

Immunization with neural derived peptides increases neurogenesis in rats with chronic spinal cord injury

Roxana Rodriguez-Barrera

Universidad Anahuac Mexico

Adrian Flores-Romero

Universidad Anahuac Mexico

Elisa García

Universidad Anahuac Mexico

Ana Maria Fernández-Presas

Universidad Nacional Autonoma de Mexico

Diego Incontri-Abraham

Universidad Anahuac Mexico

Lisset Karina Navarro-Torres

Universidad Anahuac Mexico

Juan José Juárez-Vignon Whaley

Universidad Anahuac Mexico

Ignacio Madrazo

Hospital de Especialidades Centro Medico Nacional Siglo XXI

Antonio Ibarra (✉ jose.ibarra@anahuac.mx)

Centro de Investigación en Ciencias de la Salud (CICSA), FCS, Universidad Anáhuac México Norte, Estado de México, México; Centro de Investigación del Proyecto CAMINA, Ciudad de México, México.

<https://orcid.org/0000-0003-2489-4689>

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Abstract

Background Immunization with neural derived peptides (INDP) has demonstrated to be a promising therapy to achieve a regenerative effect in the chronic phase of the spinal cord injury (SCI). Nevertheless, to date INDP-induced neurogenic effects in the chronic stage of SCI has not been explored. In the present study, we first analyzed the effect of INDP on both motor and sensitive function recovery; afterwards, we assessed neurogenesis and determined the production of cytokines and neurotrophic factors. We analyzed motor and sensitivity recovery using the BBB score and the Von Frey Hair test, respectively. Neurogenesis was evaluated by immunofluorescence. Finally, we examined the expression of IL-4, IL-10, BDNF and GAP-43 using an ELISA assay.

Results During the chronic stage of SCI, rats subjected to INDP showed a significant increase in both motor and sensitive recovery when compared to the control group. Moreover, we found a significant increase in neurogenesis, mainly at the central canal and both the dorsal and ventral horns of INDP-treated animals. Finally, INDP induced a significant production of anti-inflammatory and regeneration-associated proteins in the chronic stages of injury.

Conclusions These findings suggest that INDP has a neurogenic effect that could improve motor and sensitive recovery in the chronic stage of SCI.

Background

Spinal cord injury (SCI) is a severe medical condition generally caused by a traumatic mechanism, such as contusion, compression or transection. This event can result in physiological, biomechanical, and psychological disorders for patients (1). SCI usually induces acute and chronic repercussions depending on both the vertebral level involved and the severity of the lesion. These possible repercussions include paraplegia, quadriplegia, neurogenic shock, Brown-Sequard Syndrome, and even death (2–4).

The pathophysiology of SCI consists of a primary and a secondary phase. The primary phase occurs immediately after the trauma to the spinal cord (SC). The secondary phase occurs within 2 hours and 6 months after injury (5, 6). During this phase, the injury increases by the action of pro-inflammatory factors, excitotoxicity, vascular alterations, oxidative stress, and ischemia. These destructive mechanisms show a gradual decrease until the chronic stage of injury, where they are almost at its minimum expression; however, there is still a progressive decline in the neurological function (7). Therefore, therapeutic approaches should be directed to restore more than to protect neural tissue. With this respect, several strategies have been tested in order to promote neural restoration (8).

Immunization with neural derived peptides (INDP), specifically with A91-peptide, has demonstrated to improve neurological recovery. This peptide is derived from 87–99 amino acids of the immunogenic sequence of the myelin basic protein. A91 modulates the inflammatory and autoreactive responses observed following SCI. These effects are carried out by inducing a Th2 response, thus promoting an M2 macrophage-phenotype and providing a permissive microenvironment for functional recovery (9, 10).

In this context, INDP has shown to induce neural restoration and functional recovery in the chronic stage of injury (11–13). This therapy induces a microenvironment characterized by a significant decrease in pro-inflammatory and an increase in anti-inflammatory cytokines. Likewise, there is an important increase in the production of neurotrophic factors. These conditions have been associated with the regeneration of serotonergic (5-HT-positive) and catecholaminergic (TH-positive) axons as well as with an improved motor recovery (12). Since neurogenesis could be another possible mechanism by which INDP improves functional recovery, it is important to assess the effect of this therapy on the appearance of newly formed neurons in the chronic stages of SCI. In fact, INDP (using copolymer-1) has already shown to induce neurogenesis after stroke in experimental models (14, 15). Hence, the aim of this study is to demonstrate whether INDP induces neurogenesis in the chronic stages of SCI.

Methods

Experimental design

Sample size for this experiment was calculated using an alpha of 0.05 and beta of 0.20. Experiments were performed 60 days after SCI, with subsequent analyses carried out over the two following months. The experiment consisted of SCI rats randomly distributed into two groups (GraphPad QuickCalcs: <http://www.graphpad.com/quickcalcs/>): (1) PBS-immunized rats; (2) INDP-immunized rats.

Locomotor function was evaluated 60 days after SCI and thereafter weekly throughout eight weeks. Sensitivity was also assessed at day 0, 60 and 120 after SCI. At the end of each experiment, rats were euthanized and the SC was then analyzed. We determined the neurogenic effect of INDP at the injured site of the SC, as well as the expression of IL-4, IL-10, TNF α , brain-derived neurotrophic factor (BDNF) and GAP-43 protein levels.

Spinal cord injury

Adult female Sprague–Dawley rats weighing between 230 and 250 g were subjected to a moderate SC contusion. Animals were anesthetized by an intramuscular injection of a mixture of ketamine (50 mg/kg) (Probiomed, Mexico City, Mexico) and xylazine (10 mg/kg) (Fort Dodge Laboratories, Fort Dodge, Iowa, USA). Skin was opened in layers and a laminectomy was performed at T9 vertebral level of the SC. Subsequently, a 10 g rod was dropped onto the SC from a height of 25 mm using the NYU impactor (NYU, New York, USA). Functional recovery of all groups was assessed using the BBB locomotor scale.

Postoperative care

After SCI, animals were housed with food and water ad libitum, and received manual bladder voiding, three times a day for 2 weeks. To avoid infection, Enrofloxacin (Marvel, Mexico City, Mexico) was diluted into their drinking water at an approximate dose of 64 mg/kg/day for 1 week. Animals were carefully

monitored for signs of infection, dehydration or auto mutilation with appropriate veterinary assistance as needed.

Antigen (A91 peptide)

A91 peptide was derived from the encephalitogenic amino acid sequence 87–99 of the myelin basic protein (MBP). A non-encephalitogenic analogue was obtained by replacing the lysine residue with alanine at position 91. The modified peptide was purchased from Invitrogen Life Technologies (San Diego, CA, USA). Reverse-Phase HPLC confirmed the purity of the A91 peptide (>95%).

Active immunization

Rats were immunized subcutaneously at the base of the tail with 200 µg of A91 dissolved in phosphate buffered saline (PBS) (experimental group), or only with PBS (control group). A91 and PBS alone were emulsified in an equal volume of complete Freund's adjuvant (CFA) containing 0.5 mg/ml of *Mycobacterium tuberculosis* (Sigma-Aldrich, St. Louis, MO, USA). Immunization was performed 60 days after SCI.

Functional recovery evaluation

To evaluate the functional recovery, we used the BBB score and the von Frey Hair tests to evaluate motor and sensitivity performance, respectively. Results were evaluated by blind observers.

