemed to have no effect in reducing microglial activation in the facial nucleus (Fig. 1B versus A) and in the hypoglossal nucleus (Fig. 2B versus A). In the oculomotor nucleus, qualitatively, the effect of DMSO and DMSO + DZX could not be distinguished (Fig. 3C versus B). The sole postoperative treatment with DMSO + DZX seemed to reduce microglial activation in the facial nucleus (Fig. 1D versus 1A), in the hypoglossal nucleus (Fig. 2D versus A) and in the oculomotor nucleus, as well (Fig. 3D versus A).

For the quantitative expression of the microglial staining in the examined motor nuclei, the density of microglial cells had to be determined within the well-defined anatomical borders of the nuclei. Conventionally, the microglial cell density is used to be determined in sections obtained at different points of the nucleus under investigation, double stained with fluorescent antibodies labeling the microglial cells and the motor neurons (see e.g. Figs. 4 and 5A). However, the distribution of the activated microglial cells is not homogeneous in the

affected brain regions, which is particularly evident in the case of the oculomotor nucleus (Fig. 4), in which the innervation of one of the external oculomotor muscles (superior rectus muscle) is crossed (Akagi, 1978; Bron et al., 1997), resulting in the occurrence of the corresponding microglial activation on the contralateral side. First, to avoid the bias due to that the apparent degree of the microglial activation depends on the actual sampling point (Fig. 6), each nucleus in the study has been systematically and exhaustively sectioned with consecutive sections in the rostro-caudal axis of the animals (Fig. 4), and values have been derived characterizing the microglial staining in the entire volume of the nuclei. Next, to circumvent the non-standardized effect of photo-bleaching, the traditional double fluorescent staining method was replaced with a combination of fluorescent and diaminobenzidine (DAB)-based staining process. As demonstrated in Fig. 5A and B, the DAB + fluorescent signal combination adequately substitutes the conventional double fluorescent imaging and can be used for anatomical orientation with the advantage of providing stable microglial staining for quantification.

Using this combined staining procedure, the position of the sections from different animals could be completely synchronized in the rostro-caudal direction according to the choline acetyltransferase (ChAT) staining. The procedure offered the possibility of determining data characterizing the increase of microglial staining on the operated sides of different animals in identical rostro-caudal positions, thus could be averaged, and plotted along a mutual rostro-caudal axis (Fig. 6). Supporting the qualitative observations, the plots demonstrate the development of the strongest microglial reaction after injury in the facial nucleus (Fig. 6B), and the mildest in the oculomotor nucleus (Fig. 6A). The graphs also illustrate that the pretreatment + postoperative treatment with DZX completely eliminated the differences in microglial staining between the control and the operated side. It is also evident from Fig. 6 that the plain postoperative DZX treatment, while was able to reduce microgliosis induced by the injury, could not block it completely. Numerically, the single values characterizing the total magnitude of microgliosis were pooled according to the investigated motor nuclei and treatments. In accordance with the qualitative observations, compared to the oculomotor nucleus, the lesion-induced microglial activation was significantly higher in both the hypoglossal- ($p = 0.012$), and the facial nucleus ($p = 6 \times 10^{-6}$). In the oculomotor nucleus, the statistical evaluation shows a significant decrease ($p = 0.037$) in microglial staining in the pretreated + postoperatively treated group and the plain postoperative treated group ($p = 0.042$) compared to the untreated control (Fig. 7). Only in the oculomotor nucleus, however, DMSO exerted a similar effect to DZX in reducing glial activation, compared to the operated control ($p = 0.03$). In the facial- and in the hypoglossal nucleus, pretreatment + postoperative treatment with DZX efficiently reduced the activation of the microglial cells ($p = 6 \times 10^{-10}$, $p = 6 \times 10^{-6}$, respectively; Fig. 7). The effect of the postoperative treatment was less prominent (Fig. 7), but still significant in the facial- ($p = 0.0025$) and in the hypoglossal nucleus ($p = 0.041$). In contrast to the oculomotor nucleus, in the facial nucleus and in the hypoglossal nucleus the effect of DMSO was not significant, with p values of 0.56 and 0.86, respectively.

According to the literature (Kawabori and Yenari, 2015) and our earlier study (Paizs et al., 2017), microglial activation starts declining by day 7 after axotomy, thus the analysis of microglial activation was extended to postoperative day 7 only. In the hypoglossal nucleus, similarly to the results obtained at postoperative day 4, the elevated microglial reaction at the operated side (1321 ± 146.69 , arbitrary unit (a.u.); $n = 3$) was decreased significantly ($p = 1.1 \times 10^{-5}$) by the combined pre + postoperative treatment with DZX (255.22 ± 65.70 , a.u.; $n = 4$) while DMSO alone did not exert significant effect ($p = 0.56$) on the microglial activation (1159.66 ± 351.88 , a.u.; $n = 3$). The plain postoperative treatment with DZX decreased the microglial activation, as well (933.15 ± 96.99 , a.u.; $n = 4$), but this change did not reach a significant level ($p = 0.054$).

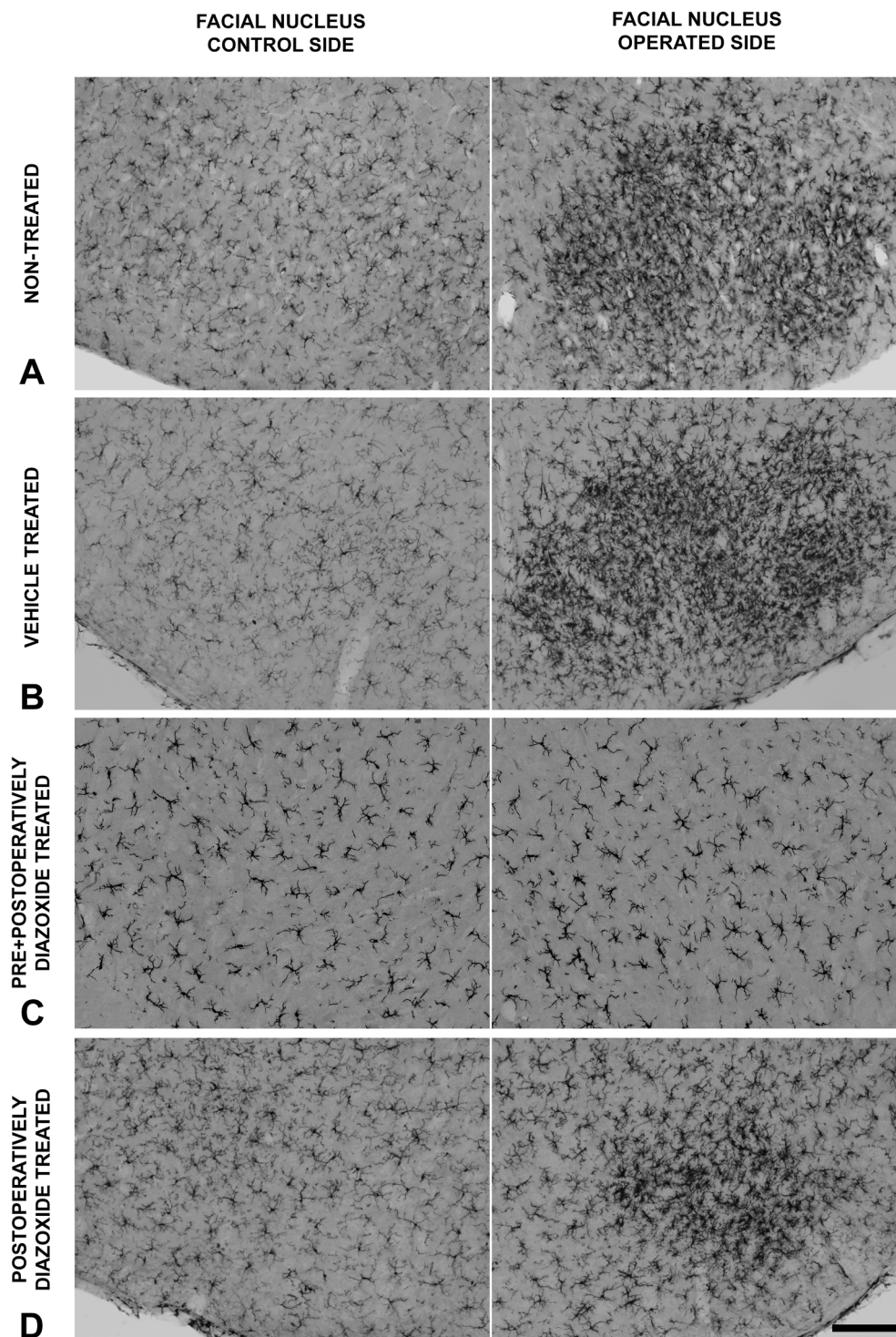


Fig. 1. Microglial activation in the facial nucleus after axotomy of the facial nerve. (A) Four days after operation, without drug treatment, strong microgliosis can be observed on the operated side (right panel) relative to the intact side (left panel). (B) DMSO, if applied preoperatively, noticeably did not affect microglial activity on the operated side (right panel). (C) DZX + DMSO treatment, if started prior to the operation, almost eliminated the activated microglial cells on the operated side (right panel) resulting in a similar appearance to the control side (left panel). (D) Postoperatively applied DZX + DMSO reduced microgliosis on the operated side (right panel), compared to the untreated condition (A, right panel) but the microglial activity could not be eliminated. Scale bar: 100 μ m.

