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TIME PROFILE OF nNOS EXPRESSION IN THE SPINAL DORSAL HORN AFTER L_5 SPINAL ROOT TRANSECTION IN RATS

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Using immunohistochemical analysis, we investigated the time profile of neuronal nitric oxide synthase (nNOS) expression in the lumbar spinal cord up to day 28 after transection of the L_5 spinal root. On day 14 after injury, we also evaluated the effect of intrathecal application of 7-nitroindazole (7-NI), a selective nNOS inhibitor (8.15 μg in 5 μl), on thermal hyperalgesia. Our results indicated that nerve transection increased the intensity of nNOS-immunoreactivity in superficial and deep laminae of the dorsal horn within a late stage (days 7 to 28) of the neuropathy model used. Furthermore, 7-NI attenuated nerve injury-evoked thermal hypersensitivity on day 14 but did not reduce it between days 2 and 5 after transection. These data suggest that nNOS overexpression is more involved in the development than in the initiation of thermal hyperalgesia in L_5-transected rats.

Keywords: neuropathic pain, spinal dorsal horn, L_5 segment, nNOS, 7-nitroindazole, thermal hyperalgesia.

INTRODUCTION

At present, central mechanisms of neuropathic pain are not fully understood. There is no sufficiently effective treatment for this debilitating disorder [1]. Neuropathic pain is characterized by exaggerated responses to painful stimuli (hyperalgesia), pain sensation in response to normally non-noxious stimuli (allodynia), and spontaneous pain [1-3]. It is accepted that nNOS is significantly involved in the regulation of several cellular processes, probably including neuropathic pain. Nevertheless, the exact contribution of nNOS to the development of pain-related behaviors remains unknown; new information in this respect may provide a novel treatment avenue. There are several controversial studies on the time profile and location of nNOS expression in the dorsal root ganglia (DRG) and spinal dorsal horn under neuropathy conditions. According to some authors, peripheral nerve lesion results in increased expression of nNOS in the DRGs but not in the spinal cord laminae [4]. Other communications, however, reported that axotomy in rats reduces the number of NOS-positive neurons in the dorsal horn [5, 6]. The above discrepancies may be related to different time intervals examined and type of neuropathy models. The first aim of our study was to further evaluate the time profile of nNOS expression in superficial and deep laminae of the rat lumbar spinal cord of rats with the spinal L_5 root transected.

In addition, pharmacological studies regarding the role of spinal nNOS in the development of pain-related behaviors are conflicting. In some studies, administration of nNOS inhibitors reduced the exaggerated pain in rodents after nerve injury [7-10]. In contrast, other studies showed that administration of nNOS inhibitors exerted no effect on diabetes-related or nerve injury-induced behaviors [4, 11, 12]. Therefore, our subsequent aim was to evaluate the contribution of spinal nNOS to the development of nerve injury-evoked thermal hyperalgesia, using an nNOS selective inhibitor.

METHODS

Animals. Male Wistar rats (Pasteur Institute, Tehran, Iran) weighting 180-200 g were used. The rats were housed in separate cages and kept in a temperature-controlled colony room under a 12/12 h light-dark cycle with free access to food and water.

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Induction of Neuropathic Pain. Neuropathic pain was induced according to the method described by Kim and Chung [13]. Briefly, animals were anesthetized with pentobarbital sodium (60 mg/kg, i.p.). The left paraspinal muscles were separated at the L_4-S_2 level, and the left transverse process of the L_6 vertebrum was removed. The L_5 spinal root was tightly ligated using a silk thread (6-0) and transected just distal to the ligature, with all fibers surely interrupted. Then, the wound was closed with 3-0 silk threads. Special care was taken to avoid any damage to the L_4 root. In the control sham group, the surgical procedure was identical to that described above, except for ligation and transection of the left L_5 spinal root. Only animals showing minimum signs of motor deficiency were included in further experimentation.

