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Research article

Neurons of the rat cervical spinal cord express vimentin and neurofilament after intraparenchymal injection of kainic acid

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HIGHLIGHTS

- Some spinal cord perikarya express VIM and NF after experimental injury.
- Expression of VIM and NF last for several days after injury.
- VIM and NF colocalize in the same perikarya after injury.
- VIM and NF expression may be a necessary change to promote recovery of the damaged tissue.

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ABSTRACT

Intermediate filaments (IF) can be altered under disorders such as neurodegenerative diseases. Kainic acid (KA) induce behavioral changes and histopathological alterations of the spinal cord of injected rats. Our goal was to evaluate the IF expression in neurons during this injury model. Animals were injected with KA at the C5 segment of the cervical spinal cord and euthanized at 1, 3 and 7 post injection (pi) days. Neuronal cell counting showed a significant loss of neurons at the injection site when compared with those of sham and non-operated animals. Immunohistochemistry for vimentin and neurofilament showed positive labeling of perikarya in sham and KA-injected animals since day 1 pi that lasted for the remaining experimental days. Colocalization analysis between enolase and vimentin or neurofilament confirmed a high index of colocalization in both experimental groups at day 1 pi. This index decreased in sham animals by day 3 pi whereas that of KA-injected animals remained high throughout the experiment. These results may suggest that perikarya initiate an unconventional IF expression, which may respond to the neuronal damage induced by the mechanical injury and the excitotoxic effect of KA. It seems that vimentin and neurofilament expression may be a necessary change to promote recovery of the damaged tissue.

1. Introduction

Intermediate filaments (IF) are the major components of the cytoskeleton, together with microtubules and microfilaments. Their expression is finely tuned depending on the cell type and development [1]. In the mammalian nervous system, mainly six types of intermediate filament proteins are known to be expressed, both at central and peripheral cells [2]: (1) peripherin, a type III IF protein expressed mainly in neurons of the peripheral nervous system and neurons of the central nervous system (CNS) that have projections toward spinal motor neurons [3]; (2) vimentin, a type III IF protein expressed in mesenchymal and neuronal progenitor cells; (3) glial fibrillary acidic protein, a type III IF expressed in mature astrocytes; (4) alpha-internexin, a class IV IF, expressed in developing neuroblasts and primarily present in neurons of the adult CNS [4] (5) neurofilament, a type IV IF, which is the major component of the cytoskeleton of mature neurons [5] and, (6) nestin, a type VI IF, implicated in the radial growth of the axon expressed in mesenchymal and neuronal progenitor cells [6]. In

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general, it has been shown that IF play an important role on cell and tissue integrities. Moreover, their functions are also specific to the tissue, i.e. neurofilaments involved in axonal growth and transport, dendritic arborization and changes in neuronal morphology [7–9].

Several IF expressed in the nervous system are altered in neurodegenerative diseases and cancer, and therefore, they can be used as biological markers. Perturbations of their metabolism and organization were found to be frequently associated with several neurodegenerative diseases, including Alzheimer disease, Parkinson disease, amyotrophic lateral sclerosis and giant axonal neuropathy [10,11].

Pathological features of many neurodegenerative diseases include synaptic loss, dendrite retraction and neuronal degeneration and death. It was reported that neuronal IF play a central role in damage-response mechanisms by activating a developmental program to differentiate neurons and to establish synaptic connections [12–14]. Levin et al. found the neuronal expression of vimentin in Alzheimer disease patients' brain, in Alzheimer disease transgenic mice as well as in adult mouse brains subjected to mechanical damage [12].

Kanic acid (KA) is a cyclic analog of the major stimulatory brain neurotransmitter glutamate. It acts on neuronal receptors inducing an excitotoxic effect, causing neuronal death and astrogliosis in different regions of the CNS [15] as well as on nervous cells cultures [16,17].

In a previous report, we observed behavioral and histopathological variations after intraparenchymal injection of Kainic acid (KA) in the C5 segment of the rat spinal cord [18]. Interestingly, there was a significant improvement at the motor and sensory responses as well as a mild increase in the neuronal cell count of the damaged segments by day 7 post injection (pi) of KA. In the present study, we aimed to determine whether modifications of histological characteristics in the spinal cord after KA exposure may include variations of the IF expression in neurons present at the cervical region.

2. Materials and methods

2.1. Animals

Young (3–4 mo. old, 300–400 g) ($n = 27$) male Sprague-Dawley rats, were used. Animals were housed in a temperature-controlled room (22 ± 2 °C) on a 14:10 h light/dark cycle. Food and water were available ad libitum. All experiments with animals were performed according to the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments (CICUAL) of Veterinary School Animal Welfare Assurance No. 49-8-15 P.