To give further clarity on the effect of DZX on microglia, their morphology was also assessed in the hypoglossal nucleus 4 days after the injury. K-means based Farthest-First cluster analysis on the output of multi-dimensional fractal analysis resulted in 2 separate cell clusters with a ramified-like (Fig. 8A) and a bushy-like morphology (Fig. 8B). 92.5% of the examined microglial cells were sorted into cluster A on the control side of the hypoglossal nucleus (Fig. 8C). This dramatically changed on the injured side, where all analyzed microglia were sorted into cluster B (Fig. 8C). A similar change was observed in the DMSO-treated injured side, where 90% of the microglia were categorized into cluster B (Fig. 8C). Pre- + postoperative and only postoperative DZX

treatment could prevent this shift to cluster B phenotype (Fig. 8C).

Besides the effect of DZX on microglia, motoneuronal survival was also assessed by stereological cell counting in the hypoglossal nucleus to determine the neuroprotective effect of DZX, which demonstrated no detectable motoneuronal loss. On day 4 after the axotomy, we counted 78737 ± 4837 motor neurons/ mm^3 at the operated side, which did not differ from the cell count at the intact side (75986 ± 7543 motor neurons/ mm^3 ; $p = 0.75$). On day 7 after the injury, the values were not significantly different either: at the operated side 72177 ± 4802 motor neurons/ mm^3 were counted, while at the control side 70258 ± 5330 motor neurons/ mm^3 were detected ($p = 0.80$). The present results of

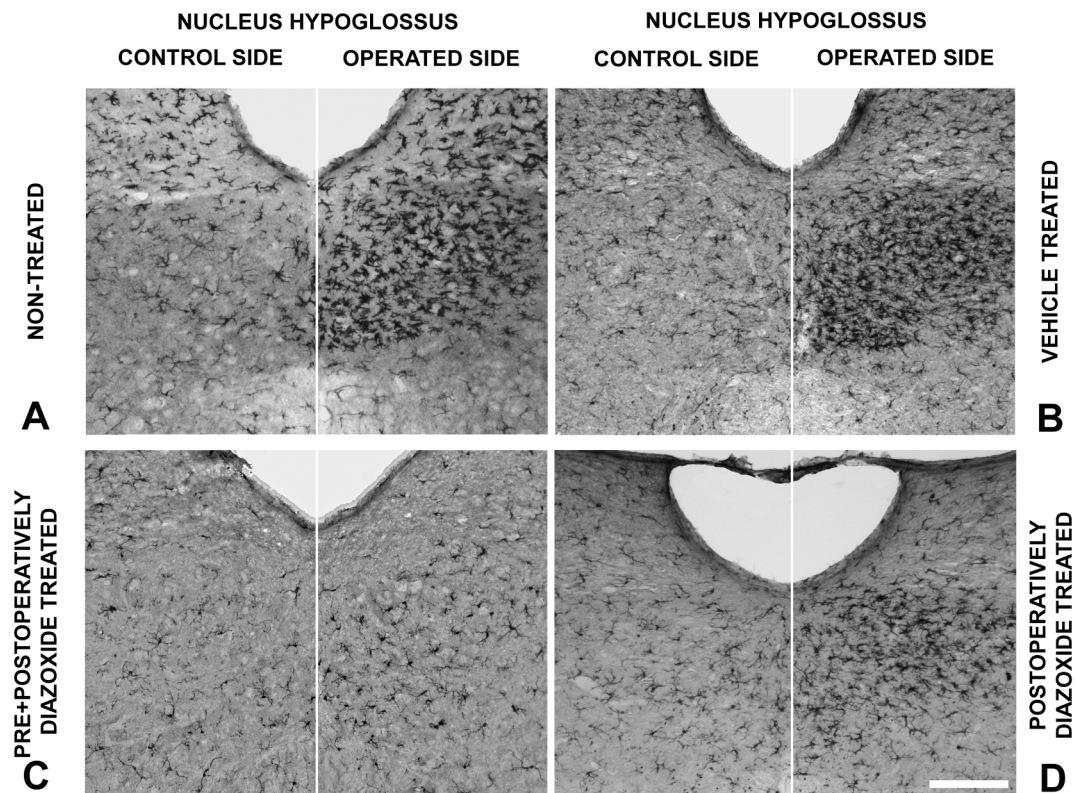


Fig. 2. Microglial activation in the hypoglossal nucleus after axotomy of the hypoglossal nerve. (A) Four days after operation, without drug treatment, strong microgliosis can be observed on the operated side (right side) relative to the intact side (left side). (B) DMSO, if applied preoperatively, did not affect considerably the microglial activity on the operated side (right side). (C) DZX + DMSO treatment, if started prior to the operation, almost eliminated the activated microglial cells on the operated side (right side) resulting in a similar appearance to the control side (left panel). (D) Postoperatively applied DZX + DMSO reduced microgliosis on the operated side (right side), compared to the untreated condition (A, right side) but microglial activity could not be eliminated. Scale bar: 100 μ m.

no detectable loss of motor neurons after axotomy in the hypoglossal nucleus are in accord with literature data describing similarly no loss of motor neurons in the facial nucleus of axotomized rats (Ichimiya et al., 2013), or enucleated adult mice (Obál et al., 2006), in the same time-frame.

Since there was no change in the number of motor neurons after axotomy, an ultrastructural analysis was conducted to examine the effect of DZX. Evaluation of mitochondrial morphology showed changes in the shape of mitochondria. The shape of mitochondria was more circular on the control side (Fig. 9A), but elongated mitochondria with ellipsoid shape and with an increased Feret's diameter could be observed on the injured side (Fig. 9B). Pre- + postoperative DZX treatment (Fig. 9C) and postoperative DZX treatment (Fig. 9D) restored altered mitochondrial morphology and the Feret's diameter of these organelles in the hypoglossal motor neurons (Fig. 9E). The ratio of the Feret's diameter of the injured motoneuronal mitochondria compared to the control side was increased in the axotomized (1.27 ± 0.05) and in the VEH control (1.29 ± 0.07) groups, implicating increased oxidative burden (Fig. 9E). This elevation could be prevented with diazoxide treatment (without treatment vs. pre- + postoperative DZX treatment: $p = 0.00488$; without treatment vs. postoperative DZX treatment: $p = 0.00422$). Microglial mitochondria were also examined but none of the measured parameters showed significant changes (Fig. 9F).

3. Discussion

DZX is a selective activator of the mitochondrial adenosine-5'-triphosphate (ATP) dependent potassium (mK_{ATP}) channel, which can be found in the inner mitochondrial membrane in several cell types, including brain cells (Bajgar et al., 2001). DZX has been shown to possess

neuroprotective potential in a variety of experimental conditions, such as in cortical neuronal cultures, *in vitro*, in which DZX has been shown to increase viability of cultured neurons against oxygen-glucose deprivation (Katakam et al., 2016) or amyloid- β ($A\beta_{1-42}$)-induced toxicity (Zhu et al., 2015). *Ex vivo*, DZX proved to be neuroprotective in neo-cortical brain slices during hypoxia (Garcia de Arriba et al., 1999). *In vivo*, in the experimental autoimmune encephalomyelitis murine model of multiple sclerosis, DZX has been shown to ameliorate disease progression and reduce the loss of neurons in the spinal cord (Virgili et al., 2011). Furthermore, in rats, it has been shown to reduce neuronal damage after middle cerebral artery occlusion (Shimizu et al., 2002), and prevent hypoperfusion-related learning dysfunction (Farkas et al., 2004).