Assessment of motor recovery.

Behavioral recovery was assessed by the BBB open-field locomotor scale method. Animals were evaluated 60 days after SCI and thereafter weekly throughout eight weeks. Three observers blinded to the treatment performed evaluations. The average of the three scores was used.

Von Frey Hair test.

The rats were placed in a clear acrylic glass enclosure on an elevated metal mesh floor and allowed to acclimate to the new environment for 15 min. The paw-withdrawal response to non-noxious mechanical stimuli was recorded using an Electronic von Frey Anesthesiometer (IITC Life Science, Inc., Woodland Hills, CA, USA). The plantar surface of each hind paw of the rats was stimulated using von Frey plastic filaments perpendicularly, and the maximum pressure required to elicit a response was automatically registered. Three scores for each paw were recorded and averaged. This sensitivity analysis was

performed before SCI (0 day) to ensure that the animals showed normal responses, and it was repeated after surgery 60 and 120 days later.

Immunofluorescence

Neurogenesis was evaluated by immunofluorescence using a double stain with anti-5-bromo-2'-deoxyuridine (BrdU) and doublecortin (Dcx) antibodies. BrdU is a synthetic nucleotide analogue of thymidine incorporated during the S phase of the cell cycle, whereas Dcx is a marker for neural progenitor cells (NPCs). Therefore, BrdU+/Dcx+ cells are a result of neurogenesis. For this assay, the rats received one injection of BrdU (Abcam, Cambridge, UK; 50 mg/kg) intraperitoneally every 12 h for 5 days. The SC were then removed (1.0 cm caudal/rostral from the injury site). SC samples were perfused and fixed with 4% paraformaldehyde. Tissues were cut transversally with the cryostat into sequential serial sections (at 0, 2, 4 and 6 mm caudal and rostral from the epicenter). Slices were 40 µm thick and a total of 48 sections per animal were counted and placed on slides using the free float method. Slides were washed twice for 10 min with PBS-Triton (PBT) and incubated with ImmunoRetriever (Bio SB, Santa Bárbara, CA, USA) for 60 min at 65°C. Afterwards, slides were washed three times for 5 min with PBS and incubated for 30 min with 1N HCl at 37°C. When completed, they were incubated for 10 min with sodium borate 0.1M and washed three times with PBT. Unspecific binding sites were blocked with standard blocking solution with fetal bovine serum for 30 min. The primary antibodies against BrdU (Roche Diagnostics, Indianapolis, USA) (mouse IgG, 1:250) and Dcx (Santa Cruz Biotechnology, Dallas, TX, USA) (goat IgG, 1:250) were allowed to incubate for 20 h overnight. The next day, the slides were washed three times for 10 min with PBT and incubated with secondary antibodies (Invitrogen, Carlsbad, CA, USA) (BrdU: donkey IgG; Dcx: rabbit IgG; all at 1:500) for 2 hours. Excess antibodies were removed by washing with PBT. Slides were counterstained with DAPI. All areas were quantified as total number of cells in all SC samples by a blinded evaluator using cell counting software ImageJ 1.52a. The total number of BrdU+/Dcx+ cells was obtained by averaging the total number of cells from 3 slides (14, 15). Confocal images were acquired using a Zeiss LSM 800 microscope.

Enzyme-linked immunosorbent assay (ELISA)

Eight weeks after PBS or A91 immunization, animals were euthanized with an overdose of sodium pentobarbital (80 mg/kg) and the SC samples were rapidly excised. Reagents, samples, and standards were prepared according to the instructions of rat IL-4 ELISA Kit (Cell Applications, San Diego, CA, USA), IL-10 ELISA kit (RayBiotech, Norcross, GA, USA), BDNF ELISA Kit (Ray Biotech, Norcross, GA, USA), neuromodulin (GAP-43) ELISA kit (CUSABIO, Houston, TX, USA) and TNF-α ELISA kit (Origene, Rockville, MD, USA). Briefly, 100 µl standard or 30µg total protein sample were added to each well and incubated for 2 hours at 37°C. The liquid of each well was removed and not washed. Afterwards, 100 µl of Biotin-antibody (1x) was added to each well and incubated for 1 hour at 37°C. Then, it was aspirated and washed 3 times. One hundred µl HRP-avidin (1x) were added to each well and incubated for 1 hour at 37°C. Subsequently, it was aspirated and washed 5 times and, 90 µl of TMB substrate were added to

each well, incubated and protected from light for 15–30 minutes at 37°C. Finally, 50 µl Stop solution were added to each well and read at 450 nm within 5 minutes.

Statistical analysis

Data is displayed as mean \pm standard deviation (SD), and statistical significance was established when $p \leq 0.05$. GraphPad Prism 8.0 (GraphPad Software, Inc. La Jolla, CA, USA) was employed in statistical analysis. Data from the assessment of functional recovery was analyzed using an ANOVA for repeated measures with Bonferroni's post hoc test (BBB test) and Student T test (von Frey test). Neurogenesis was analyzed by One-way ANOVA followed by Tukey-Kramer post hoc test. ELISA assay was evaluated using a Student T test.

Results

Immunization with A91-peptide improves motor recovery after chronic SCI

In order to test the effect of INDP on neurological recovery, we first evaluated the motor performance of SCI rats compared to the PBS group. Eight weeks after PBS or A91 immunization, rats submitted to INDP showed a significant increase in motor recovery (7.97 ± 0.87) when compared to those immunized with PBS alone (6.37 ± 0.27 ; $p < 0.05$, Two-way ANOVA for repeated measures with Bonferroni's post hoc test; Figure 1).

Sensitive function improves when immunizing with A91-peptide in the chronic stages of SCI.

As the SCI and the subsequent inflammatory response trigger a nociceptive hypersensitivity reaction (16), we evaluated the development of mechanical hypersensitivity (MH). In this case, the lower is the damage, the less is the MH and thereby, the withdrawal threshold is greater (the animal needs higher levels of pressure to stimulate withdrawal response). Therefore, hind paw MH was assessed by measuring withdrawal threshold mechanical stimulation with von Frey filaments.

Evaluation of sensitivity function before therapeutic intervention demonstrated that the mechanical withdrawal threshold was similar in both groups before (PBS: 52.19 ± 5.7 , INDP: 51.53 ± 5.2 , mean \pm SD) and 60 (PBS: 16.71 ± 5.5 , INDP: 15.52 ± 3.5) days after SCI. After 120 days, rats treated with INDP showed a significant increase (31.68 ± 3.9) in mechanical withdrawal threshold when compared to those with PBS-immunization (21.97 ± 2.4 ; $p = 0.02$, student T test; Fig. 2).

Neurogenesis is an active phenomenon in the chronic stages of SCI that can be increased by immunizing with A91-peptide.

In order to assess the number of NPCs in the area of injury, we labeled BrdU+/DCX+ cells at different sites from the epicenter of the lesion. We found an active formation of newly formed neurons even in the SC of rats treated with PBS (see Fig. 3A). Nevertheless, the amount of double positive cells was much higher in the group subjected to A91-immunization (Fig. 3B). This effect was mainly observed at the caudal stump (at the central canal as well as at the dorsal and ventral horns); showing similar results from the epicenter towards 6 mm from this site (Fig. 4). The count of BrdU+/DCX+ cells revealed a higher number of NPCs in the group immunized with A91-peptide when compared to the one treated with PBS alone ($p < 0.05$, One-way ANOVA followed by Tukey's test; Fig. 5).