2.2. Toxin preparation and surgical procedure for injection

Before surgery, KA (Sigma-Aldrich, Inc., St. Louis, MO, USA) was dissolved in 0.9% saline and kept at 4 °C until use. On experimental day 0, rats were anesthetized with ketamine hydrochloride (40 mg/kg; ip) plus xylazine (8 mg/kg; im) and placed in prone position. Intraparenchymal injection of KA (1 mM) (KA-injected group) or saline (sham group) was performed as previously described [18]. Briefly, to gain access to the C5 segment trepanation at the C4–C5 fibrous joint was performed. For injecting the solution, a 10 μl Hamilton® syringe fitted with a 26 G needle was hand-held. The needle was vertically introduced 1.5 mm down on the right side of the spinal cord to reach the Lamina-VI of that side (ipsilateral). Once introduced, the needle was held in place for 2 min. Discharge of the solution lasted for 5 min. Before withdrawal, the needle was held in place for 2 more min to avoid leaking of the solution. Five μl either of the KA solution or saline were discharged at that point at a rate of 1 μl/min. Injection of KA induced a reversible loss of motor activity of the ipsilateral forelimb as previously reported [18]. Injected rats were euthanized at days 1, 3 and 7 pi. For each time point five KA-injected or three sham rats were used. One normal rat per euthanasia day was killed serving as an intact control (control group).

2.3. Specimen collection and processing

Euthanasia was performed according to the Guidelines for the Use of Animals in Neuroscience Research (the Society of Neuroscience) and the Research Laboratory Design Policy and Guidelines of NIH. Immediately before euthanasia rats were placed under general anesthesia by injection of ketamine hydrochloride (40 mg/kg, i.p.) plus xylazine (8 mg/kg; i.m.) and then intracardiacally perfused with a buffered saline-paraformaldehyde 4% solution. The vertebral column was removed and postfixed in 10% buffered formaldehyde for 24 h. The spinal cord was then dissected, immersed in cryopreservation buffer (sucrose 30%; polyvinylpyrrolidone 1%; ethylene glycol 30% phosphate buffer 1 M 1%; DW to 100 ml) and stored at −20 °C until use.

Coronal sections of C5 segment were performed under a magnifying glass as previously described [18]. Sections were serially cut into 20 μm thick coronal sections using a vibratome (Leica VT 1000S, Germany) and mounted on jellified slides. From each block, three to five sections, 120 μm apart, were analyzed.

2.4. Immunohistochemistry (IHC)

Spinal cord sections were incubated with 0.03% H_2O_2 in PBS for 30 min at room temperature. Sections were then rinsed twice in PBS and exposed to microwave antigen retrieval using a buffer citrate solution (PBS), pH 6.0. Later, sections were washed twice in PBS and incubated with 1% bovine serum albumin (BSA) in PBS for 30 min, followed by overnight incubation either with anti-neuronal nuclear antigen (NeuN, monoclonal, mouse Clone A60, Millipore, CA, USA; diluted 1:200), anti-vimentin (VIM, monoclonal, DakoCytomation, Carpinteria, CA, USA), or anti-neurofilament (NF, monoclonal, DakoCytomation,) antibody. The EnVision detection system + HRP system labeled anti-mouse or anti-rabbit polymer (DakoCytomation) was applied for 30 min. Sections were then rinsed threefold in PBS. Liquid 3,3-diaminobenzidine tetrahydrochloride (Vector Laboratories Inc., CA, USA) was used as chromogen and Hill's hematoxylin for counterstaining. Control negative sections were prepared by omitting primary antibody.

2.5. Immunofluorescence

Spinal cord sections were rehydrated with PBS containing 0.05% Tween-20 for 10 min at room temperature. Sections were exposed to microwave antigen retrieval using a buffer citrate solution (pH 6.0). Then, sections were incubated with 1% BSA in PBS for 30 min, followed by overnight incubation with neuron specific enolase (NSE, polyclonal, Sigma-Aldrich, St Louis, MO, USA), in combination either with anti-VIM or anti-NF antibodies. Sections were then rinsed threefold in PBS and incubated with 1:1000 Alexa Fluor 488-conjugated anti-mouse or Alexa Fluor 555-conjugated anti-rabbit (Invitrogen, Thermo Fisher Scientific Inc.) secondary antibody for 45 min. Then, sections were rinsed threefold in PBS and counterstained for 15 min with the fluorescent DNA stain 4′,6-diamidino-2-phenylindole. Control negative sections were prepared by omitting primary antibody.