Although DZX may target mitochondrial Complex II of the respiratory chain (Hanley et al., 2002), or plasmalemmal ATP-dependent potassium channels (Hatlapatka et al., 2009), its protective effect might be attributed to its action as an opener of the mK_{ATP} channel, because its neuroprotection can be antagonized with mK_{ATP} channel inhibitors (Domoki et al., 2005; Shimizu et al., 2002; Son et al., 2018). Since in a wide variety of insults, such as ischemia/reperfusion (Dux et al., 1987; Murphy and Steenbergen, 2008), traumatic brain injury (Scholpa and Schnellmann, 2017), nerve transection (Li et al., 2014), aging and neurodegeneration (Strehler and Thayer, 2018; Pivovarova and Andrews, 2010; Patai et al., 2017), mitochondrial calcium overload may play a central pathogenic role (Berridge et al., 1998), saving mitochondria from excess calcium may serve as a protective tool. Indeed, it has been proven experimentally that either in amyloid- β -induced toxicity (Son et al., 2018), or during ischemia/reperfusion injury (Domoki et al., 2004) the neuroprotective effect of DZX paralleled the reduced mitochondrial calcium uptake, and the augmented preservation of the mitochondrial structure.

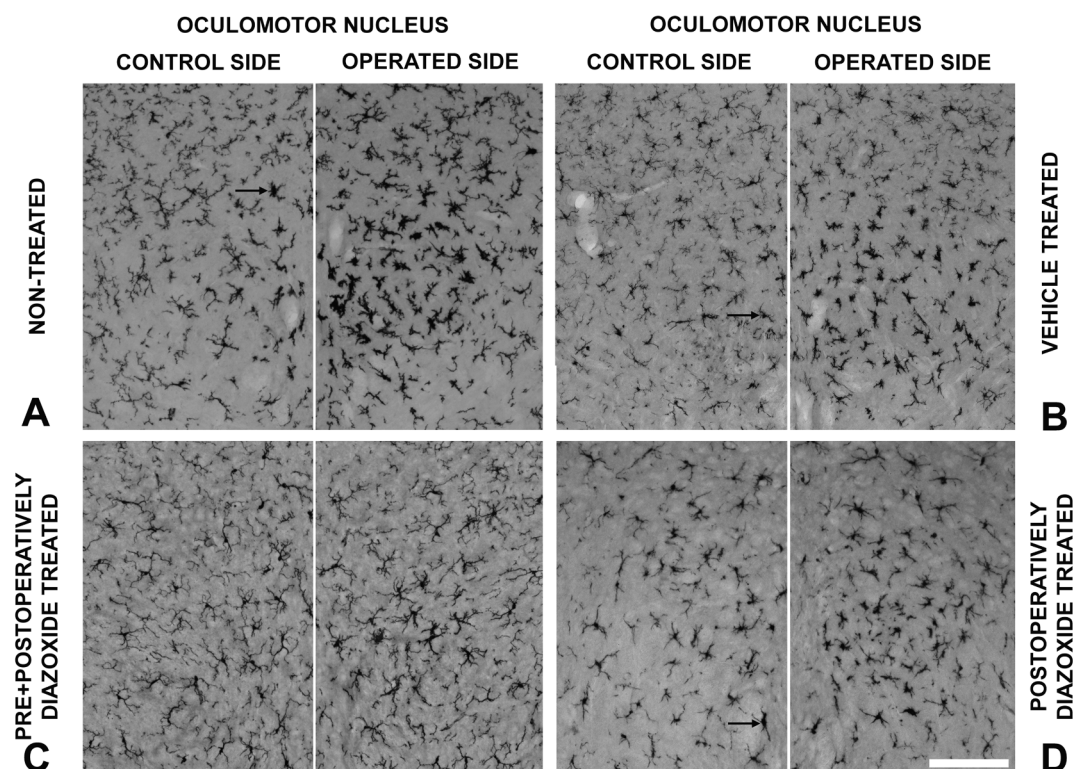


Fig. 3. Microglial activation in the oculomotor nucleus after unilateral enucleation. (A) Four days after operation, without drug treatment, moderate microgliosis can be observed on the operated side (right side) relative to the intact side (left side). (B) DMSO, if applied preoperatively, strongly decreased microglial activity on the operated side (right side). Some microglial cells exhibiting activated morphology can also be observed in the left sides of panels (A), (B) and (D), due to the contralateral innervation of the superior rectus muscle (arrows). (C) DZX + DMSO treatment, if started prior to the operation, decreased microgliosis similarly to DMSO alone, as observed by comparing the right sides of panels (B) and (C). (D) Qualitatively, postoperatively applied DZX + DMSO reduced microgliosis on the operated side (right side) to the same degree as the prophylactically applied drugs (compare right sides of panels (B), (C) and (D)). Scale bar: 100 μ m.

In the nervous system, the neuroprotective effect of DZX is accompanied by an anti-inflammatory property, which has been convincingly evidenced in a model of multiple sclerosis, an acquired inflammatory demyelinating disorder (Virgili et al., 2011). Theoretically, the anti-inflammatory action of DZX could be considered a secondary consequence of neuroprotection, as reduced damage to nerve cells might lead to an abbreviated release of chemokines, attracting a reduced number of microglial cells, as was shown in axotomized motor neurons with upregulated parvalbumin (Paizs et al., 2017). The primary role of DZX as a neuroprotective drug has gained further support from excitotoxicity experiments performed in organotypic hippocampal cultures depleted of microglia, in which the neuroprotective effect proved to be independent of microglial cells (Virgili et al., 2013). On the other hand, since DZX has been documented to inhibit rotenone-induced neuroinflammation by interacting with K_{ATP} channels present on microglial cells (Zhou et al., 2008), a synergism between its anti-inflammatory and neuroprotective effect may exist. However, the anti-inflammatory effect of DZX after acute injury has not been analyzed systematically. With this aim, in the present study, motor neurons with different susceptibility to injury have been axotomized, and the microglial reaction in the appropriate motor nuclei has been quantitatively determined postoperatively at day 4, an early time point, when the full-blown inflammatory reaction was expected already to be present (Streit et al., 1999; Kalla et al., 2001).

Regarding the quantification of the microglial activation, two major aspects should be considered which may considerably affect the precision and the reproducibility of the results: uneven distribution of the activated microglial cells within the examined brain region and stability of the staining pattern for microglia under the microscope. Indeed, as demonstrated in Fig. 6, the density of microglial cells on the operated side relative to the intact control side cannot be considered evenly

distributed along the examined nucleus either in the hypoglossal- or the facial-, or the oculomotor nucleus (upper row). This inhomogeneity in the distribution of the activated microglial cells is most obvious within the oculomotor nucleus (Fig. 6A, upper row), in which the innervation to superior rectus muscle is crossed, resulting in negative-going values on the diagram representing the most caudal part of the nucleus (Bron et al., 1997; Remington, 2012). Consequently, if using a single, or low number of sections to estimate the degree of microgliosis, the results would largely depend on the actual sampling positions. To avoid this, a systematic sampling protocol was applied by serial sectioning the entire volume of the examined nuclei permitting the determination of the increased microglial staining on the operated side relative to the intact side globally in the entire volumes. The net differences between the staining in the operated and the control side calculated in the sections were summed up through the series to arrive at a single number representative for microgliosis in each treatment group in each of the examined nuclei (Fig. 7), which could be used for statistical analysis (Fig. 7).