Immunohistochemistry of nNOS. Tissue samples were collected from the lumbar enlargement of animals of the sham-operated (n = 5) and neuropathic rats (6 groups, n = 6 in each). Rats were deeply anaesthetized with pentobarbital sodium (80 mg/kg, i.p.) and perfused transcardially with heparinized 0.9% saline followed by cold 4% paraformaldehyde in 0.1 M phosphate buffer saline (PBS, pH 7.4). The lumbar enlargement of the spinal cord was quickly removed and post-fixed in the same fixative for 24 h at 4°C. Fixed tissues were processed by graded alcohols and xylenes and paraffin-embedded the next day. Five-µm-thick transverse spinal; cord sections were cut on a microtome and mounted onto slides.

The sections were deparaffinized, rehydrated sequentially in graded alcohols, treated with freshly prepared 0.3% hydrogen peroxide for 30 min (to eliminate intrinsic peroxidase activity), and washed out three times in PBS (pH 7.4). Microwave antigen retrieval was performed in 0.01 M citrate buffer (pH 6.0) at 85°C for 3 min, cooled in the same solution for 2 min (replicated three times), and rinsed three times in PBS. Non-specific binding was reduced by using a blocking solution of 5% normal goat serum (NGS) for 30 min. Then, the sections were incubated with rabbit polyclonal anti-nNOS primary antibody (abcam, ab95436, 1:1000 dilution using distilled water) at 4°C overnight. After rinsing three times with PBS, the sections were exposed with a mouse- and rabbit-specific HRP/DAB (ABC) detection kit (abcam, ab64264) for 60 min at room temperature and washed out three times in PBS. After this, the sections were exposed to diaminobenzidine tetrahydrochloride for 10 min. Immunostained sections were counterstained with hematoxylin, dehydrated by increasing alcohol concentrations, rinsed in xylene, and coverslipped with Entellan. Control sections were treated in the same way except for the exposure to primary antibody.

Counting Procedure and Image Analysis. Labeled cell bodies were viewed and digitized under a light microscope (Olympus, Japan). At least 80 sections from the L_5 spinal segment of both sides (two randomly selected sections per animal) were photographed and counted. The number of nNOS-ir cells in laminae I-V of the dorsal horn was counted within a test-frame of 500 µm × 500 µm in each section. The number of nNOS-ir cells in lamina X was counted within a circle 500 µm in diameter around the center of the central canal in each section. The analyses were performed by an observer masked to the experiment.

Behavioral Study. We used the plantar test to confirm the successful induction of neuropathy before immunohistochemical study. Sham-operated (n = 8) and neuropathic rats (n = 8 in each group) were examined for the development of thermal hyperalgesia one day before and 2, 5, 7, 14, 21, and 28 days after section of the L_5 spinal root. Immunohistochemical analyses showed that nerve damage produced maximum increases in the number of nNOS positive neurons on day 14 after the neuropathy-inducting operation. Therefore, the intrathecal effect of 7-NI on thermal hyperalgesia was evaluated on day 14. The neuropathic rats (n = 8) received intrathecal infusion of 7-NI (8.15 µg in 5 µl) or of an equal volume of 0.9% NaCl saline. 7-NI was injected over a period of 60 sec, and introduction was followed by 5 µl of 0.9% saline to flush the catheter. Thermal hyperalgesia was evaluated just before administration and 30, 60, 90, 120,150 and 180 min after 7-NI or saline administration. The dose of drug administration was based on the data of the earlier study [9].

Thermal Hyperalgesia. The paw withdrawal latency, PWL, in response to application of radiant heat was assessed using the plantar test apparatus (Ugo Basile, Italy). Briefly, the animal was placed in a Plexiglas box with a glass floor and allowed to acclimate 30 min before the experiment. A mobile radiant heat source was positioned under the plantar surface of the left hind paw, and the PWL value was measured. The injured paw was tested three times with 5-min-long intervals, and the average value of the consecutive testes was calculated. The cut-off time was 33 sec.