2.6. Quantification of IHC positive cells

For NeuN positive cells counting a digital image analyzer (cellSens Dimension, V1.7, Olympus Corporation, Japan) was used. For each spinal cord section, three areas of analysis were considered: dorsal gray matter (Paxinos Laminae I-V), central gray matter (Paxinos Laminae VI and X), and ventral gray matter (Paxinos Laminae VII-IX) [18,19]. Seven fields (0.12 mm^2/field) were analyzed for the dorsal and ventral areas, whereas only three were considered in the central area. The average counting of NeuN positive cells in each of the three selected areas was compared among groups.

The total number of VIM or NF positive cells to total number of NeuN positive cells ratio was calculated and expressed as percentage to estimate the proportion of positive VIM or NF neurons after injury.

2.7. Immunofluorescence colocalization analysis

Colocalization analysis of NeuN and Enolase was carried out to determine the specificity of both antibodies to neuronal cells. Colocalization analysis of VIM and NF labeling was performed on NSE stained spinal cord segments to determine the variability of their expression on neurons through the neurotoxic process. Briefly, confocal (FV100, Olympus) monochrome images obtained in the selected pi days were pseudo–colored. Image-J software was used to perform image analysis and channel imaging. For each immunofluorescence combination (NSE-NF and NSE-VIM) at least 30 neurons per animal were analyzed. The Intensity Correlation Analysis Plugin (ICA) was used to calculate Mander's overlap coefficients (R) (value 0.0 for no colocalization; 1.0 for 100% colocalization of two intensity patterns [20,21] according the following formula:

$$R = \frac{\sum_i Ch1_i \cdot Ch2_i}{\sum_i (Ch1_i)^2 \cdot \sum_i (Ch2_i)^2}$$

where Ch1 represents the green staining (VIM or NF) and Ch2 represents the red staining (NSE). The overlap coefficients M1 and M2 were also calculated to determine the contribution of each antigen to the colocalized areas, according the following formuli:

$$M_1 = \frac{\sum_i Ch1_{i,coloc}}{\sum_i Ch1_i} \qquad M_2 = \frac{\sum_i Ch2_{i,coloc}}{\sum_i Ch2_i}$$

where

$$Ch1_{i,coloc} = Ch1_i \quad if\ Ch2i > 0$$

$$Ch2_{i,coloc} = Ch2_i \quad if\ Ch1_i > 0$$

where M1 represents the contribution of green (VIM or NF) to the colocalized area while M2 represents the contribution of red (NSE) to the colocalized area. Values: 0 to 1; i.e. M1 = 1 and M2 = 0.5 for a dye pair means that 100% of Ch1 pixels colocalize with Ch2, but only 50% of Ch2 pixels colocalize with those of Ch1 [21].

2.8. Statistical Analysis

Data expressed as mean ± SEM were analyzed by Student's t-test and one-way analysis of variance (ANOVA). Fisher's LSD for multiple comparisons was used as a post hoc test. For non-parametric values, the Kruskall-Wallis test was used. Significance was assumed at values of $p < 0.05$.

3. Results

3.1. Immunohistochemistry

3.1.1. Number of NeuN positive cells

Section of KA-injected animals showed a significant loss of neurons throughout the experiment in comparison to sham animals at the dorsal, medial and ventral areas of the ipsilateral side of C5 segments ($p < 0.05$) (Fig. 1). No neuronal loss was observed at the contralateral side among groups. Control animals showed the same pattern as the contralateral side of both KA-injected and sham animals (data not shown).

3.1.2. Neuronal expression of NF and VIM

Anti-NF antibody showed positive labeling for neuronal projections while anti-VIM antibody labeled ependymal and endothelial cells in control group sections (Fig. 2A and B). In this group, no perikarya was labeled neither with VIM nor NF. Sham group showed the same pattern, although labeling of perikarya at day 1, 3 and 7 pi was also observed (Fig. 2C and D). On the other side, perikarya of some neurons in KA-injected animals showed positive labeling for VIM and NF throughout the experiment (Fig. 2E and F).

