Fluoro-Jade B Evidence of Induced Ischemic Tolerance in the Rat Spinal Cord Ischemia: Physiological, Neurological and Histopathological Consequences

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Abstract. Fluoro-Jade B, a marker of degenerating neurons, was used to label histopathological changes in the rat spinal cord after transient ischemia and ischemic preconditioning (IPC). To characterize postischemic neurodegenerations and consequent neurological changes, a particular attention was paid to the standardization of ischemic conditions in animals of both groups. 1. The control ischemic rats were submitted to a reversible occlusion of descending aorta by insertion and subsequent inflation of a 2F Fogarty catheter for 12 min. 2. In the IPC rats, an episode of short 3 min occlusion and 30 min reperfusion preceded the 12 min ischemia. Postischemic motor function testing (ambulation and stepping) was provided repeatedly for evaluation of neurological status 2 h and 24 h after surgery and at the end of postischemic survival, i.e. after 48 h. Fluoro-Jade B staining was used to demonstrate degenerated neurons. In the control rats, neurological consequences of histopathological changes in lumbosacral spinal cord, manifested as paraplegia, were present after 12 min ischemia. Thus, numbers of degenerated Fluoro-Jade B positive cells were visible in gray matter of the most injured L_4–S_2 spinal cord segments. Slight motor function impairment, consequential from significant decreasing in Fluoro-Jade B-positivity in the L_4–S_2 spinal cord segments of the IPC rats, was considered the pathomorpfological evidence that IPC induces spinal cord tolerance to ischemia. Our results are consistent with the previously published silver impregnation method for histopathological demonstration of ischemic degeneration.

Key words: Degeneration — Improvement — Locomotion — Fluoro-Jade B — Quantitative analyses

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Introduction

Ischemic preconditioning (IPC) has been defined as an endogenous cellular protective mechanism, evoked by a brief ischemic period (Ishida et al. 1997; for review see Lukáčová 1999). Recently, it has been well documented that IPC renders the nervous tissue more resistant to the subsequent severe ischemic insult (Kitagawa et al. 1991; Zvara et al. 1999). In the brain, IPC offers significant neuroprotection after focal and global ischemia (Pérez-Pinzón et al. 1997; Masada et al. 2001; Burda et al. 2003; Danielisová et al. 2003; Némethová et al. 2003; Radoňák et al. 2003) and also in the spinal cord, the IPC has been shown to induce ischemic tolerance (Abraham et al. 2000; Orendáčová et al. 2004). In spite of many literary data, the mechanism underlying the increased resistence of the nervous tissue to severe ischemia by a preceding mild ischemic exposure remains unclear. Although a similar protective effect of the IPC has been observed even after experimental traumatic brain injury (Pérez-Pinzón et al. 1999), it has been documented that IPC does not improve the outcome after spinal cord compression injury, and even worsens the damage of the spinal cord white matter in rat (Ondrejčák et al. 2004).

Previously, degeneration of spinal cord neurons and corresponding loss of motor and/or sensory function have been shown by silver impregnation methods in mammals (Marsala et al. 1989, 1997; Vanický et al. 1992; Marsala and Yaksh 1994; Saganová and Maršala 1994; Farkas et al. 2003). At present, a relatively novel fluorescent dye, Fluoro-Jade B, is successfully used as a marker of dying, apoptotic and necrotic neurons (Schmued et al. 1997; Schmued and Hopkins 2000; Ballok et al. 2003). Here we used Fluoro-Jade B labeling to detect neuropathological changes, manifested as motor function impairment, after severe spinal cord ischemia in the rats. In addition, the reliability of the Fluoro-Jade B staning was tested in the model of ischemic tolerance induced by IPC in the rat spinal cord.

Materials and Methods

In this study, 23 male Wistar rats were used. Special attention was taken to include only animals with similar body weights (strictly within the range between 350–375 g) to assure high reproducibility of the outcomes. The animals were randomly selected into the control ischemic group ($n = 11$) and IPC group ($n = 12$). 1. Animals in the control group underwent only 12 min ischemia. 2. Preconditioning in the IPC group was induced by transient 3 min spinal cord ischemia followed by 30 min of reperfusion prior to the 12 min ischemia. The surgical procedures including anesthesia, blood withdrawal and drug administration were identical in animals of both groups.