Immunization with A91-peptide induces a permissive microenvironment for neural restoration.

The evaluation of gene expression (using qRT-PCR) has shown that INDP induces a permissive microenvironment for neural restoration in the chronic stages of injury (12, 13). Based on these findings, we explored the induction of this microenvironment by directly analyzing the protein expression of anti-inflammatory (IL-4; IL-10) and pro-inflammatory (TNF- α) cytokines. Additionally, we assessed the production of some regeneration-associated proteins (BDNF and GAP-43).

Figures 6 and 8 show that INDP elicited a significant production of anti-inflammatory and regeneration-associated proteins in the chronic stages of SCI. Rats immunized with A91-peptide showed a significant increase in IL-4 (15.85 ± 1.39 ; $p = 0.0001$, Student T test; Fig. 6A) and IL-10 (4.18 ± 0.29 , $p = 0.0409$, Student T test; Figure 6B) when compared to PBS-immunized ones (IL-4: 0.85 ± 0.36 ; IL-10: 3.03 ± 0.80). As expected, A91-immunization induced a significant decrease in TNF- α (53.62 ± 4.051) when compared to the group immunized with PBS alone (71.19 ± 5.02 , $p = 0.0092$, Student T test; Figure 7). In regard to BDNF (Fig. 8A) and GAP-43 (Fig. 8B), a significant increase of these molecules was observed in A91-immunized rats (BDNF: 7068 ± 73.73 , $p = 0.0001$, Student T test; GAP-43: 1392 ± 155.50 , $p = 0.0001$ Student T test) when compared to those immunized with PBS alone (BDNF: 6034 ± 15.73 , $p = 0.0001$, Student T test; GAP-43: 0.85 ± 1.47 , $p = 0.0001$ Student T test).

Discussion

Previous studies in our laboratory have shown that INDP induces axonal regeneration and functional recovery in the chronic phase of SCI (12). On this basis, we then tried to elucidate whether INDP is also capable of inducing neurogenesis. As shown, after SCI there is an important formation of neuroblasts,

even in chronic stages of injury particularly at the central canal but also at the dorsal and ventral horns. These newly formed neurons were significantly increased when rats were subjected to INDP.

There are well-defined neurogenic areas in the adult CNS: the subventricular and subgranular zones (SVZ and SGZ, respectively) (17). For a long time, it was conjectured that neurogenesis takes place only in these two areas. Nevertheless, recent reports suggest that neurogenesis in the adult state can be found in other regions of the CNS, such as the amygdala, neocortex (18), cerebellum, striatum, and substantia nigra (19, 20). Additionally, neurogenesis is also amplified after mechanical damage due to traumatic brain injury (21) or SCI (22).

Regarding the latter, induction of neurogenesis after injury has already been documented. For instance, previous studies have shown that SCI activates neurogenic processes, especially at acute phases; however, little is known about these events in the chronic stages of SCI. In this study, we demonstrate that neurogenesis, is a process that continues to be activated even at 120 days after SCI. This is an important finding since it reveals that even at chronic stages of injury, there are mechanisms that aim to restore neural tissue. The presence of neurogenesis long after injury, is a totally feasible event since, ependymal cells – a potential origin of endogenous NPCs- are actively proliferating even several weeks after SCI (23–25). Therefore, it is plausible to expect the appearance of newly formed neurons derived from ependymal cells in the chronic stages of injury.

Neurogenesis in chronic SCI is a topic that deserves to be further studied since it represents an important process being activated—even long after injury- as an effort to restore neural connections. Unfortunately, under pathophysiological conditions (with no therapeutic intervention) this restoration attempt does not contribute to neurological functional recovery. For that reason, neural restoration necessarily requires to be boosted with additional therapeutic approaches that in some way improve events as neurogenesis, inducing then functional recovery after SCI.

In the present study, we intended to boost neurogenesis using INDP, a therapeutic approach that has previously shown to increase the formation of neuroblasts after CNS disorders, such as stroke (15, 26). Our results show that INDP is capable of increasing neurogenesis. This increase was significantly associated with a better sensory and motor recovery. With this regard, previous studies have shown that INDP induces both motor and sensitive restauration in both acute (27, 28) and chronic (12, 13) stages of injury. These beneficial effects could be the result of the formation of new fibers, as demonstrated by previous research (12). Nevertheless, as was shown in this study, neurogenesis could reinforce the formation of connections between interneurons at the ventral and dorsal horns. Thus, providing the ability to convey efficient information through afferent or efferent fibers (29, 30).

Neurogenesis after SCI is a phenomenon that has already been documented. Almost two million of new cells are produced at the site of injury (31). Moreover, the induction of proliferation, migration, and differentiation of NPCs towards NeuN+ mature neurons after SCI in mice has been described as well (32). Nevertheless, the origin of these cells is still unknown and has not been well documented. Currently, there is not much information about the existence of any neurogenic niche in the SC. A particular emphasis

has been placed on the ependymal channel, since proliferation, migration, and differentiation of ependymal cells into NPCs was reported in SC regions after injury (23, 33). Other studies have shown that ependymal tanycytes have a neurogenic potential (34). Therefore, the ependymal channel is emerging as the main source of neurogenesis; however, neuroblasts could also derive from meningeal cells (35) or even from very faraway places like hippocampus (36). This is a topic that should be further addressed to gain new insights into NPCs origin following A91 therapy in chronic SCI.

On the other hand, the mechanisms by which INDP induces neurogenesis are also a topic for further analysis. After SCI, INDP induces the activation of Th2-lymphocytes, a phenotype that releases high concentrations of IL-4 and IL-10 (12, 37). These cytokines have been strongly associated with the induction of neurogenesis (26, 38, 39). It has been shown that IL-4 increases neurogenesis via STAT6 phosphorylation (40). IL-10 has the same effect by modulating extracellular signal-regulated kinases (ERK) and Signal transducer and activator of transcription 3 (STAT3) activity (41). In the present investigation, we show that INDP effectively induces the release of high concentrations of IL-4 and IL-10. In addition, INDP decreases pro-inflammatory cytokines as well. All of these findings have already been reported in both acute and chronic phases of injury, and have been proposed as important factors that contribute to the formation of a permissive microenvironment, which favors neural tissue restoration (12, 37).

In order to better understand the way by which INDP is boosting neurogenesis, we also determined the concentrations of BDNF and GAP-43, two molecules strongly associated to neurogenesis (42). Regarding this, we found a significant increase of both molecules after INDP. This finding supports the fact that INDP is providing a permissive microenvironment for inducing neurogenesis. Several studies have previously shown that BDNF/TrkB signalling pathway is strongly involved in the induction of neurogenesis (42, 43). Similarly, GAP-43 upregulation contributes to neurogenesis as it is involved in the orientation of cell division and is also required for the maturation of neurons (44-47).

Conclusions

The results of this study show that neurogenesis is a phenomenon that is still activated even several weeks after injury. In addition, we demonstrate that INDP is capable of increasing neurogenesis in the chronic stages of injury. This beneficial effect is associated with a better motor and sensitive recovery. Further studies are needed in order to better understand the origin, dynamics and functionality of this neurogenic phenomenon induced by INDP after SCI.