Quantification of VIM and NF positive neurons showed that about 8 to 10% of the neurons of the sham group were positive for VIM by days 1 and 3 pi, although this percentage was significantly reduced by day 7 pi both at the ipsilateral and at the contralateral sides (Fig. 3A). Nevertheless, at the ipsilateral side, the number of positive neurons was significantly higher than that at the contralateral side, at least for the first day pi. As for the KA-injected group, only 4% to 6% of the neurons were positive for VIM, and no differences were detected neither at the ipsilateral nor at the contralateral side throughout the experiment. Moreover, no differences were observed in the number or positive cells of crossing sides at every analyzed day pi (Fig. 3A). When the amount of VIM positive cells was compared between groups, significant differences were found: at the ipsilateral side, sham counting was higher than that of KA for days 1 and 3 pi. At the contralateral side, differences were only observed on day 3 pi. No significant differences were observed in the amount of VIM positive neurons by day 7 pi.

In the sham group, NF positive neurons were observed by day 1 pi (3 to 5%), but this number significantly decreased by days 3 and 7 pi (2% to 1%, respectively), in both sides of the segment (Fig. 3B). Sections of KA-injected animals showed a 3 to 6% of perikarya labeling. No differences in percentage were observed throughout all the experimental days and for both sides. When the amount of NF positive cells was compared between groups, significant differences were found: for both sides, KA counting was higher than that of sham for days 3 and 7 pi.

3.2. Quantitative Colocalization Analysis

To further analyze the expression of markers in the perikarya, colocalization analysis was carried out. Double immunofluorescence labeling for NeuN and NSE showed over a 90% colocalization index in the cytoplasm of all neurons present in the control group (Fig. 4A). NSE labeling was entirely expressed on the neuron cytoplasm whereas NeuN labeling was also present at the nuclei.

Colocalization also revealed that some neurons showed positive labeling for VIM-NSE and NF-NSE in both sham and KA-injected groups. Immunofluorescence labeling for VIM and NF showed a scattered labeling pattern over the neuron cytoplasm whereas NSE was widespread all over the perikarya. At day 1 pi, sham group showed an average of M1 = 0.8 and M1 = 0.85 for VIM-NSE and NF-NSE ($R = 0.926$ and $R = 0.884$), respectively (Fig. 4B) whereas KA-injected group showed a colocalization of M1 = 0.85 and M1 = 0.55 ($R = 0.816$ and $R = 0.776$), respectively (Fig. 4C). By day 3 pi, sham

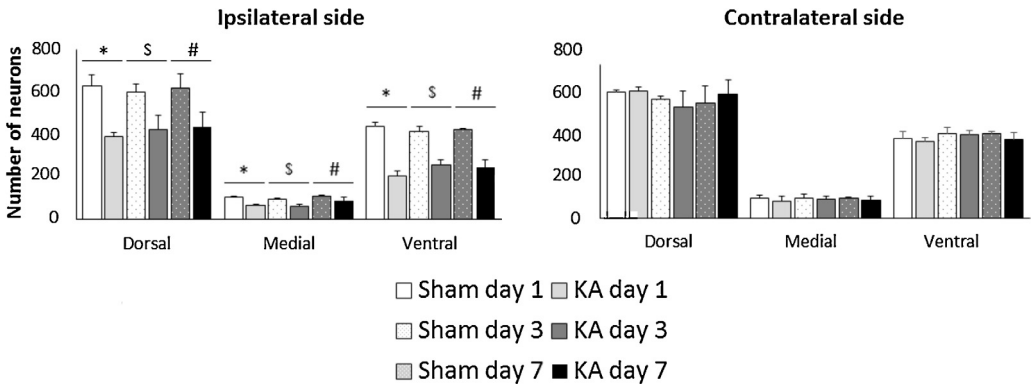

Fig. 1. Quantification of NeuN positive cells. C5 segment of KA-injected group showed a significant loss of neurons from days 1 to 7 pi in comparison to sham group at the ipsilateral side for dorsal, medial and ventral areas. No significant loss of neurons was observed at the contralateral side of the corresponding segment. Data are expressed as mean ± SEM. *, $, # $p < 0.05$.