Ischemic model – descendent aorta occlusion

The model of spinal cord ischemia described previously in detail by Taira and Marsala (1996) has been used. Briefly, the tail artery was cannulated with a 22-gauge polytetrafluoroethylene (PTFE) catheter for monitoring of distal arterial

pressure (DAP) and for intra-arterial administration of heparin. The right carotid artery was cannulated with an 18-gauge PTFE catheter for monitoring of proximal arterial pressure (PAP) and for blood withdrawal. The right femoral artery was exposed, and 2F Fogarty catheter was inserted cranially (11.1 cm from the site of insertion) into the thoracic aorta, so that the tip of the catheter reached the left subclavian artery. After completion of arterial cannulations, all animals received 200 U of heparin (0.2 ml). The carotid artery cannula was connected to a heated blood-collection circuit (37.5 ℃) that included a 54 cm vertical column filled with heparinized saline (1 U/ml). For induction of spinal cord ischemia, the balloon was inflated with 0.05 ml of saline and aortic occlusion was confirmed by reduction of the DAP. Simultaneously, to control the PAP during occlusion, the blood from the carotid artery was allowed to flow into a heparinized reservoir maintaining PAP at 5.3 kPa (40 mm Hg). At the end of ischemic period, the balloon was deflated, and the collected blood was reinfused slowly within 60 s. In the control group, the aorta was occluded for 12 min. The IPC rats were submitted to a short 3 min period of occlusion followed by 30 min of reperfusion before the 12 min ischemia. During reperfusion, the animals in IPC group were monitored and maintained under anesthesia. After removal of the catheters, surgical wounds were closed and 4 mg (0.4 ml) of protamine sulfate was delivered intraperitoneally.

In addition, animals undergoing the same surgical procedure without the ischemic insult, with the same post-injury survival, served as sham control.

Neurological status assessment

The animals were allowed to survive for 48 h after the completion of surgical procedures. The first open field locomotion testing was performed 2 h after ischemic injury, the next after 24 h, and the last, 48 h after ischemic injury. An observer unaware of experimental conditions performed testing. Neurological status of each animal was evaluated using two concurrently performed behavioral tests, described in details by Marsala and Yaksh (1994): 1. Ambulation – scale ranging from 0 to 4 was used to assess the locomotion of rats, i.e. their ability to use hindlimbs for walking. 2. Stepping – scores from 0 to 2 were assigned with regards to the rat's competence to drag the hindlimbs over an obstacle (table edge). For ambulation and stepping tests, 0 reflected normal status whereas the total loss of function was rated with the score of 4 or 2, respectively.

Tissue sampling for Fluoro-Jade B staining

The rats were perfused with 4 % paraformaldehyde in a deep anesthesia. The lumbosacral spinal cord was removed from the backbone, postfixed, cryoprotected in 30 % sucrose and cut into 10 equidistant blocks. From each block, frozen 40 μm thick coronal sections were cut and collected in phosphate buffer. Fluoro-Jade B was used as a marker of neurons undergoing degeneration. The sections mounted on 2 % gelatin coated slides and air-dried were heated at 50 ℃ for at least half an hour before staining. The slides were immersed in absolute alcohol for 3 min, for 1 min in 70 % alcohol and for 1 min in distilled water. Then the slides were transferred to

the solution of 0.06 % potassium permanganate for 15 min and rinsed in distilled water for 2 min. After 30 min in the staining solution, prepared according to the Schmued and Hopkins (2000), 3 × 1 min rinses in distilled water followed. The slides were dried at room temperature, cleared by xylene and cover slipped with Fluoromont. Results were assessed with blue excitation light of Olympus BX51 reflected fluorescent microscope.

Statistical analyses of neurological data

For each hind limb, i.e. ipsilateral (operated) or contralateral (intact) hind limb, scores from both tests were summed to obtain a single value of motor deficit index (MDI). For final examination performed 48 h, postoperatively intergroup differences were analyzed by non-parametric test (Mann–Whitney U test). Data are presented as means ± standard error (SEM) of the mean; p value of less than 0.05 was considered statistically significant.