Double staining immunohistochemistry is a common approach for visualizing the co-distribution of certain cell types in anatomically defined brain regions (Chen et al., 2010). When localizing the region of interest, first, one must get an appropriate orientation on the section, locate landmarks, which may require illumination of tissue regions for variable periods at varying intensities due to the application of different magnifications. Although the commonly used chromophores may have different resistances to photo-bleaching (Mahmoudian et al., 2011), a 10–20 sec illumination might be sufficient to lose more than 50% of the fluorescence intensity (Gallardo-Escárate et al., 2007). Since this period is comparable with the time required for the proper localization of a region of interest to be photographed, the consequent fading of fluorescence may lead to certain cellular profiles becoming unrecognizable if

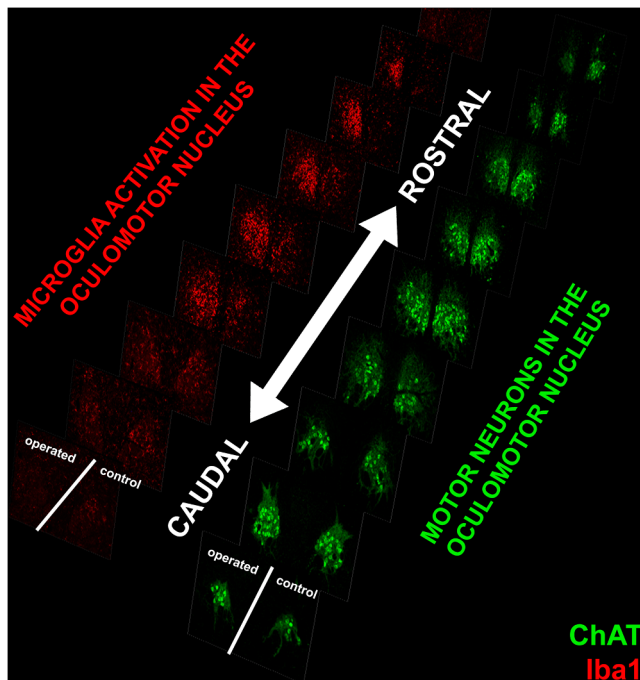


Fig. 4. Uneven distribution of the microglial cells within the entire volume of the oculomotor nucleus after the enucleation of the left eye. While ChAT-positive (green) motor neurons can be used to delineate the borders of the oculomotor nucleus at different rostro-caudal positions, Iba1 positive (red) signals demonstrate the corresponding microglial activation. Clearly, the intensity of staining is not uniform along the rostro-caudal axis, furthermore, the effect of decussation of the nerve innervating the superior rectus muscle is clearly visible.

their staining intensity falls below the detection threshold. This effect makes quantification unpredictably erroneous. This was circumvented in the present study by substituting photosensitive immunofluorescent staining of microglial cells to be quantified, with photostable DAB-based staining (Fig. 5).

The microglial activation induced by the experimental lesion of the motor axons was least prominent in the oculomotor nucleus (Fig. 6A). This might be partially explained by the contralateral innervation of the superior rectus muscle (Akagi 1978; Bron et al., 1997; Remington, 2012). However, the values representing the parts of the oculomotor

nucleus ipsilaterally innervating the inferior rectus, the inferior oblique and the medial rectus muscles of the eyeball are still lower than the characteristic values in the hypoglossal and the facial nucleus (Fig. 6B, 6C, upper row). An explanation of these results might be based on that the oculomotor neurons, probably due to their higher calcium buffering capacity, are more resistant to injury (Vanselow and Keller, 2000; Paizs et al., 2010; Obál et al., 2006; Mosier et al., 2000). Consequently, their reduced cellular damage may lead to reduced emission of cytokines (Raivich et al., 1999), working as distress signals, leading to reduced attraction of microglial cells (Paizs et al., 2017). Indeed, calcium-binding proteins, such as calbindin D_{28k} or parvalbumin was shown to denote motor neurons at low risk of degeneration during motor neuron disease (Laslo et al., 2000; Alexianu et al., 1994; Comley et al., 2015), furthermore, parvalbumin was accepted as a marker of motor neurons resistant to degeneration (Elliott and Snider, 1995).

Regarding the possible combined effect of DZX and its solvent, DMSO, DZX was shown to have a neuroprotective effect (Domoki et al., 2004), however, DMSO alone is able to provide certain neuroprotection (Di Giorgio et al., 2008). Thus, it is reasonable to assume, that during the injury of the more resistant oculomotor neurons, the solitary effect of DMSO might be sufficient to reduce the magnitude of the stress in these cells, resulting in a lowered release of chemoattractants, and a consequent reduced microglial activation (Fig. 7). Nevertheless, in the present systematic analysis of microglial activation after acute injury, DZX was proven to completely block the inflammatory reaction, if applied prophylactically. The present data, that posttraumatically applied DZX also significantly reduced inflammation, may support its possible therapeutic applications.

4. Experimental procedure

4.1. Experimental animals and treatments

Inbred adult male Balb/c mice (RRID: IMSR_JAX:000651; mean body weight of 22 ± 5 g) obtained from the conventional animal facility of the Biological Research Centre (Szeged, Hungary) were used for the study. The animals were housed in plastic cages (2–3 animals per cage, at most) in a temperature-controlled (23 ± 2 °C) room under a 12-hours light/dark cycle, with access to regular rodent chow and water *ad libitum*. Seventy-seven mice were used in the study sorted into 3 groups, according to the type of surgery, then assorted into subgroups according to the treatments (Table 1). Since the VEH, the organic solvent of DZX, DMSO was shown to influence

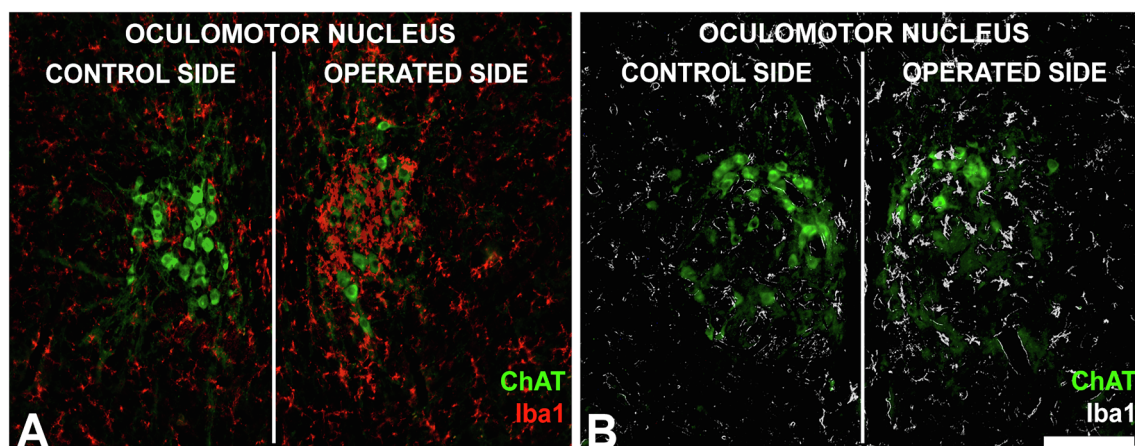


Fig. 5. Comparison of the conventional double immunofluorescence staining (A) with the double staining based on the combination of the immunofluorescence- and diaminobenzidine (DAB) methods (B). In (A) and (B), the left panels show motor neurons and microglial cells of the intact oculomotor nucleus, the right panels display the same nucleus after enucleation of the eye. By comparing (A) and (B), it can be concluded that while the green channel showing the ChAT staining can be used for anatomical orientation, it delineates the border of the oculomotor nucleus, it does not interfere with the DAB-based microglial staining, the amount of which can be determined using a different, photo-stable color channel. Scale bar: 100 μ m.