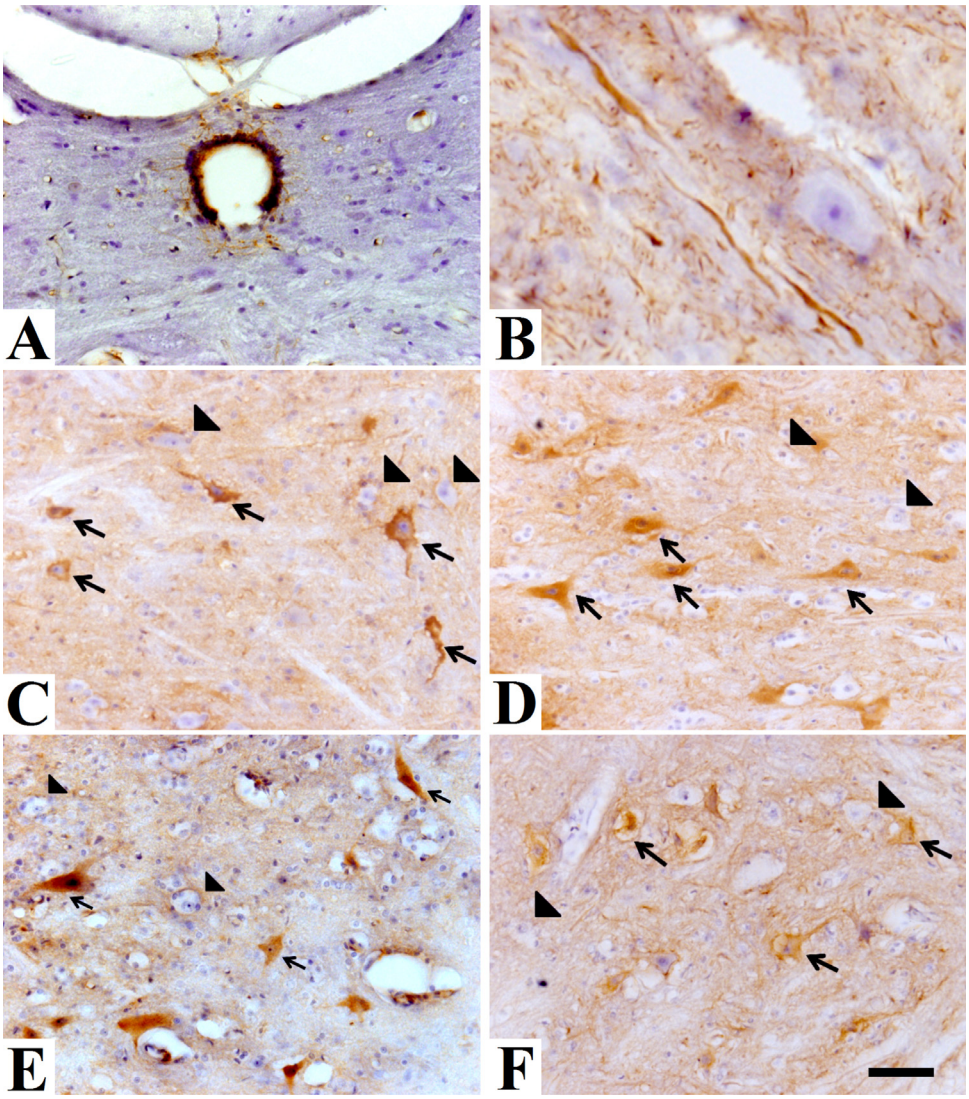

Fig. 2. Immunodetection of VIM and NF. (A) Ependymal and endothelial cells positive labeling for anti-VIM antibody and **(B)** neuronal projections labeled with anti-NF antibody were observed in control group. Anti-VIM (**C**) and anti-NF (**D**) positive labeling (arrows) was observed in the perikarya of neurons of sham animals. Positive labeling for anti-VIM (**E**) and anti-NF (**F**) (arrows) was seen in KA-injected animals. Negative neurons of sham and KA-injected animals are indicated with arrowheads. Bar for panels A,C-F = 50 μm; B = 25 μm.

group values for VIM-NSE and NF-NSE decreased to M1 = 0.53 and M1 = 0.60 ($R = 0.896$ and $R = 0.837$), respectively whereas for the KA group values were M1 = 0.85 and M1 = 0.70 ($R = 0.911$ and $R = 0.778$), respectively. By day 7 pi, double immunofluorescence labeling for VIM-NSE and NF-NSE decreased significantly in the sham group (M1 = 0.08 and M1 = 0.09; $R = 0.634$ and $R = 0.469$, respectively) but