Semi-quantitative assessment of histopathological data

Ten representative sections were selected from the whole lumbosacral spinal cord according to principles of systematic random sampling. The sections were transformed into digital images by the Olympus reflected fluorescence system U-RFL-T, the Olympus BX51, the digital camera DP50 and the visual communication suite Olympus DP-soft, version 3.0. The number of Fluoro-Jade B positive cells was counted within 0.3 mm^2 area of the ventral horn, intermediate zone and dorsal horn (Fig. 3). In order to determine the number and optical density of Fluoro-Jade B-exhibiting neurons in the L_4–S_2 spinal cord, the UTHSCSA Image Tool and Microsoft Excel were used in both experimental groups. The gray scale values relative to the highest concentration of Fluoro-Jade B in degenerating neurons and the lack of the fluorescent marker in the section was defined as the 100 % and 0 % density values (Kuchárová et al. 2001). The results were statistically evaluated by t-test and have been given as means ± SEM.

Results

Physiological variables during ischemia

During the operation, several physiological parameters were measured, i.e. rectal temperature, PAP, DAP and blood volume collected in the extracorporeal circuit during the ischemia. No significant changes in body temperature were observed during the experiment (Table 1). In sham-operated animals, only rectal temperature and PAP and DAP was measured and no significant differences in comparison to the experimental animals occurred.

During aortic occlusion, PAP was maintained close to 5.3 kPa (40 mm Hg) in both groups. Resting DAP before ischemia was 12.59 ± 0.79 kPa or 12.37 ± 1.41 kPa in the control group and the IPC, respectively. In the IPC group, after the induction of 3 min ischemia, DAP dropped to 0.99 ± 0.33 kPa and during the

Table 1. Physiological variables during the operation

Parameter/Group		Control	IPC
Rectal temperature (℃)	Preconditioning	*	37.2 ± 0.20
	Reperfusion	*	37.05 ± 0.42
	Ischemia	37.2 ± 0.33	37.11 ± 0.38
DAP (kPa)	Baseline	12.59 ± 0.79	12.37 ± 1.41
	Preconditioning	*	0.99 ± 0.33
	Reperfusion	*	11.19 ± 1.43
	Ischemia	0.63 ± 0.28	0.81 ± 0.13
Vented blood volume (ml)	Preconditioning	*	10.9 ± 0.94
	Ischemia	11.5 ± 2.01	9.80 ± 0.60

Rectal temperature was continuously measured and quoted during 3 min preconditioning, reperfusion and 12 min ischemia. Distal arterial pressure (DAP) was recorded before and during the blood drainage. Volume of temporary withdrawn blood was measured just before its redelivery. In the control group, where only 12 min ischemia was induced, variables related to the ischemic preconditioning (IPC) were not measured (asterisks). Data are shown as group means ± SEM.

reperfusion period it stabilized at 11.19 ± 1.43 kPa. During the 12 min ischemia, DAP dropped to 0.63 ± 0.28 kPa in the control group, whereas in the IPC group it fell to 0.8 ± 0.13 kPa. In the control group, the blood volume collected during 12 min ischemia was 11.5 ± 2.01 ml. In the IPC group, the blood volume accumulated in the extracorporal collector was 10.9 ± 0.94 ml during 3 min ischemia and 9.80 ± 0.60 ml during 12 min ischemia. The values of collected blood volume during the severe 12 min ischemia did not differed significantly between the two groups.

Neurological status assessment

After recovery from anesthesia, all animals exhibited marks of impaired locomotor function. 2 h after intervention, all but one rat in the control ischemic group were paraplegic with no recovery observed by the end of survival (Fig. 1). Similarly, the locomotor performance of rats in the IPC group as well as the sham control animals was deteriorated when measured 2 h after the severe ischemia, but they, however, exhibited noticeable recovery during the survival. The final MDI score measured 48 h after the operation was 5.66 ± 1.03 in the control ischemic group (incidence of complete paraplegia 81%) and 3.07 ± 1.12 in the IPC group (complete paraplegia observed in 27% of animals). Statistical evaluation of the final MDI (scores averaged across the hind limbs) revealed a statistically significant intergroup difference ($p = 0.003$, Mann–Whitney U test). Similarly, when comparing the MDI scores separately for ipsilateral and contraleteral hind limbs between the two groups, the differences were statistically significant ($p < 0.01$). The differences of MDI scores between the right (ipsilateral) and left (contralateral) hind limb mea-