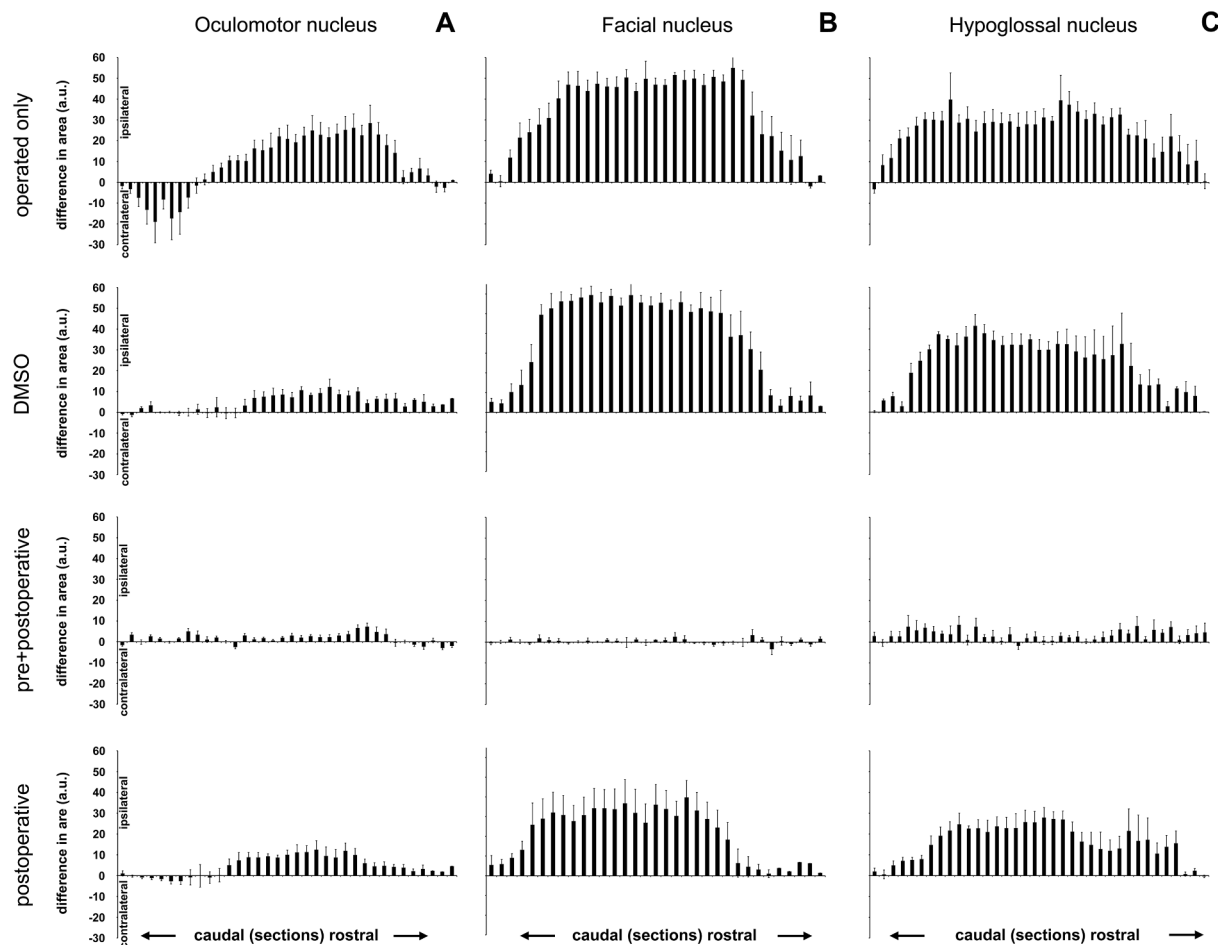


Fig. 6. The difference of microglial staining between the operated (ipsilateral) and the non-operated (contralateral) sides at different rostro-caudal positions from the oculomotor- (A), facial- (B) and hypoglossal (C) nucleus. The algebraic differences between the areas with positive microglial staining on the operated and control sides in each section were calculated, then these numbers, obtained at identical rostro-caudal positions from different animals were averaged ($n = 4$, data are shown as mean $\pm$ s.e.m.). These average values were plotted according to the rostro-caudal coordinates. Axotomy induced the smallest microglial activation in the oculomotor nucleus (A, upper panel), and the largest in the facial nucleus (B, upper panel). Note the varying magnitudes of microglial activation along the rostro-caudal axis. The negative-going part of Fig. 6, upper row, represents the crossed innervation of the superior rectus muscle. Pretreatment with DMSO decreased microglial activation in the oculomotor nucleus (second row), furthermore, DZX + DMSO practically eliminated microglial activation in each nucleus (third row). In the postoperative treatment paradigm (bottom row) a reduced, but detectable microglial activity could be demonstrated. a.u.: arbitrary units.

neuroinflammation after injury (Farkas et al., 2004; Farkas et al., 2005; Rivers-Auty and Ashton, 2013), a VEH-treated set of animals was formed, as well.

Pretreatments started 7 days prior to the surgery and the treatments were continued for 4 postoperative days. Our earlier study (Paizs et al., 2017), in agreement with other literature data (Kawabori and Yenari, 2015) documented that microglial activation in the affected brain region after nerve injury is transient, with a peak intensity at day 7 after the surgery, but as early as postoperative day 4 a full-blown microglial response was present. Thus, animals were sacrificed 4 days after the operations. DZX (1.25 mg/kg; in 10 mg/ml DMSO in distilled water) and VEH (10 mg/ml DMSO in distilled water) were administered once per day regularly at the same time by intraperitoneal injections. After obtaining the results from all groups at 4 days of survival, the hypoglossal nucleus was selected to examine the changes 7 days after the injury. All animal experiments were approved by the Ethical Committee for the Protection of Animals in Scientific Research at the Biological Research Centre (approval No. 72-45/b/2001 and No. 03876/0014/2006), which conforms to the Hungarian governmental (XXVIII. chapter IV. paragraph 31) and international (EEC Council Directive 86/609, OJ L 358, 1 DEC. 12, 1987; NIH Guide for the Care and Use of Laboratory Animals, United States National Research Council, revised 1996) laws and policies related to animal protection. All efforts were

made to minimize animal suffering, thus multiple surgical treatments were avoided.

4.2. Surgical procedures

Unilateral surgical interventions were performed under deep and reversible anesthesia with Avertin (tribromoethanol, Fluka, Buchs, Switzerland; 240 mg/kg body weight in a 0.5 ml volume) administered intraperitoneally. For target deprivation of the oculomotor neurons, the right eyeball was carefully removed, then the orbit was cleared of the remaining extraocular muscles and nerve segments. For the hypoglossal axotomy, the nerve was transected laterally to the hyoid bone and a 2–3 mm nerve segment was dissected to prevent regeneration. For the facial nerve axotomy, an incision was made under the ear canal, and the trunk of the facial nerve was cut, then a 2–3 mm nerve segment was removed. In each case, the non-operated side served as an internal control. Following surgery, the animals were allowed to recover, then were returned to their cages.

4.3. Tissue preparation for immunohistochemistry/cell counting and electron microscopy

Under irreversible anesthesia with Avertin (Fluka), mice were

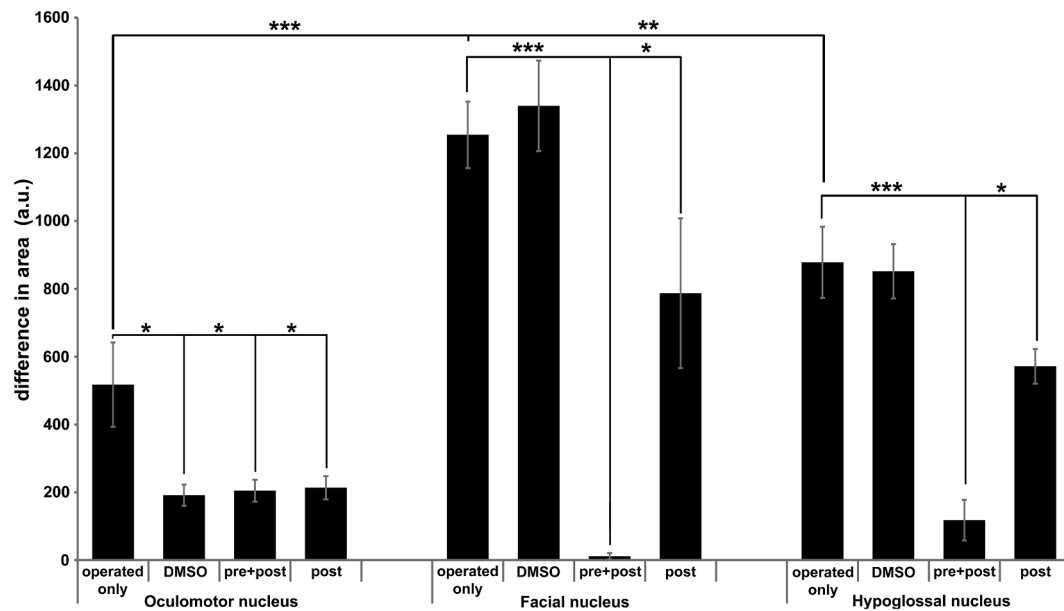


Fig. 7. Values characterizing the magnitude of microglial activation were pooled according to the investigated motor nuclei and treatments and plotted as bar graphs. Significant reduction of microglial activation can be observed in all anatomical regions after DZ treatment, however, the postoperative paradigm resulted in a smaller neuroprotective effect. Data are shown as mean $\pm$ s.e.m. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

transcardially perfused with 10 mM phosphate buffered saline (PBS; pH 7.4) followed by 4% paraformaldehyde (Sigma, St. Louis, MO, USA) in 10 mM PBS (pH 7.4). The entire brains were removed and fixed overnight in the same fixative at 4 °C. Then the samples were cryoprotected in 30% sucrose (Sigma), dissolved in 10 mM PBS, for 1 day at 4 °C and embedded in optimal cutting temperature medium (Tissue-Tek). A series of 30 μ m thick consecutive coronal sections were cut through the entire anatomical regions of interests with a cryostat (Slee MNT, Mainz, Germany), collected individually in 10 mM PBS in wells of tissue culture plates and stored at 4 °C until processed for immunohistochemical staining.