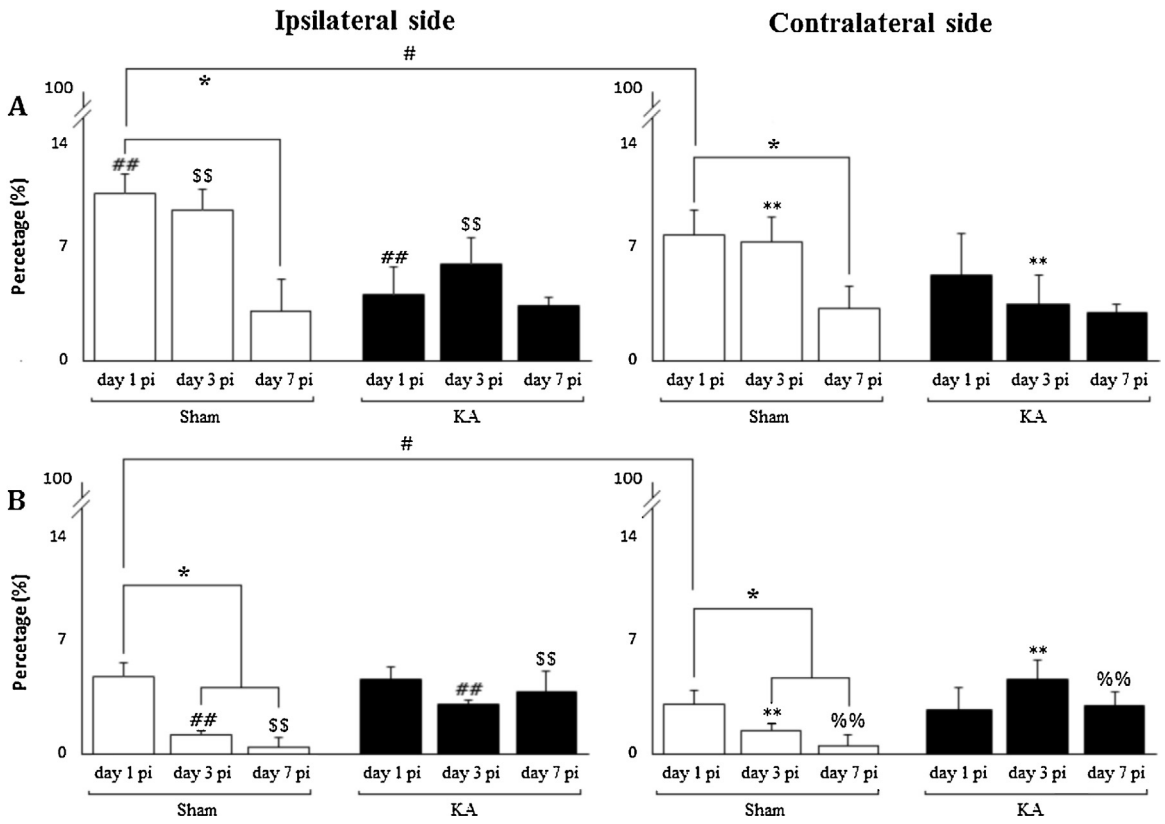

Fig. 3. Quantitative analysis of VIM and NF immunodetection. Histograms represent the percentage of VIM **(A)** and NF **(B)** positive labeled neurons over the remaining neurons at the ipsilateral and contralateral sides. Data are expressed as mean ± SEM. *, #, $p < 0.05$. **, ##, $$, %% differences between pairs $p < 0.05$.

it remained high for the KA-injected rat (M1 = 0.81 and 0.73; $R = 0.77$ and $R = 0.873$, respectively) (Fig. 4D).

4. Discussion

Intermediate filaments are primary components of the cytoskeleton of all eukaryotes, together with microtubules and microfilaments. The expression of several IF proteins in neurons depends on the neuronal type, their localization and stage of development of the nervous system. Although IF are present in perikarya and dendrites, they are particularly abundant in large myelinated axons, where they are essential for axon radial growth during development and axon caliber maintenance [1,22].

As we have reported in a previous study, the intraparenchymal delivery of KA induces neuronal damage at the injection site [18]. Immunohistochemistry analysis of spinal cord sections of KA-injected group showed positive labeling of the perikarya for VIM and NF antigens throughout the experiment, whereas in the sham group this dual expression was observed mainly during the first three days pi. In intact rats (controls), neither VIM nor NF expression was detected in the perikarya.

Vimentin is a 57 kDa intermediate filament expressed primarily in cells of mesenchymal origin [23,24]. It is widely expressed in almost all cells during embryonic development and later replaced by others IF [13,24]. Thus, VIM is initially expressed by nearly all neuronal precursors in vivo, and replaced by NF shortly after immature neurons become post-mitotic [25,26]. Studies performed on cultured neurons and differentiating neuroblastoma cells showed that VIM is required for axonal initiation [24,27]. It was reported that VIM initially concentrates within axonal neurites being subsequently replaced by NF [28,29].