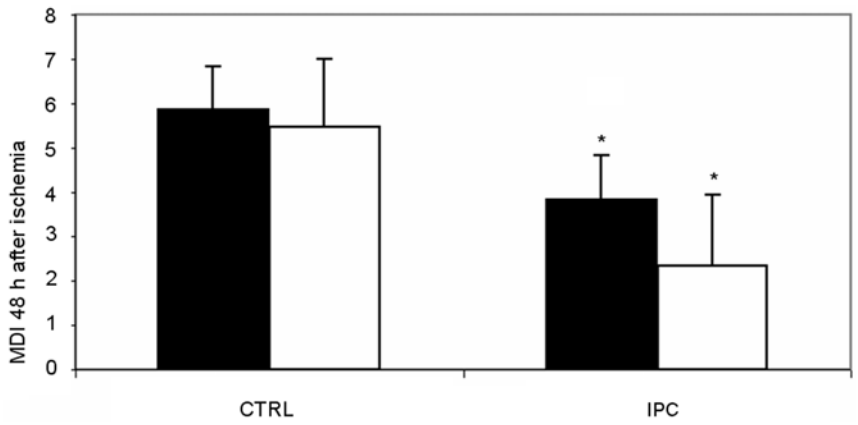

Figure 1. Neurological status of control ischemic (CTRL) and ischemic preconditioning (IPC) rats, obtained 48 h after the operation by assessment of both ambulation and stepping separately for ipsilateral (black column) and contralateral (white column) hind limb, is expressed as motor deficit index. Value of 6 represents total loss of ability to use hind limbs. Zero belongs to the rats without any neurological consequences. Asterisks indicate statistically significant difference ($p < 0.01$) of motor deficit index (MDI) scores between the control and IPC group. Data are expressed as means ± SEM.

sured in individual groups did not reach the level of statistical significance after 48 h (Fig. 1).

Histopathological outcomes

12 min ischemia: Pathomorphological signs of ischemic degeneration were fully developed in the most injured L_4–S_2 spinal cord segments of the control rats subjected to 12 min ischemia and 48 h surviving. Widespread neuronal dying appeared as numbers of Fluoro-Jade B positive cells in a distinct, rostro-caudally demarcated area of L_4–S_2 spinal cord segments. The sections displayed numbers of green yellowish fluorescence of multipolar cells, visible as light structures on black and white figures, and identifiable by their apparent neuronal-like morphology as neurons. The labeled cells were distributed particularly throughout the dorsal horn and intermediate zone (Fig. 2A). The upper segments of lumbar spinal cord showed omissible fluorescence.

Ischemic preconditioning: Pathomorphological signs of ischemic degeneration in spinal cord neurons of IPC rats were minimal within the L_4–S_2 spinal cord segments. A few Fluoro-Jade B positive cells were visible as green yellowish fluorescence (the light structures on black and white figures) situated haphazardly in the intermediate zone (Fig. 2B).

Sham control animals: The presence of fluorescent cells was much frequent than after ischemic preconditioning, thus it was considered omissible for quantitative analysis.