Implantation of an Intrathechal Catheter. Under pentobarbital sodium anesthesia (60 mg/kg, i.p.), an intrathecal cannula was implanted according to the

method of Storkson et al. [14]. Concisely, a PE-10 polyethylene tube was inserted between the L_5 and L_6 vertebrae and carefully threaded into the subarachnoid space in the cranial direction to reach the lumbar enlargement. The outer part of the catheter was plugged and fixed onto the skin. The correct subarachnoid position was assured by a typical tail or hindpaw flick. Also, the catheter placement was verified by observing transient hindpaw paralysis induced by intrathecal injection of 10 μl 2% lidocaine. Only the rats showing complete paralysis of both hindlimbs and tail after lidocaine administration were used for the subsequent experiments. At the end of each experiment, the position of the PE tubing in the intrathecal space at the lumbar enlargement was visually verified by exposing the lumbar spinal cord.

Statistics. All data are presented below as means ± ± s.e.m. In the immunohistochemistry study; one-way ANOVA followed by the Tukey *post-hoc* test was used. In the behavioral study, the data were analyzed by one-way repeated-measures and two-way ANOVA followed by the Bonferroni *post-hoc* test. Graphics and statistical analysis were performed using Graphpad Prism, version 5.0 (Graphpad Prism software, USA).

RESULTS

Time Profile of nNOS-Immunoreactivity in Superficial and Deep Laminae of the Dorsal Horn in Neuropathy. We examined the time profile of nNOS expression in the L_5 segment of the spinal dorsal horn in neuropathic rats. The nNOS-ir neurons at the ipsilateral (left) and contralateral (right) sides were calculated in neuropathic rats, sham rats, and control (naive) animals. In the latter (naive and sham) rats taken as the controls for neuropathic animals, immunohistochemical nNOS staining did not reveal significant differences in the number of positive neurons on both sides (ipsilateral and contralateral) of the dorsal horn. After transection of the L_5 spinal root, significant increases in the number of nNOS-ir cells on both ipsilateral and contralateral sides of the L_5 segment were observed, as compared to the control. This increase was observed in the superficial laminae (I-II), deep laminae (III-V) of the dorsal horn (Figs. 1-3), and also around the central canal (lamina X; Figs. 4 and 5). This elevation became significant on day 7, reached the maximum level on day 14, and stayed at a high level up to 28 days after induction of neuropathy, as compared to the control (Table 1).