Declarations

List of abbreviations

BDNF: brain-derived neurotrophic factor; BrdU: 5-bromo-2'-deoxyuridine; CFA: complete Freund's adjuvant; Dcx: doublecortin; ERK: extracellular signal-regulated kinases; GAP-43: growth-associated

protein; INDP: immunization with neural derived peptides; MBP: myelin basic protein; MH: mechanical hypersensitivity; NPCs: neural progenitor cells; PBS: phosphate buffered saline; PBS-Triton (PBT); SC: spinal cord; SCI: spinal cord injury; SD: standard deviation; SGZ: subgranular zone; SVZ: subventricular zone; STAT3: Signal transducer and activator of transcription 3.

Ethics approval and consent to participate

All procedures were carried out in accordance with the National Institutes of Health Guide for the care and use of laboratory animals, and the Mexican Official Norm on Principles of Laboratory Animal Care (NOM 062-ZOO–1999). In addition, the Animal Bioethics and Welfare Committee approved all animal procedures (ID: 178544; CSNBTBIBAJ 090812960). All experiments were designed and reported according to the ARRIVE guidelines.

In order to perform euthanasia, animals were previously anesthetized by an intramuscular injection of a mixture of ketamine (50 mg/kg) and xylazine (10 mg/kg).

Consent for publication

Not applicable

Availability of data and material

The dataset supporting the conclusions of this article is included within the article.

Competing interests

The authors declare no competing financial or non-financial interests.

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Author's Contributions

RRB contributed to the concept, design of experiments, as well, substantially contributed to the acquisition, analysis, interpretation of data and drafting of the manuscript. AFR contributed to acquisition and interpretation of data, surgical procedures and postoperative care of the experimental animals. EG contributed to the surgical procedures and postoperative care. AMFP was involved in the drafting of the manuscript. DIA was involved in the drafting of the manuscript and postoperative care. LKNT contributed to the drafting of the manuscript and acquisition of data. JJJVW was substantially involved in the drafting of the manuscript. MI was substantially involved in the drafting of the manuscript. AI contributed to the conception and design of this project, general supervision of the research group and gave final approval of this manuscript. All authors read and approved the final manuscript.

Author details

¹. Centro de Investigación en Ciencias de la Salud (CICSA), FCS, Universidad Anáhuac México Campus Norte, Huixquilucan, Edo. de México, CP 52786, México. ². Proyecto CAMINA A. C., Tlalpan 4430, CP 14050, Ciudad de México, México. ³. Departamento de Microbiología y Parasitología, Facultad de Medicina, Av. Universidad 3000, Col. Universidad Nacional Autónoma de México, CP 04510, Ciudad de México, México. ⁴. Unidad de Investigación Médica en Enfermedades Neurológicas, CMN Siglo XXI, IMSS, Ciudad de México, México.

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Figures

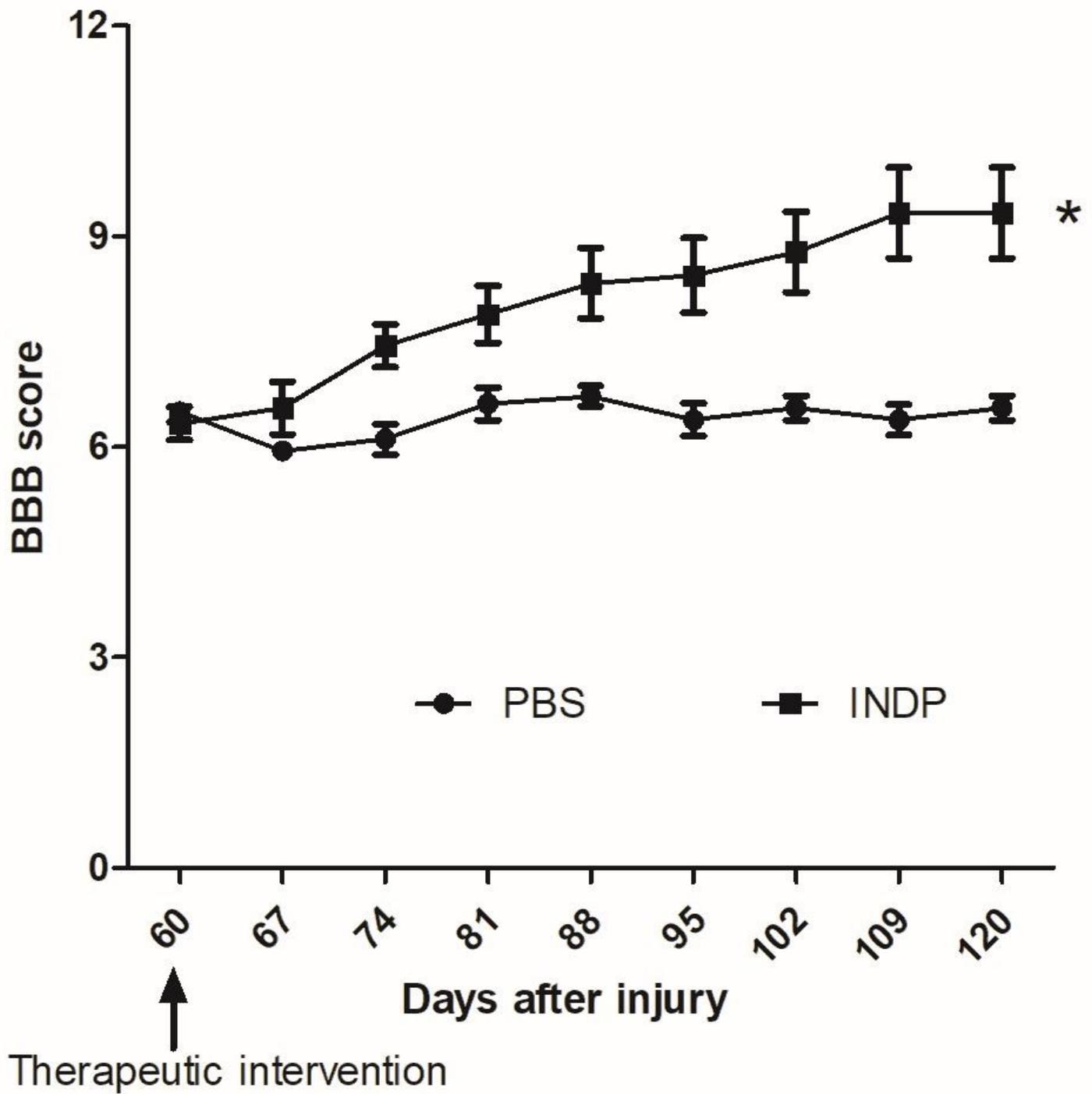


Figure 1

INDP induced a significant increase in motor recovery in the chronic stages of SCI. After intervention, a significantly better motor recovery was observed in the INDP group when compared to the PBS-immunization one. * $p < 0.001$, Two-way ANOVA for repeated measures with Bonferroni's post hoc test. Each point represents the mean \pm SD of 9 rats.