For electron microscopic evaluation animals were transcardially perfused with 10 mM PBS then fixed in Karnovsky solution (Karnovsky, 1965). Hypoglossal nuclei were removed and fixed in the same fixative for 4 h at room temperature, rinsed and postfixed in 2% osmium tetroxide solution (Millonig, 1961). After dehydration with an ascending series of ethanol, the samples were embedded in epoxy resin (Durcupan ACM, Sigma, USA) and polymerized at 56 °C for 2 days. Ultrathin sections (50 nm) were prepared and contrasted with uranyl acetate (Hayat, 1970) and lead citrate (Reynolds, 1963).

4.4. Double staining for fluorescent immunohistochemistry

To illustrate and qualitatively describe the distribution of microglial cells within the selected brainstem nuclei, a combination of a motoneuron-specific (ChAT) and a microglia-specific (ionized calcium-binding adaptor molecule 1; Iba1 – the best and most widely used microglia marker (Korzhenskii and Kirik, 2016)) antibodies was used. For double staining, 30 μ m thick free-floating cryostat sections were first rinsed in 10 mM PBS (3 changes, 5 min each) then non-specific staining was blocked with 2% normal horse serum (Vector Laboratories, Burlingame, CA, USA; Cat# S-2000, RRID: AB_2336617) in 10 mM PBS containing 0.2% Triton X-100 (Sigma) (TPBS), for 1 h. Sections were then incubated in the primary antibody cocktail of rabbit-anti-Iba1 (Wako Chemicals, Neuss, Germany; Cat# 019-19741, RRID: AB_839504; 1:200) and sheep-anti-ChAT (Merck Millipore, Billerica, MA, USA; Cat# AB144P, RRID: AB_2079751; 1:200) in 10 mM TPBS at 4 °C overnight. After washing in 10 mM PBS (3 changes, 5 min each) sections were incubated for 1 h at room temperature with donkey-anti-

sheep Alexa Fluor 488 antibody (Jackson ImmunoResearch Labs, West Grove, PA, USA; Cat# 713-545-003, RRID: AB_2340744) dissolved in 10 mM TPBS (1:100), then blocked for 1 h with a mixture of 1%-1% normal goat (Vector Laboratories, Cat# S-1000, RRID: AB_2336615) and normal donkey serum (Sigma, Cat# D9663, RRID: AB_2810235) in 10 mM TPBS, for 1 h. Afterwards, sections were incubated in goat-anti-rabbit Alexa Fluor 546 antibody (Invitrogen, Waltham, MA, USA; Cat# A-11035, RRID: AB_143051; 1:400), dissolved in 10 mM TPBS (1:100). Finally, sections were washed in 10 mM PBS (3 changes, 5 min each), mounted on silane coated glass slides, covered with Gel/Mount (Bio-medica, Foster City, CA, USA) and visualized under a fluorescent microscope (Eclipse 80i, Nikon, Tokyo, Japan, RRID: SCR_015572) equipped with a 2560 $\times$ 1920 pixel MicroPublisher 5.0 real-time viewing (RTV) charged-coupled device (CCD) camera (Teledyne QImaging, Surrey, Canada). To improve quality and reduce noise, the captured digital images were uniformly processed with the two-dimensional blind deconvolution module of the AutoQuant X program (Media Cybernetics, Rockville, MD, USA, RRID: SCR_002465).

4.5. Combined fluorescence- and diaminobenzidine-based immunohistochemistry

A cocktail of the same motoneuron-specific (ChAT) and microglia-specific (Iba1) antibodies was used, however, the secondary fluorescent antibody against Iba1 was replaced with a biotinylated one. Immunostaining was performed on 30 μ m thick free-floating cryostat sections, which were rinsed, blocked to reduce non-specific staining in the same way as was done with the conventional fluorescent staining, with an additional step to block endogenous peroxidase activity with 0.6% hydrogen peroxide in 10 mM TPBS. This was followed by overnight incubation at 4 °C in a mixture of the primary antibodies, containing a polyclonal rabbit-anti-rat antibody against Iba1 (Wako Chemicals, Cat# 019-19741, RRID: AB_839504) and a polyclonal sheep-anti-ChAT antibody (Merck Millipore, Cat# AB144P, RRID: AB_2079751) diluted to 1:3000 and to 1:500, respectively, in 10 mM TPBS. After washing in 10 mM PBS (3 changes, 5 min each), sections were incubated at room temperature in a donkey-anti-sheep Alexa Fluor 488 (Jackson ImmunoResearch Labs, Cat# 713-545-003, RRID: AB_2340744) fluorescent secondary antibody for 1 h (1:100 in 10 mM