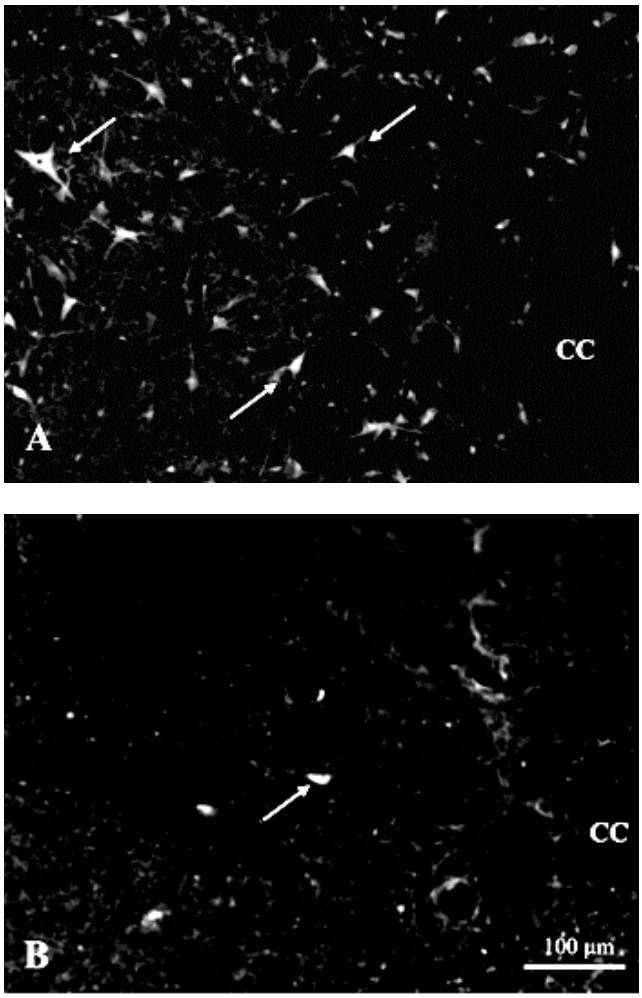

Figure 2. Representative fluorescence micrographs show Fluoro-Jade B-labeled neurodegenerations in the L_4 spinal cord segments after 48 h survival. **A.** The control group: arrows indicate numbers of fluorescent cells in the dorsal horn and intermediate zone. **B.** The IPC group: only a few fluorescent cells (arrow) are visible in the intermediate zone. CC, central canal.

Semi-quantitative assessment of histopathological data

Quantitative analysis of Fluoro-Jade B sections showed a significant increase in fluorescent cells throughout the gray matter of L_4–S_2 spinal cord segments in the control ischemic group in comparison to the IPC rats (Fig. 3). In the rats submitted only to the 12 min ischemia, the highest number of Fluoro-Jade B-exhibiting cells was present in the dorsal horn and intermediate zone. In the IPC rats, only a

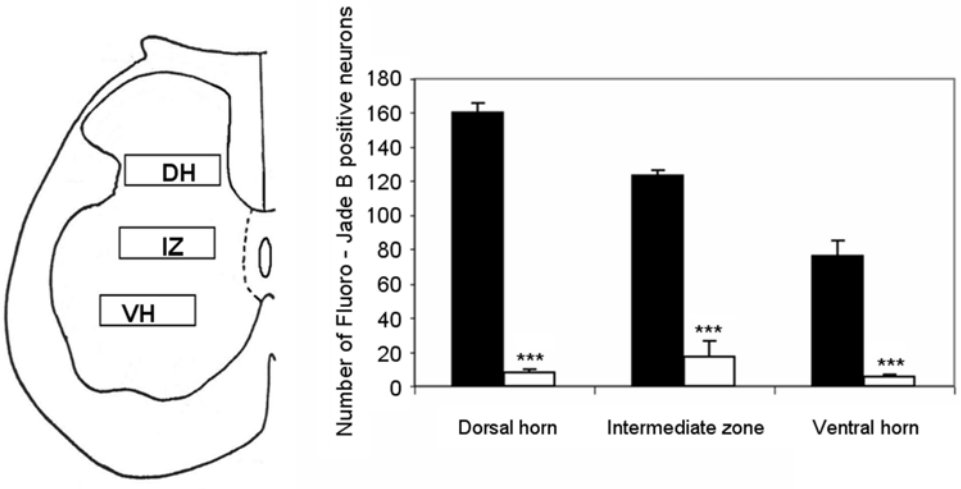

Figure 3. Schematic drawing indicates dorsal horn (DH), intermediate zone (IZ) and ventral horn (VH); the selected areas are shown as boxed areas and represent regions of 0.3 mm^2, from which the quantitative analyses were made. Beneficial effect of ischemic preconditioning (IPC, white column) to the 12 min ischemia (I, black column) is evaluated quantitatively as significant decreasing of the numbers of Fluoro-Jade B positive neurons in the dorsal horns, intermediate zone and ventral horns. Asterisks indicate significant differences ($p < 0.001$) of Fluoro-Jade B positive cells number between the control and IPC group. Data are expressed as means ± SEM.

few Fluoro-Jade B-expressing cells were distributed in the intermediate zone. The optical density value, reflecting concentration of Fluoro-Jade B in degenerating cells was similar, between 63 and 65, in the control and IPC group.