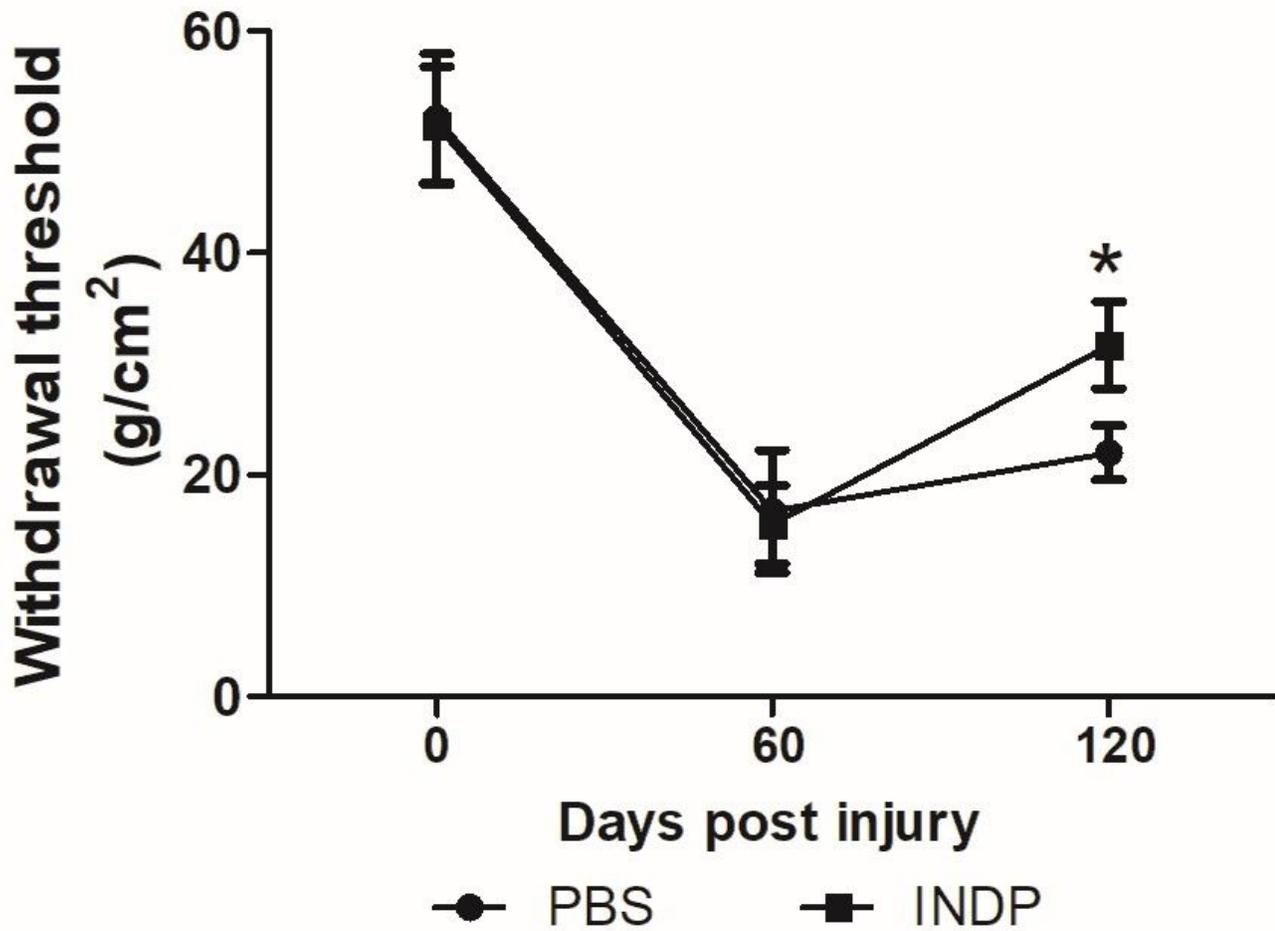


Figure 2

A91-immunization improved sensitivity function in the chronic stages of SCI. After intervention, a significantly better withdrawal threshold was observed in the INDP group when compared to the PBS one. Each point represents the mean \pm SD of 9 rats. * Different from PBS group; $p = 0.02$, Student T test.

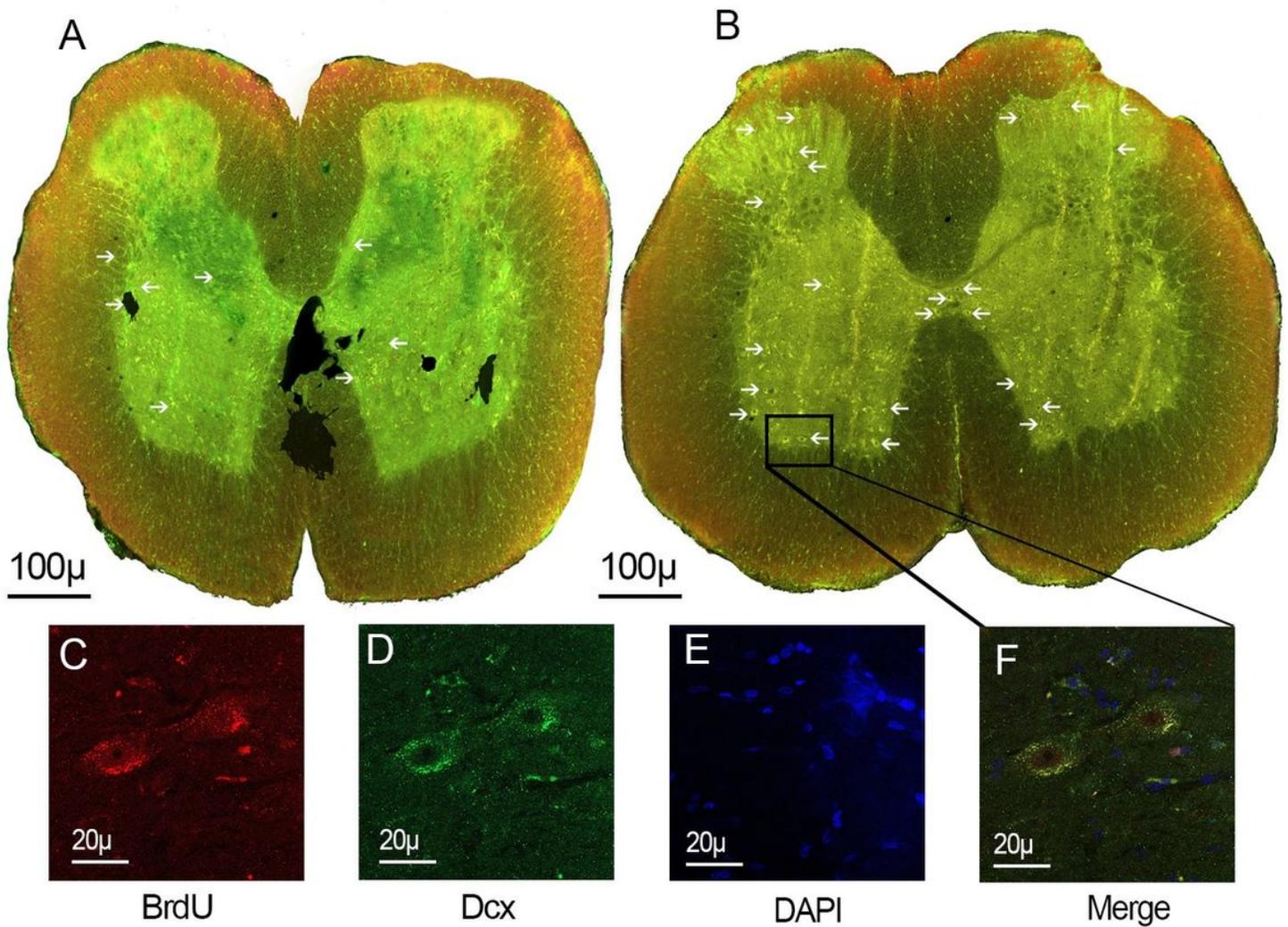


Figure 3

Representative microphotograph of BrdU+/DCX+ cells at the central canal, dorsal and ventral horns, and injury site of SCI rats after therapeutic intervention. A) PBS and B) INDP. Both groups showed BrdU+/DCX+ cells at the central canal, dorsal horn, and injury site. This is one representative photograph of three experiments. Scale bar 100 μm (A and B); 20 μm (C, D, E and F).