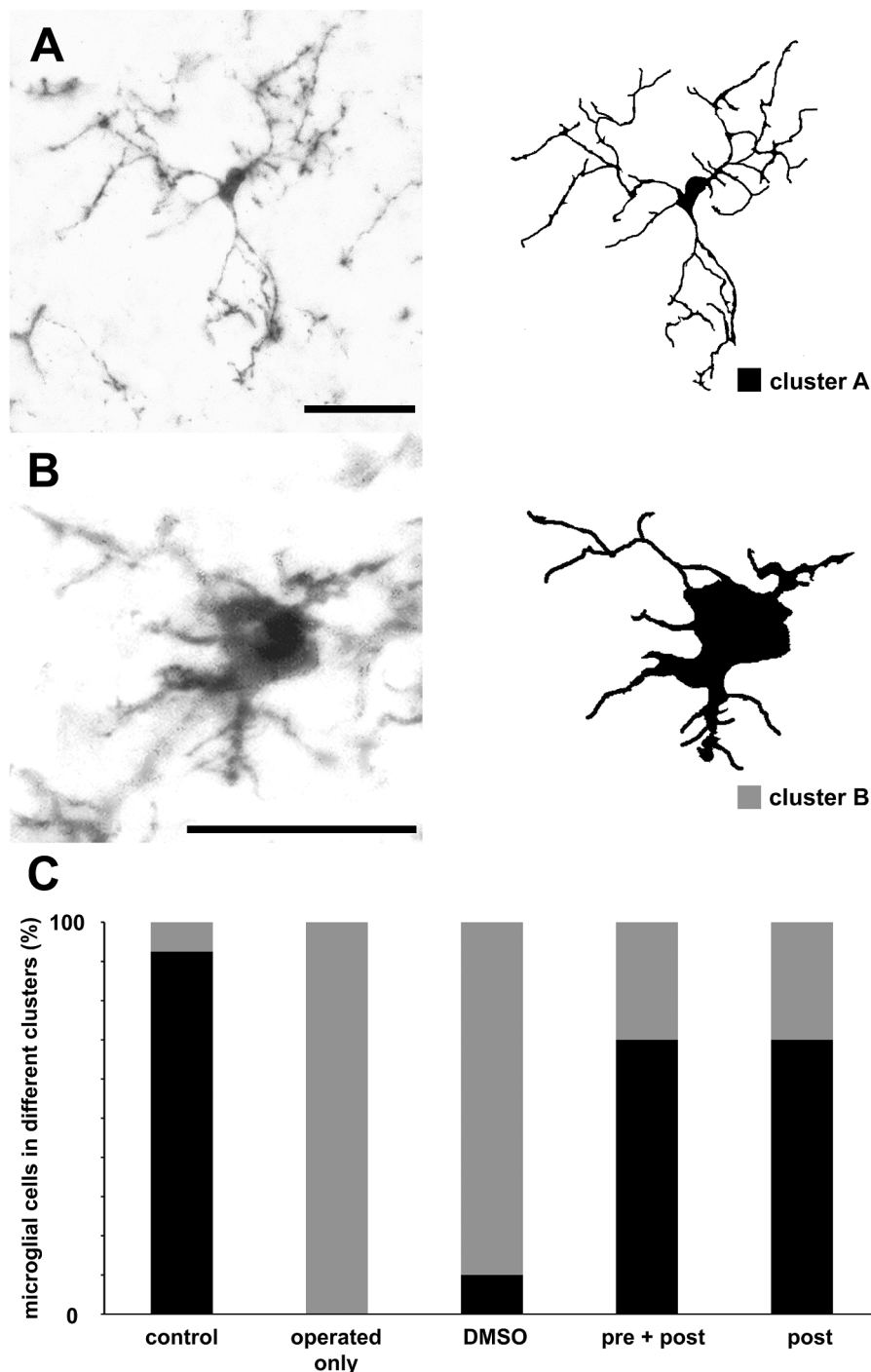


Fig. 8. Qualitative and quantitative changes in microglial morphology. A ramified morphology of a single microglia cell with a small perinuclear area and long and branching processes from the control side is shown as a microscopic image and in a binarized form (A). Activated microglial morphology is represented by the bushy-like shape with hypertrophic cytoplasm (B). Quantitative evaluation was conducted on microglia, then cluster analysis was used to determine the ratio of different morphology (C). 92.5% of all microglia from the control sides were classified into cluster A, represented by the ramified morphology on panel A. All microglia on the injured side without any treatment were sorted into cluster B, with characteristic bushy-like shape represented on panel B. In the DMSO-treated group, 90% of all examined microglia classified into cluster B. Regardless of the DZX treatment paradigm, 70% of the microglia on the injured side showed cluster A morphology, which represents the ramified-like phenotype. Scale bars: 25 μ m.

TPBS). Afterwards, non-specific staining was blocked for 1 h with a mixture of 1%-1% normal goat (Vector Laboratories, Cat# S-1000, RRID: AB_2336615) and normal donkey serum (Sigma, Cat# D9663, RRID: AB_2810235) in 10 mM TPBS, for 1 h, then sections were incubated in a biotinylated goat-anti-rabbit antibody (Vector Laboratories, Cat# BA-1000, RRID: AB_2313606; 1:400). Next, the sections were rinsed in 10 mM PBS (3 changes, 5 min each) and incubated in the avidin-biotin complex (Vector Laboratories; Cat# PK-6100, RRID: AB_2336819; 1:1600 in PBS) for 1 h at room temperature. Finally, after washing in 10 mM PBS, the reactions were visualized by incubation in 5% diaminobenzidine tetrahydrochloride (DAB; Sigma) in 10 mM PBS for 15 min. Finally, sections were washed in 10 mM PBS (3 changes, 5 min each), mounted on silane-coated glass slides, covered

with Gel/Mount (Biomedex) and visualized under a bright field/fluorescent microscope (Eclipse 80i, Nikon, RRID: SCR_015572). The method resulted in photo-stable staining for microglial cells and simultaneous fluorescent visualization of motor neurons. Thus, reproducible quantification of the number of microglial cells could be achieved while the borders of the investigated motor nucleus could be marked without affecting the quantification of the distribution of the microglial cells.

4.6. Quantitative evaluation of the activation of the microglial cells

For the quantitative assessment of the overall increase in microglial staining in the injured motor nuclei, combined fluorescent and DAB-

HYPOGLOSSAL NUCLEUS

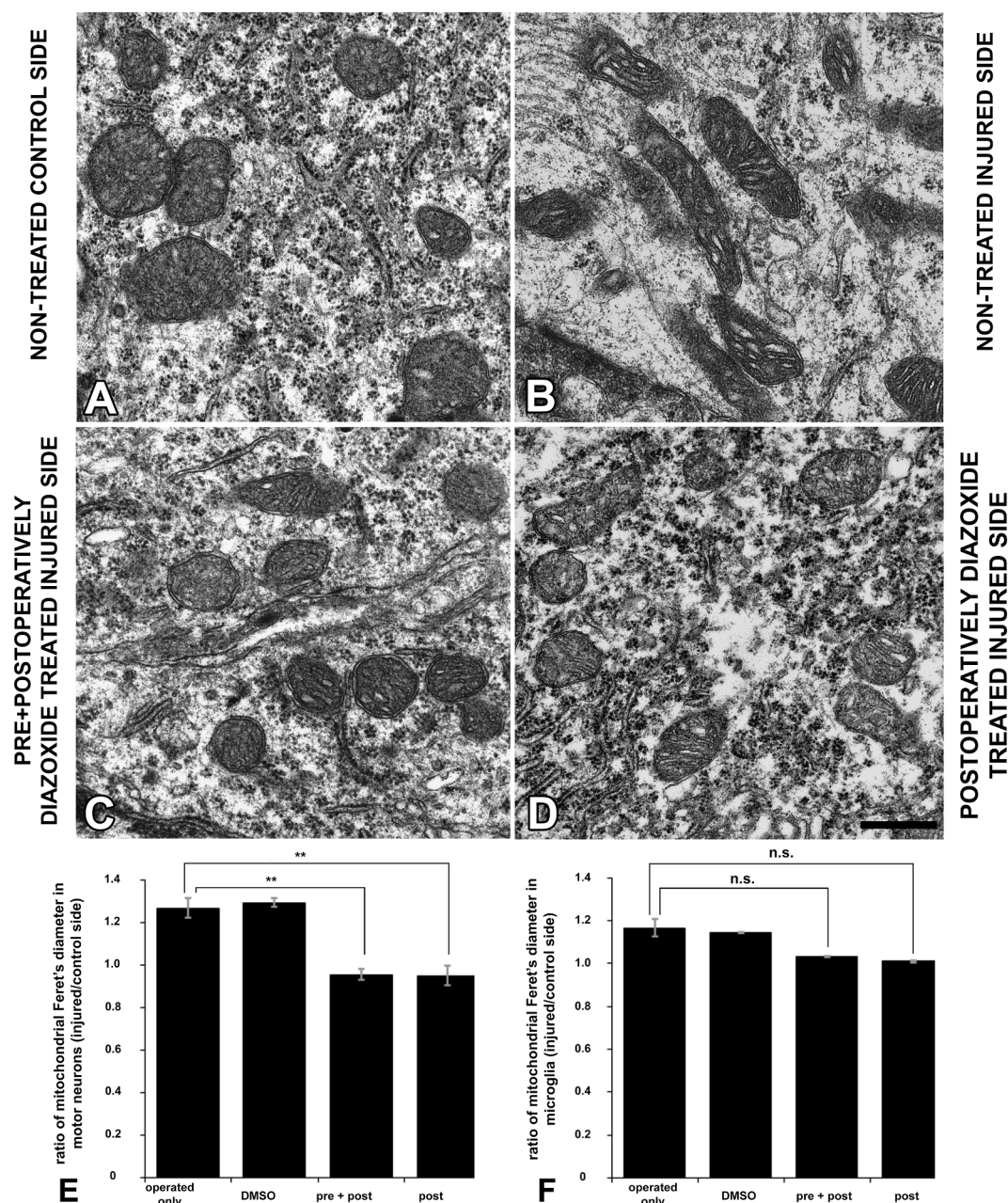


Fig. 9. Changes in the mitochondrial morphology in hypoglossal motoneurons and in microglia after axotomy. Representative image from the control hypoglossal nucleus without any treatment (A) shows round mitochondria with small Feret's diameter. A similar motoneuron from the non-treated operated side (B) shows more ellipsoid morphology with increased Feret's diameter. Besides the changes in the shape of these organelles, dilated cristae in the mitochondria could be noted on the operated side (B). After DMSO + DZX treatments (C: pre- and postoperatively DMSO + DZX-treated; D: postoperatively DMSO + DZX-treated), mitochondria regained their physiological morphology represented by their round shape and small Feret's diameter. The ratio of mitochondrial Feret's diameter (injured/control side) was measured in motoneurons (E) and microglia (F). Mitochondria from axotomized animals without any treatment, or after DMSO-treatment showed an elevated Feret's diameter in motoneurons. A marginal, non-significant increase could be noted in microglia. DZX successfully reduced this elevation in the motoneurons both in the pre + postoperatively and postoperatively treated groups but this effect was not significant in the microglia. **: $p < 0.001$; n.s.: non-significant; scale bar: 500 nm.

based immunohistochemically stained consecutive coronal sections were used. The number of sections was, in each case, high enough that the series contained the entire nucleus under investigation in the rostro-caudal direction. First, in the fluorescent mode of the microscope, the motor nuclei were located, guided by the anti-motoneuronal (ChAT) signal. Next, the identified fields of view were photographed at the same magnification to capture the fluorescent images, containing the information for marking the boundaries of the motor nuclei (ChAT),

and to record the bright field images, indicating the distribution of the microglial cells (Iba1). Using an interactive program module, developed in our laboratory for the Image-Pro Plus (Media Cybernetics, RRID: SCR_007369) image analysis software (Paizs et al., 2009), the amount of significant Iba1 staining was determined in the investigated nucleus on the operated and the contralateral control sides, in each section, expressed in area with positive Iba1 staining in a.u.. These data, representing the consistently segmented profile areas integrate both the

Table 1
Groups and sub-groups of the experimental animals used in the study.