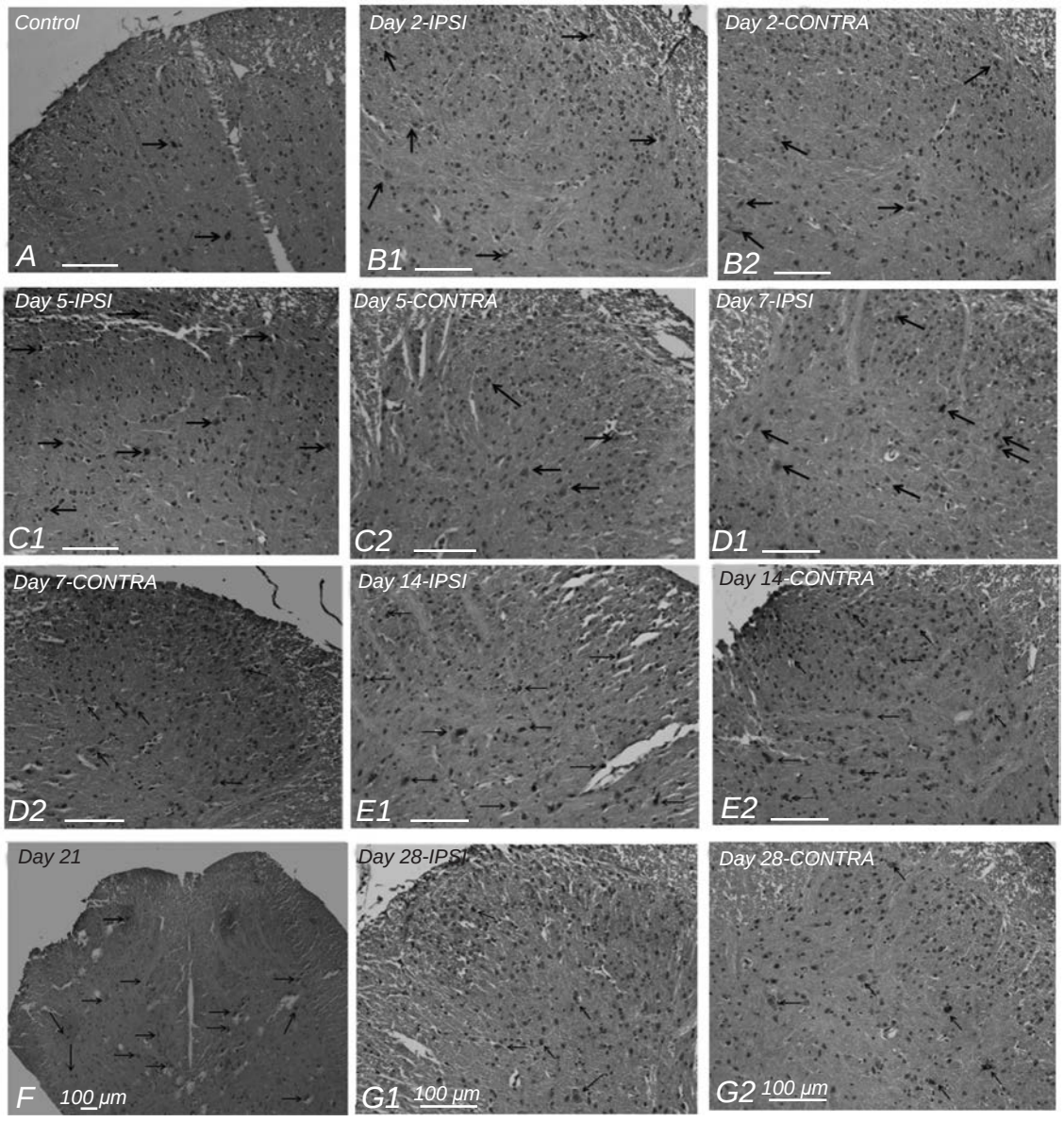
Effect of Intrathecal Infusion of the nNOS Inhibitor on Thermal Hyperalgesia. Thermal hyperalgesia after transection of the L_5 spinal nerve became obvious on day 2, reached the maximum on day 7, and then remained relatively steady up to 28 days, as compared to the pre-injury baseline latencies (Fig. 6A). Noxious thermal stimulation before spinal nerve transection elicited lifting of the hindpaw with the mean latency of 17.03 ± 0.48 sec, and the development of neuropathy produced a dramatic decline in the withdrawal latency (8.97 ± 0.26 sec) on day 14 after the above transection. As expected, sham operation did not produce any reliable change in the baseline values within the entire observation period. To define the effect of inhibition of nNOS on thermal sensitivity at the spinal level, we examined the effect of intrathecal injection of 7-NI, a selective nNOS inhibitor, on nerve injury-related behavioral changes and induction of thermal hyperalgesia on day 14 after

Normalized numbers (%) of nNOS-ir cells at the ipsilateral and contralateral sides of laminae I-II, laminae III-V, and also lamina X of the L_5 segment in neuropathic rats

Нормовані кількості (%) nNOS-імунореактивних клітин у різних пластинах сегмента L_5 щурів із нейропатією

Groups (days after root transection)	Laminae					
	I-II		III-V		X	
	ipsilateral	contralateral	ipsilateral	contralateral	ipsilateral	contralateral
D-2	85.71±20.82	14.28±13.36	107.69±27.19	76.92±19.61	27.73±18.24	8.76±18.24
D-5	92.85±18.21	21.42±18.21	115.38±22.42*	15.38±12.16	82.48±36.49	64.23±34.94
D-7	185.71±15.97***	71.42±20.82	161.53±14.39***	115.38±15.38*	173.72±34.94***	9.48±21.07
D-14	428.57±24.22***	264.28±18.21**	507.69±33.08***	176.92±22.42***	301.45±36.49***	137.22±18.24**
D-21	200.00±26.24***	128.57±30.72***	323.07±19.61***	300.00±27.19***	283.21±34.94***	155.47±36.49**
D-28	192±85±20.82***	128.57±26.72**	169.23±27.19***	92.30±27.19	210.21±18.24***	137.22±18.24**