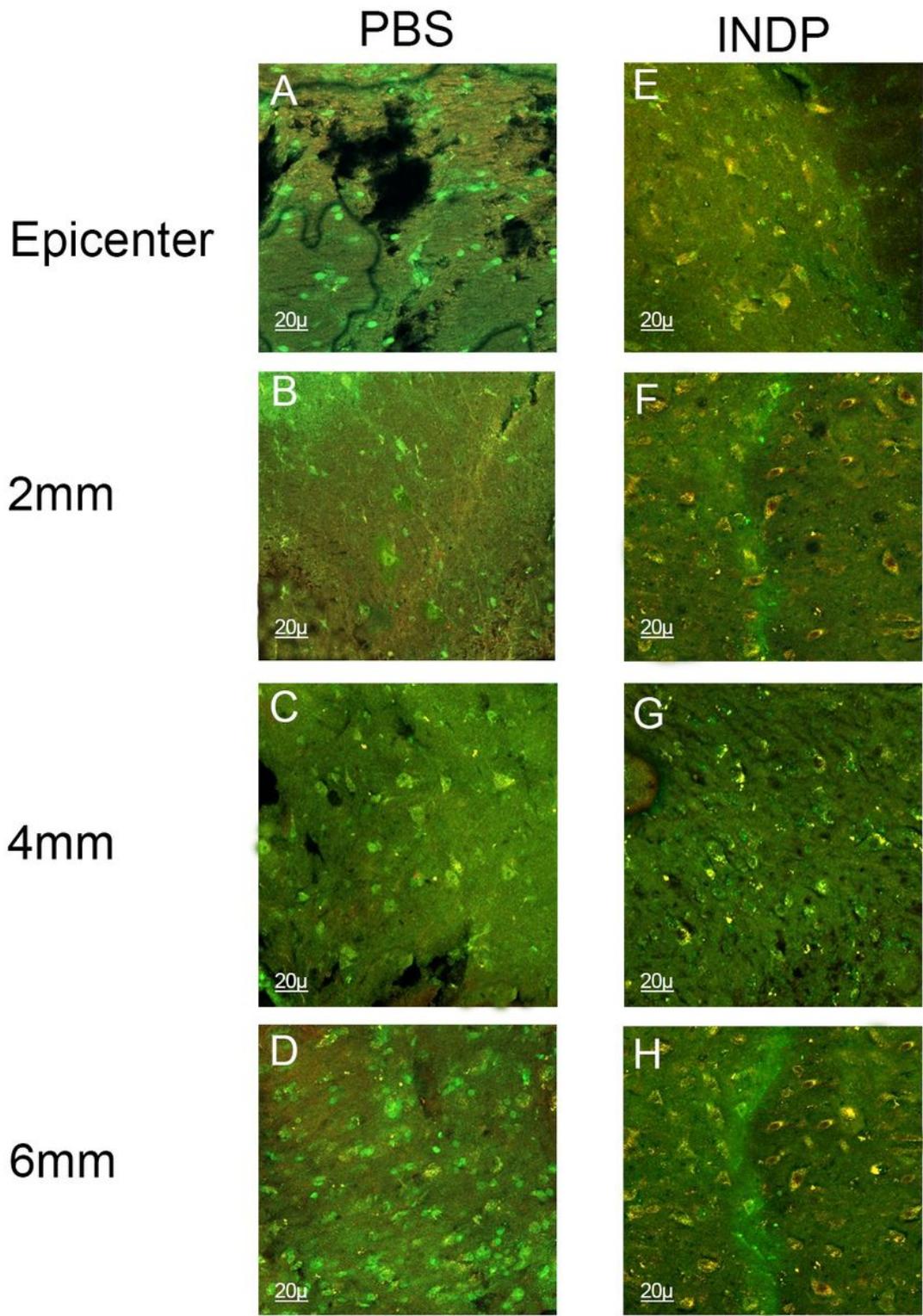


Figure 4

Representative microphotograph of BrdU+/DCX+ cells at the epicenter and towards 2, 4 and 6 mm of the caudal stump of SCI rats after therapeutic intervention. PBS group: A, B, C and D. INDP group: E, F, G and H. This is one representative photograph of three experiments. Scale bar 20 µm.