Discussion

Standardization of physiological parameters in the spinal cord ischemia model prevents from appearance of controversial neuropathological changes (Zivin and DeGirolami 1980; DeGirolami and Zivin 1982). Although the preconditioning episodes of short duration ischemia have provided a significant histological protection of CA1 pyramidal cells against subsequent severe ischemic insult, this has not resulted in a complete behavioral protection (Corbett and Crooks 1997). This finding, one of others similar, indicates importance of correlation of the morphological findings with functional outcomes. In our experiment we intended to find morphological basis of functional impairment after ischemia or morphological evidence of IPC beneficial effect by showing the neurological consequences of morphological changes.

To guarantee the reproducibility of the data, first we paid attention not only to the physiological parameters measurement during ischemia but also to the age, sex, habitus and body size of experimental animals. This selection increased probability

that the tip of Fogarty catheter, introduced into the thoracic aorta 11.1 cm cranially from the site of insertion, reached the level of left subclavian artery. Thus we provided control of the occlusion level. Also the volume of vented blood, needed for assurance of the blood hypovolemy during the ischemic period, was controlled to be similar in all experimental animals. These requirements for experimental condition were considered very important and together with simultaneous measurement of physiological parameters gave us the evidence about the standards of ischemic conditions in each experimental animal.

Although the monitored spinal cord ischemia is considered reliable to induce paraplegia in rabbits (Johnson et al. 1993) and rats (Marsala and Yaksh 1994; Taira and Marsala 1996) it is needed to find the shortest survival time at which the neurological deficit reflects pathomorphological changes rather than postoperative discomfort of animals. Previously it has been documented that selectively affected Nauta positive neuronal pools, characteristic as somatodendritic argyrophilia, are fully developed after 48 h of postischemic recirculation in rabbits (Saganová and Maršala 1994). In addition, 48 h survival after ischemia had been shown as sufficient to produce argyrophilia also in the spinal cord neurons of the rats (Ondrejčák 2001; Orendáčová et al. 2004). Our neurological findings, that immediately after recovery from anesthesia all animals, ischemic and IPC as well, exhibited marks of impaired motor function accent the importance of postinjury survival duration to distinguish neurological impairment due to the ischemic degeneration from the transient postoperative locomotion discomfort. Therefore we evaluated neurological status separately at the right hind limb, i.e. at the side of Fogarthy catheter insertion, and at the left hind limb, i.e. the intact contralateral one, after 2, 24 and 48 h postinjury. The differences of MDI scores between the right (ipsilateral) and left (contralateral) hind limb measured in individual groups did not reach the level of statistical significance after 48 h. The final examination of motor deficit index, done 48 h after surgery, put the evidence about the saved and/or impaired neurological status. We consider the time of 48 h survival after surgical intervention as sufficient to make distinction between functional impairment of ischemic control animals and improvement of IPC rats.

Kainic acid exposure was the first neurotoxic insult systematically examined by the Fluoro-Jade B (Schmued and Hopkins 2000). Despite the incomplete knowledge of this fluorochrome staining mechanisms, it is evident that this relatively simple method reliably detects dying neurons, regardless of the cell death cause. Up to now, new experiments have brought new facts about this fluorochrome using, what is not surprising. Thus, for example, recently there has been no doubt about reliability of Fluoro-Jade B using for detection of apoptotic cells in the nervous tissue due to the simultaneous using of Fluoro-Jade B and the technique of TdT-mediated dUTO-biotin nick end labeling (TUNEL) (Simpson et al. 2001; Ballok et al. 2003). Also the results indicating neurodegeneration in the brain tissue after traumatic injury (Sato et al. 2001) and after middle cerebral artery occlusion (Butler et al. 2002) are clear and undoubted. On the other hand, the results concerning the spinal cord are vague. Anderson et al. (2003) stressed null Fluoro-