Levin et al. [12] found VIM positive labeling in large pyramidal neurons in the frontal, temporal and entorhinal cortices, as well as in the hippocampus and cerebellum in Alzheimer's disease brains. This labeling was preferentially localized in the perikarya and dendritic compartments of neurons. Indeed, they showed that mouse brain subjected to transection results in abundant expression of VIM in many neurons of cerebral cortex and hippocampus, thus suggesting a direct association between physical damage to the dendrite and the expression of VIM within affected neurons [12]. These results support our finding of VIM expression in the neurons of injected animals (KA and sham) here reported.

Colocalization results were coincident with the immunohistochemical detection of VIM and NF in the spinal cord of KA-injected and sham animals at the three time points studied. Mander's coefficient (R) and overlap coefficient (M1) analysis showed a sustained expression of both VIM and NF in KA-injected animals from day 1 to 7 pi, which may correspond to a transitory co-expression occurring in damaged neurons [25,26]. It is known that NF gene transcriptional regulation is crucial for NF protein expression, especially in axonal regeneration and degenerative diseases [13]. Moreover, VIM filaments form a scaffold from the nuclear envelope to the inner plasma membrane [30], and perikaryal VIM filaments can also elongate into filopodia-like extensions to provide temporal stabilization of putative neurites, yet maintaining an appropriate plasticity [25]. Therefore, VIM filaments may serve as a temporary guide for NF delivery into the developing axon. The improvement of motor and sensitive performance of KA-injected animals observed by day 7 pi and reported in our previous work [18] may be associated with molecular changes in which the activation of NF protein expression is crucial. How the change in the expression of VIM to NF is carried out by neurons remains unknown, but it is clear that transitional expression of VIM is relevant for axonal outgrowth and neuritogenesis [25].

We also found VIM and NF expression in the spinal cord of sham animals throughout the experiment, but unlike KA-injected