Balb/c mice (n=77)	Facial axotomy (n=17)	D4 operated only (n=5)
		D4 pre- and postoperatively treated with VEH (n=4)
		D4 pre- and postoperatively treated with VEH+DZX (n=4)
		D4 postoperatively treated with VEH+DZX (n=4)
	Hypoglossal axotomy (n=43)	D4 operated only (n=11)
		D4 pre- and postoperatively treated with VEH (n=6)
		D4 pre- and postoperatively treated with VEH+DZX (n=6)
		D4 postoperatively treated with VEH+DZX (n=6)
		D7 operated only (n=3)
		D7 pre- and postoperatively treated with VEH (n=3)
		D7 pre- and postoperatively treated with VEH+DZX (n=4)
		D7 postoperatively treated with VEH+DZX (n=4)
	Eye enucleation (n=17)	D4 operated only (n=5)
		D4 pre- and postoperatively treated with VEH (n=4)
		D4 pre- and postoperatively treated with VEH+DZX (n=4)
		D4 postoperatively treated with VEH+DZX (n=4)

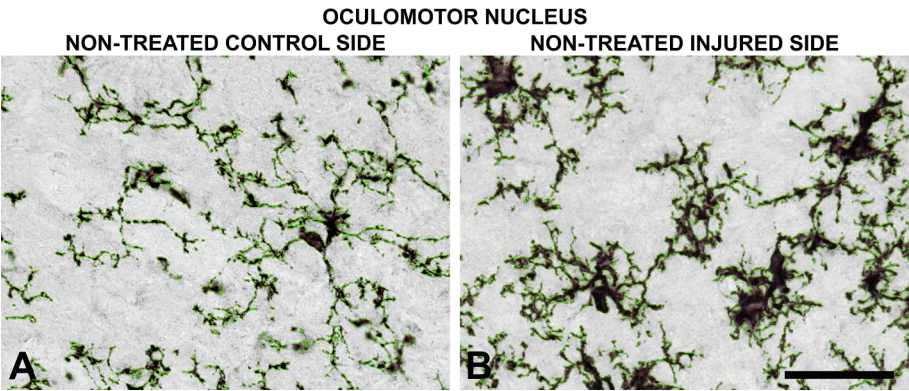


Fig. 10. Segmented profiles (green outlines) of resting microglia on the control side (A) and activated microglial cells on the injured side (B) of the oculomotor nucleus. The sum of the outlined areas relative to the area of the whole field of view is a parameter sensitive to the product of the number and the size of the individual microglial cells. In the illustrated example from the oculomotor nucleus, activated microglial cells with phagocytic and ramified phenotype at the operated side (B) display 50% larger area coverage than the resting microglia at the control side (A). Scale bar: 50 μ m.

changes in the number of positive microglial cells and in their morphological appearance, as illustrated in Fig. 10. Finally, the algebraic differences of the values obtained at the operated side and the non-operated side were formed to express the increase of staining due to the surgery. These differences were plotted along the rostro-caudal axis, to demonstrate the rise and decay of microglial activation within the nucleus along this axis. Alternatively, these values were summed up throughout the sections of the series to arrive at single values characterizing the magnitude of microgliosis after injury in each investigated motor nucleus of each animal, which were used for statistical analysis.

4.7. Quantification of neuronal survival and changes in mitochondrial morphology

Motor neurons were counted under Eclipse 80i (Nikon, Tokyo, Japan) light microscope in a set of equidistantly (at 60 μ m) placed sections, selected from the collection of consecutively cut sections from the hypoglossal nucleus of each animal. The motor neurons were identified by their size, their location in the coronal section and their morphological features. To satisfy the selection criterion of the disector method, i.e., to identify the object to be counted with its single, point-like structure, motor neurons with discernible nucleus were counted. From each animal (n = 4/group), 10–10 sections were screened for the presence of motor neurons. Motor neuronal counts were pooled for animals and expressed as a volume density (number/mm³), according to the total examined volume (total area examined multiplied by a 30 μ m section thickness) of the hypoglossal nucleus in each animal. The average number of motor neurons was determined for each treated

group at each time point.

Alterations of the mitochondria are routinely detected with morphometric parameters such as perimeter, area or Feret's diameter, the longest distance between any two points of the mitochondrial external perimeter (Errea et al., 2015; Picard et al., 2013). Since increased Feret's diameter could be observed in oxidative stress (Federico et al., 2017; Demeter-Haludka et al., 2018), we selected this parameter to test whether reduced oxidative stress could be achieved by DZX treatment. Transmission electron microscope (JEM-1400Flash; JEOL, Tokyo, Japan) was used in conventional transmission mode (120 kV) to capture mitochondria inside motor neurons and inside microglia, using a scientific complementary metal-oxidesemiconductor (sCMOS) camera (Matataki Flash; JEOL) at 12 000 $\times$ magnification. 10–10 images were taken from different motor neurons and microglia from both sides. Images obtained with electron microscopy were used for examining the changes in mitochondrial morphology. Each mitochondrion from all images was segmented and morphometric parameters were measured with the built-in functions of Image-Pro Plus (Media Cybernetics).

4.8. Morphometry of the microglial cells

Quantitative morphometry was examined according to the work of Fernández-Arjona and her colleagues (Fernández-Arjona et al., 2017). Sections were screened under Eclipse 80i light microscope (Nikon) on 40 $\times$ magnification. 10–10 microglial cells were identified and captured with extended depth of field imaging from the control and from the injured side of the hypoglossal nucleus to visualize the complete arborization of the microglial processes. Images were then processed in Photoshop (Adobe, San Jose, CA, USA) to extract single microglial cells.

Contrast and brightness were adjusted to achieve the best cell structure visibility. Images were then converted to binary images in ImageJ and further corrected in Photoshop. From the segmented microglia, area and perimeter of the cell were extracted from ImageJ and the following parameters were extracted with the use of Box Counting Fractal Analysis method using FracLac plugin in ImageJ: cell area, cell perimeter, fractal dimension, lacunarity, convex hull (CH) span ratio, CH area, CH perimeter, CH circularity, max/min radii, mean radius, diameter of bounding circle (Karperien et al., 2013). Density, cell circularity, and roughness were calculated. All data were normalized in order to serve as an input to clustering algorithms, then processed with Weka Explorer (Witten and Frank, 2000) open-source data mining software package. Data were automatically divided into two different clusters with the k-means based Farthest-First clustering algorithm (Hochbaum and Maass, 1985).

4.9. Statistical analysis

Values, characterizing the magnitude of microgliosis, the number of motor neurons and mitochondrial Feret's diameter were pooled according to the investigated motor nuclei and treatments and were expressed as mean $\pm$ s.e.m. Differences among the mean values were assessed by one-way analysis of variance with least significant difference post-hoc test. All statistical analyses were performed with R version 3.6.1 statistical computing software (The R Foundation, Vienna, Austria, RRID: SCR_001905) with RStudio Integrated Development Environment (RStudio, Boston MA, USA, RRID: SCR_000432).

CRedit authorship contribution statement

Bernat Nogradi: Investigation, Resources, Writing - original draft. **Valeria Meszlenyi:** Investigation, Visualization. **Roland Patai:** Investigation, Supervision, Project administration, Formal analysis. **Tamas F. Polgar:** Investigation, Data curation, Visualization, Formal analysis. **Krisztina Spisak:** Investigation, Visualization. **Rebeka Kristof:** Investigation, Visualization. **Laszlo Siklos:** Conceptualization, Methodology, Funding acquisition, Validation, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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