Jade B positive neurons after contusion injury in rodent spinal cord. Instead, they presented Fluoro-Jade B labeled astrocytes in uninjured control and injured animals. The authors confirmed the specificity of astrocytes staining by co-localization of fluorescein with glial fibrilar acid protein. Opposing to that findings, some of the up-to-date papers demonstrated Fluoro-Jade B-positivity in neurons. Nguyen et al. (2004) demonstrated neurodegeneration by presence of the fluorochrom in neuronal cell bodies and axons in ventral horns of L5 spinal cord level after robust inflammatory reaction in mouse model of amyotrophic lateral sclerosis. Simpson et al. (2001) demonstrated by Fluoro-Jade B labeling neuronal cell death of caspase 3 deficient peripheral nervous system, trigeminal and dorsal root ganglia neurons but not central nervous system during prenatal development. The authors suggest that peripheral nervous system neurons are dependent on caspase 3 for execution of apoptosis.

To be sure that the labeled cell are neurons and not astrocytes, first we compared Fluoro-Jade B staining with the previously published results of ischemic neurodegeneration demonstrated by silver impregnation methods (Marsala et al. 1989). The distribution pattern, size and shape of Fluoro-Jade B positive cells coincided with the silver impregnated, i.e. degenerated neurons. Our results strengthen the observations with simultaneous using of the both methods (Poirier et al. 2000; Schmued and Hopkins 2000; Ye et al. 2001). In addition, it should be stressed that Fluoro-Jade B staining has some advantages in comparison to the silver impregnation. Non-specific staining does not appear (Ballok et al. 2003), no special care has to be taken to avoid artificial changes, e.g. following inadequate perfusion or in situ fixation, as it is necessary to keep during the silver impregnation.

To identify the labeled cells type, we accomplished comparison of Fluoro-Jade B- and glial fibrilar acid protein (GFAP)-fluorescent immunohistochemistry. Cizkova et al. (2004) in a parallel study, which we consider crucial, demonstrate GFAP-negativity in the most injured regions of rat lumbar spinal cord after 10 min ischemia and 24 h survival. GFAP positive cells, demonstrated in noninjured regions, have different shape of body due to their numerous processes in comparison to the larger, mostly triangular perikarya and long tiny processes of the Fluoro-Jade B positive cells. It should be stressed that morphology of Fluoro-Jade B-labeled cells present in spinal cord after ischemia correspond to morphology of the spinal cord fluorescent cells visualized by NeuN, a specific marker of neurons (Kakinohana et al. 2004). Taking together, these results ensure us to identify the Fluoro-Jade B cells as neurons.

In our laboratory we have demonstrated reliability of Fluoro-Jade B staining also for detection of dying cell associated with cell proliferation within the rostral migratory stream in control neonatal rats (Martončíková et al. 2003) and in adult bulbectomized rats (Mitrušková et al. 2005). Here we demonstrate similar results by the Fluoro-Jade B labeling as they have been published by the silver impregnation after 12 min ischemia in rats (Ondrejčák 2001). Moreover, the distribution of the labeled fluorescent cells within the spinal cord gray matter as well as rostro-caudal demarcation of the most injured spinal cord segments copy

the spatial distribution of argyrophilic Nauta positive neurons (Orendáčová et al. 2004).

Fluoro-Jade B staining showed its reliability also in the IPC rats, where only minimal number the Fluoro-Jade B positive cells was present in the L_4–S_2, which means absence of severe degeneration. We interpret this finding as beneficial effect of ischemic preconditioning in the IPC rats. It was expected, because attenuation of the neurological impairment strongly indicated this beneficial effect of the 3 min preconditioning in the IPC rats. In addition, the quantitative analyses confirmed improvement of neurological status and histopathological outcomes of the IPC rats as significant.

In conclusion, we consider the significant decreasing of Fluoro-Jade B labeling in the spinal cord of IPC rats in comparison to the ischemic rats, as pathomorphological evidence that a short ischemic preconditioning protects rats against ischemic neuronal damage.

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