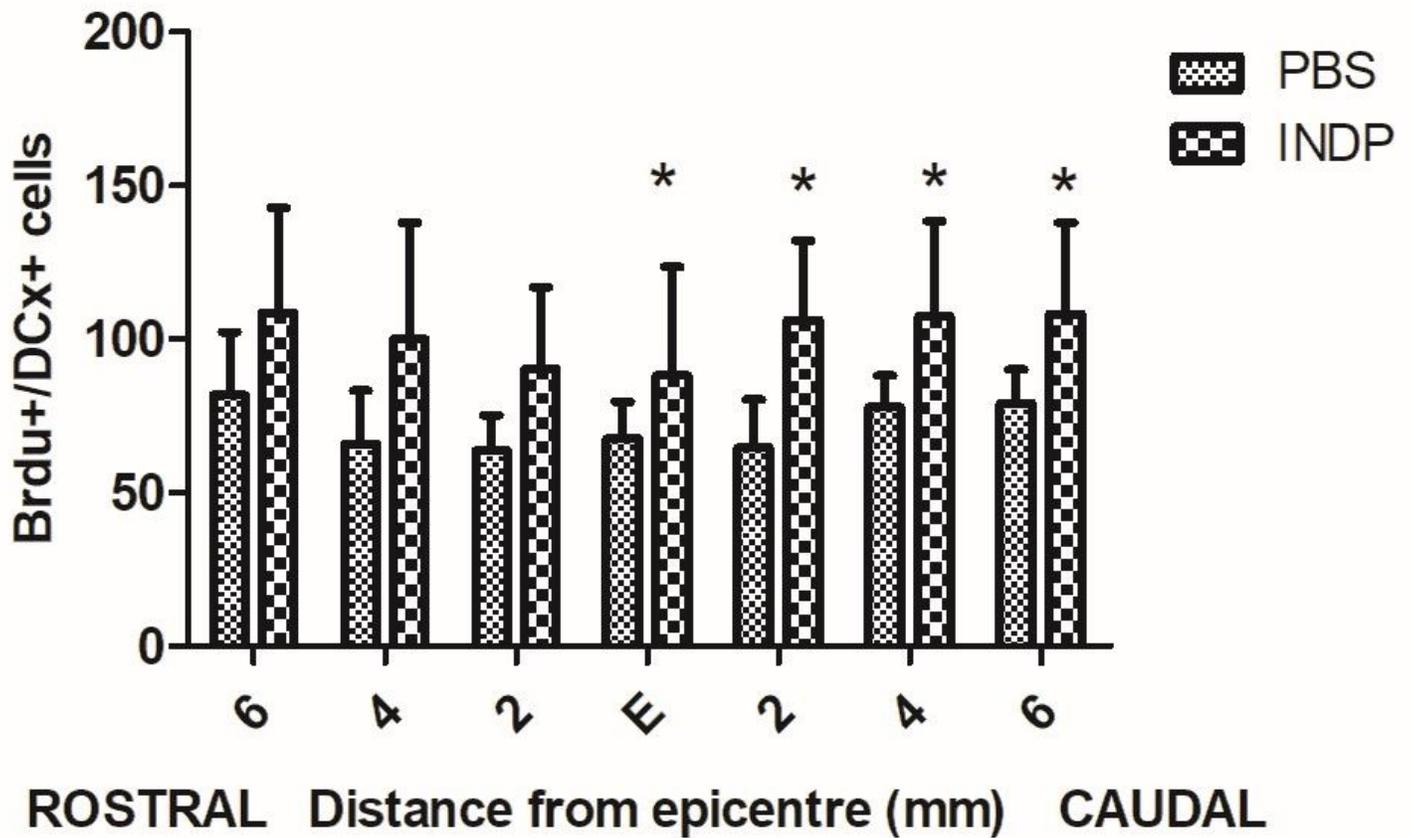


Figure 5

Number of BrdU+/DCX+ cells at the epicenter (E), rostral, and caudal stumps of the SC. INDP group showed a significant increase in the total number of BrdU/DCX labeled cells (neuroblasts) compared to the PBS group. Bars represent the mean \pm SD of 5 rats. This is one representative graph of three experiments. * Different from PBS, $p < 0.05$; one-way ANOVA followed by Tukey's test.

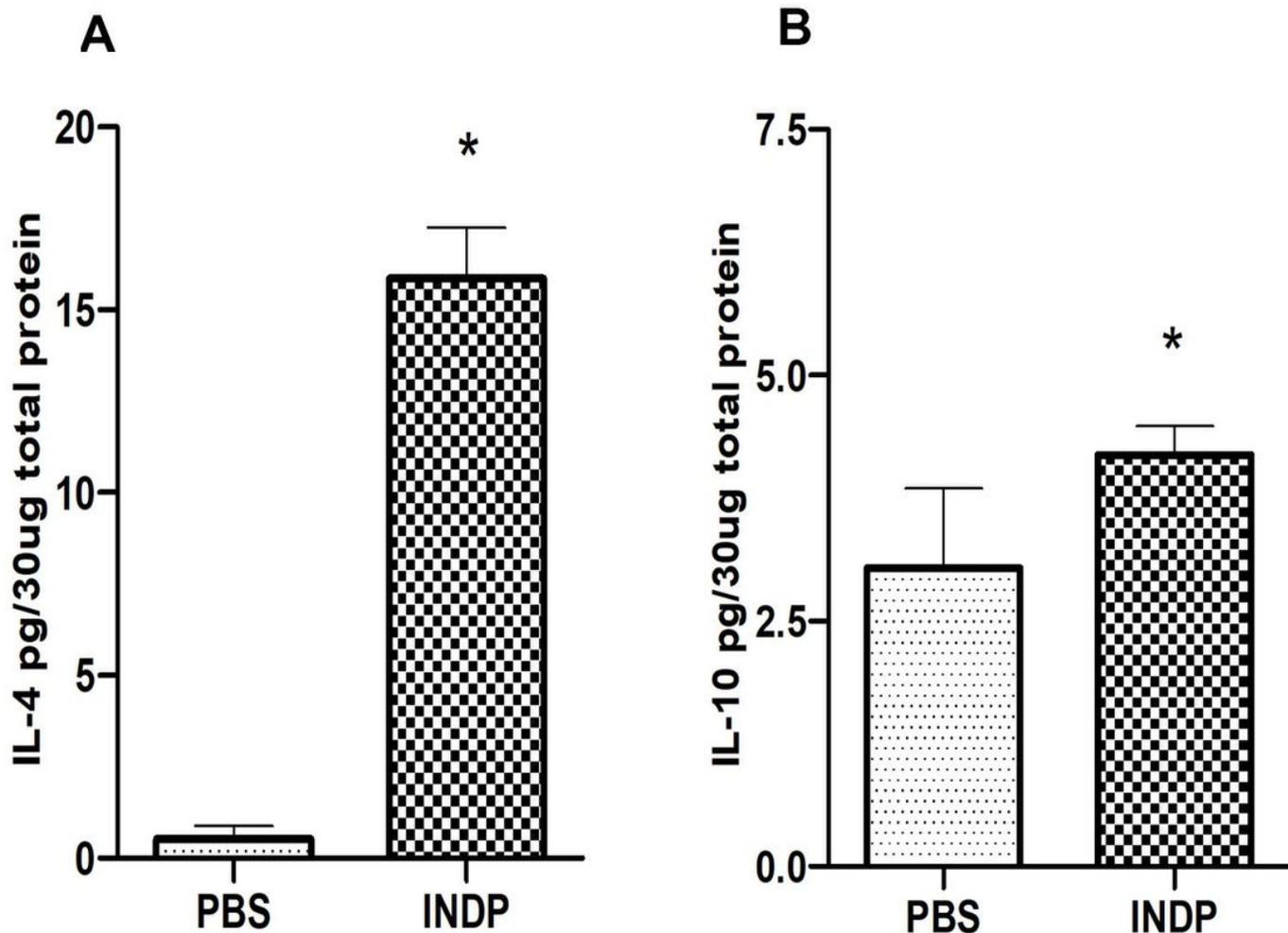


Figure 6

Concentration of anti-inflammatory cytokines 120 days after SCI. The levels of IL-4 (A) and IL-10 (B) in A91-immunized rats were significantly higher than those immunized with PBS alone. Bars represent the mean \pm SD of 5 rats. This is one representative graph of three experiments. * Different from PBS, $p < 0.05$; Student T test.