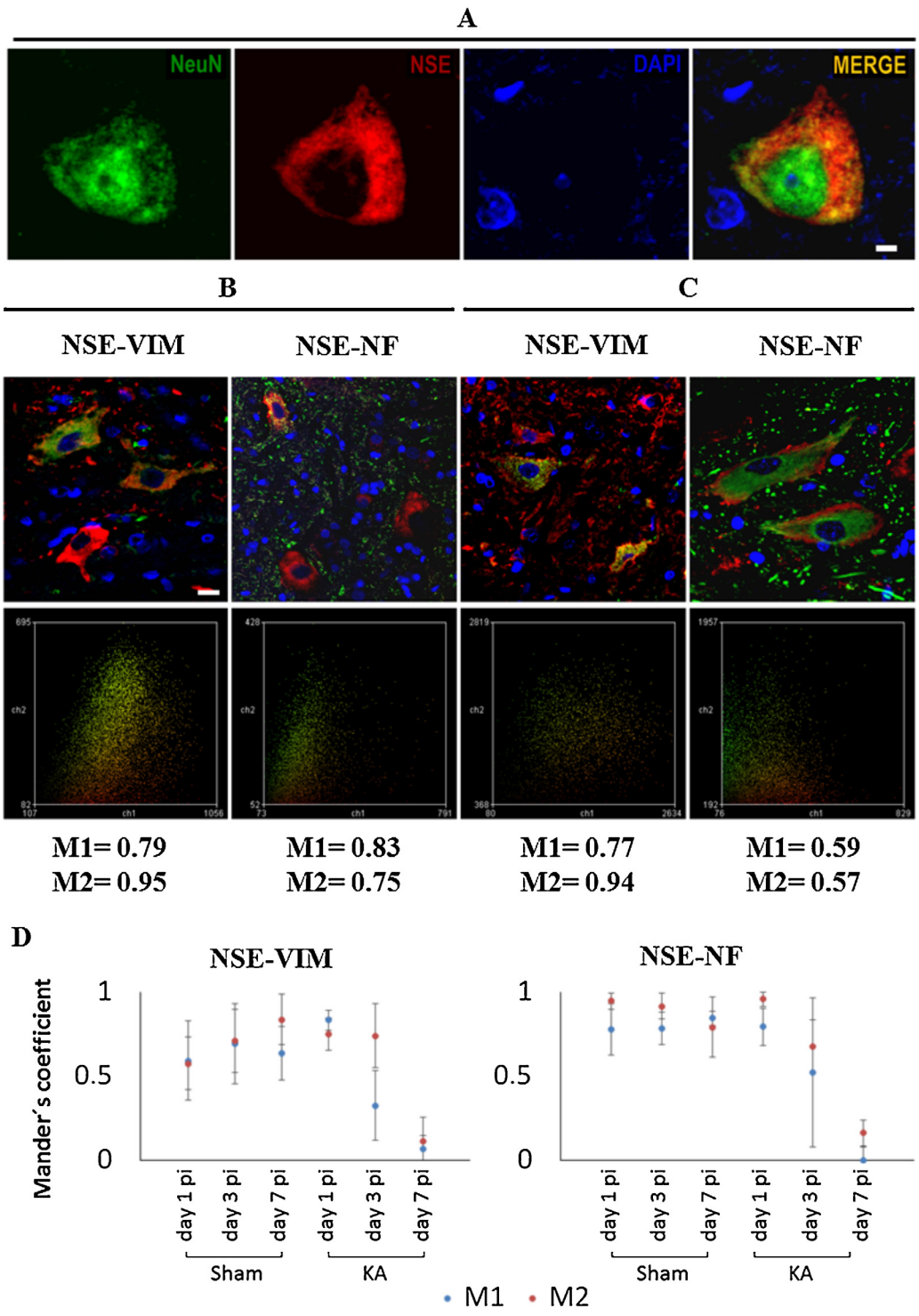

Fig. 4. Colocalization analysis of VIM-NSE and NF-NSE labeled neurons. (A) Double labeling of a neuron with NeuN and NSE showing colocalization at the perikarya. Bar = 5 μm. Representative merged images for sham **(B)** and KA **(C)** at day 1 pi and their corresponding scatterplot, with the overlap coefficients (M1 and M2) indicated. Green channel for VIM or NF; red channel for NSE. Scale bar = 10 μm. **(D)** Summarized data for M1 and M2 for NSE-VIM and NSE-NF along time. Data are expressed as mean ± SEM. $^{*}p < 0.05$.

rats this co-expression decreased by day 3 and it was significantly reduced at day 7 pi. We suggest that this pattern may be the result of the mechanical damage caused by the introduction of the needle, which may trigger the activation of a cytoskeletal remodeling process by the damaged tissues, instead of being the result of a specific process of axonal outgrowth and neuritogenesis. This hypothesis would justify the absence of expression of IF in the perikarya of control rats.

After neonatal rat spinal cord injury, the remodeling of axonal projections is regulated by the availability of neurotrophic factors. It is likely that adult spinal cord injuring would also require trophic factors from the environment and from post synaptic neurons for axonal remodeling under pathological conditions. These stimuli may cause the expression of neonatal IF in adult neurons to return to an immature fashion-like status [31]. Thus, the presence of neurotrophic factors may increase axonal growth after injury in the

adult by acting at the level of the cell body to upregulate cellular programs associated with a regenerative response [32,33]. Moreover, the release of neurotrophic factors by the injured tissue would explain the presence of positive VIM and NF neurons in the contralateral side of the lesion, as those factors would gain the systemic routes [34].

Nevertheless, intrinsic neuronal and extrinsic environmental influences, such as a low expression of growth-associated proteins, the presence of myelin-associated inhibitors of axonal growth, alterations in astrocytes and the presence of extracellular matrix molecules that restrict growth may contribute with the attenuation of the growth capacity in the mature CNS [31]. The lower proportion of VIM positive neurons in KA-injected animals in comparison to sham group may be explained by the reduction in the number of neurons in KA-injected animals, possibly due to a lower amount of neurotrophic factors released to the system. On the other hand, the sustained expression of VIM and NF in KA-injected animals may indicate an active replacement of IF.

Recent data suggest that KA induce an increment of proliferation of glial cells [17] as well as the production of cytokines by these cells [35] that would participate in the recovery of the damaged tissue. In a previous report, Shigyo et al. [29] showed that after spinal cord injury astrocytes release VIM to the extracellular space and stimulates the axonal regrowth by acting directly on IGF-1 receptors. They also considered that astrocytes and microglia may secret IGF-1 and both, together with the extracellular VIM, play a key role in promoting axonal elongation in injured CNS. As Levin et al. [12] showed at the cerebral cortex, here we showed that spinal cord injury promotes the expression of VIM in neurons which is afterwards replaced by NF. These results may also explain our previous result in which rat's motor and sensitive performance were improved by day 7 after intraparenchymal injection of KA in the spinal cord [18]. The unconventional expression of IF may be implicated somehow in the reconnection of nerve endings disrupted after KA-induced injury.

Despite axonal plasticity is spatially restricted in mature CNS after lesions, in the present study we showed that in the perikarya of treated rats there is a non-stereotyped IF expression. The mechanical injury by the needle and the excitotoxic effect of KA may induce the transitory expression of specific IF. It seems that VIM and NF expression may be a necessary change to promote recovery of the damaged tissue.

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