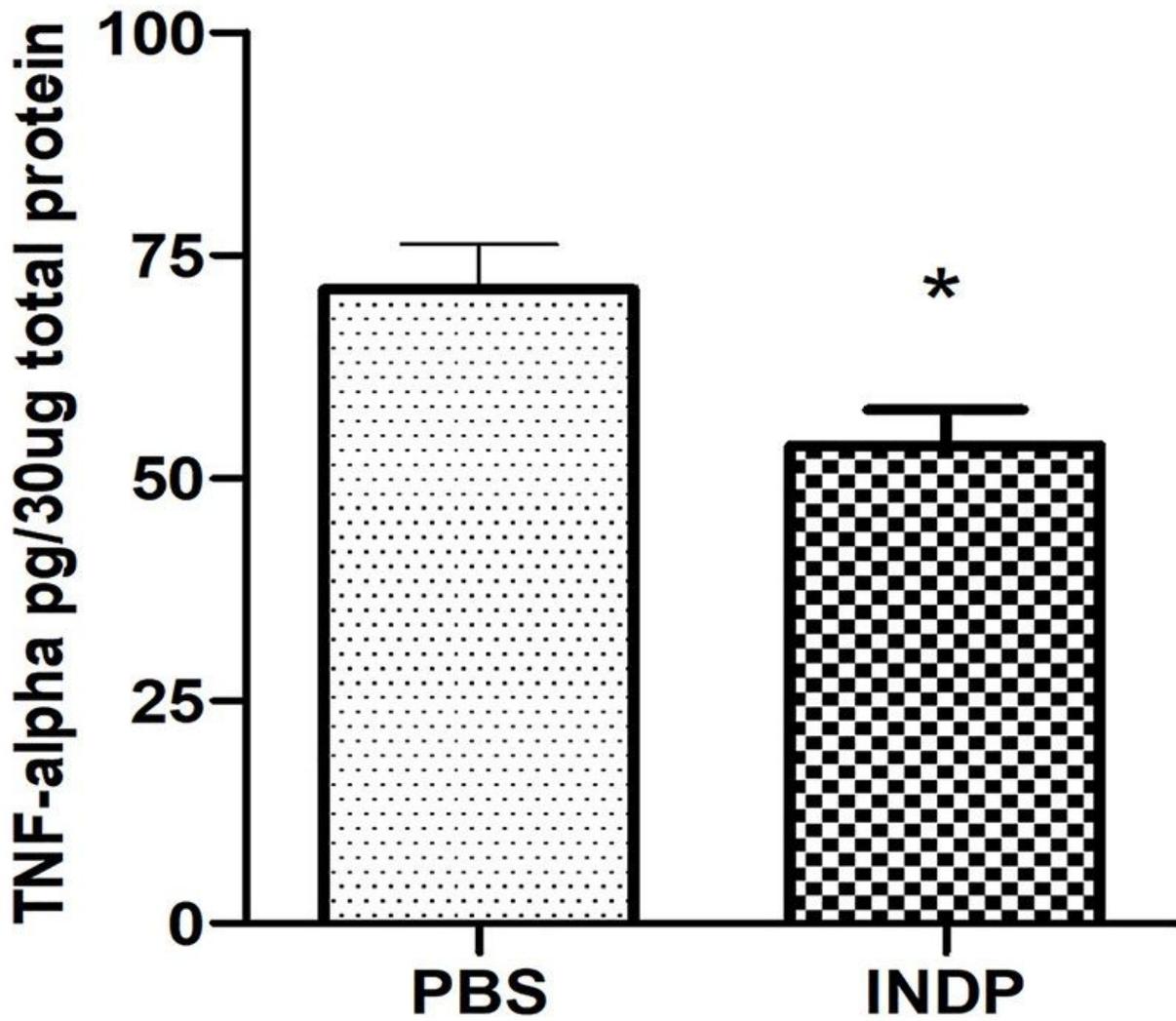


Figure 7

Concentration of TNF-alpha 120 days after SCI. The levels of TNF- α in A91-immunized rats were significantly lower than those immunized with PBS alone. Bars represent the mean \pm SD of 5 rats. This is one representative graph of three experiments. * Different from PBS, $p < 0.05$; Student T test.

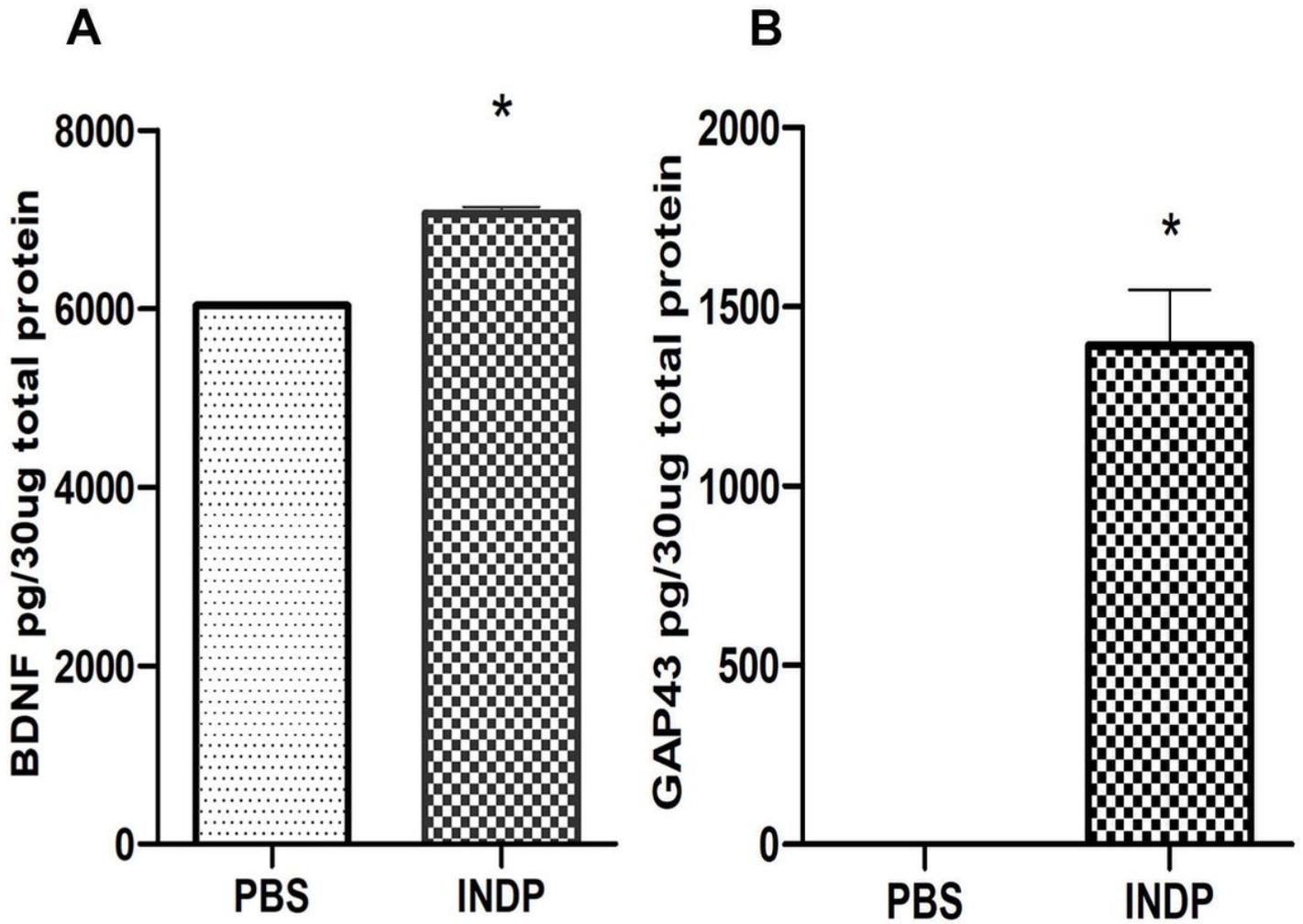


Figure 8

BDNF and GAP-43 concentrations 120 days after SCI. The levels of BDNF (A) and GAP-43 (B) in A91-immunized rats were significantly higher than those immunized with PBS alone. Bars represent the mean \pm SD of 5 rats. This is one representative graph of three experiments. * Different from PBS, $p < 0.05$; Student T test.