Footnotes: Indices in the naive animals are taken as the control (100%). Values represent means ± s.e.m. (eight rats per group). Statistical analysis was performed using one-way ANOVA and Tukey's *post-hoc* test.$^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ indicate significant differences between neuropathy and sham-operated animals.



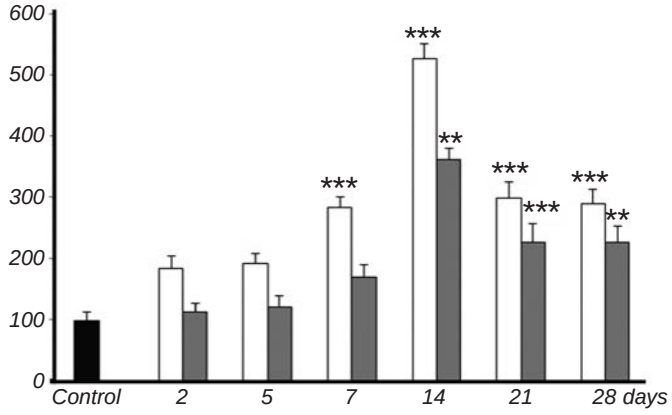
F i g. 1. Representative photomicrographs showing nNOS-ir neurons (arrows) at the ipsilateral and contralateral sides of the dorsal horn of the L_5 segment in neuropathic rats. *A*) In control animals. *B1-G2*) Images made at different times after transection of the L_5 spinal root (2, 5, 7, 14, 21, and 28 days, respectively).

Р и с. 1. Мікрофотографії розподілу nNOS-імунореактивних нейронів (вказані стрілками) в іпси- та контралатеральному дорсальних рогах спінального сегмента L_5 щурів із нейропатією.

neuropathy. Intrathecal injection of 7-NI significantly decreased thermal hyperalgesia in the ipsilateral hindpaw 60 min after administration (the mean paw lifting latency increased to 12.43 ± 0.60 sec), which persisted for 120 min after treatment (13.74 ± ± 0.56 sec) and became closer to the pre-drug administration level after 150 min (12.04 ± 0.50 sec); the mean pre-drug administration value in these series was 9.96 ± 0.68 sec (B).

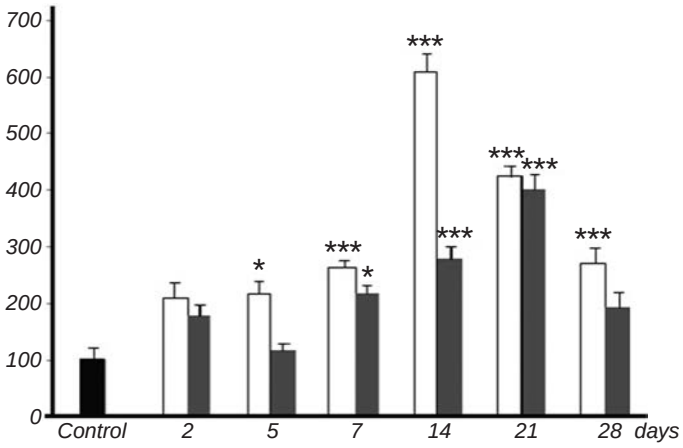
DISCUSSION

We demonstrated that L_5 root transection is accompanied by profound alterations of nNOS expression in the spinal dorsal horn. Such transection increased the number of nNOS-ir cells in the superficial and deep laminae of the L_5 segment of the dorsal horn and also in the lamina X, but this occurred mostly within the late stage (days 7 to 28) of



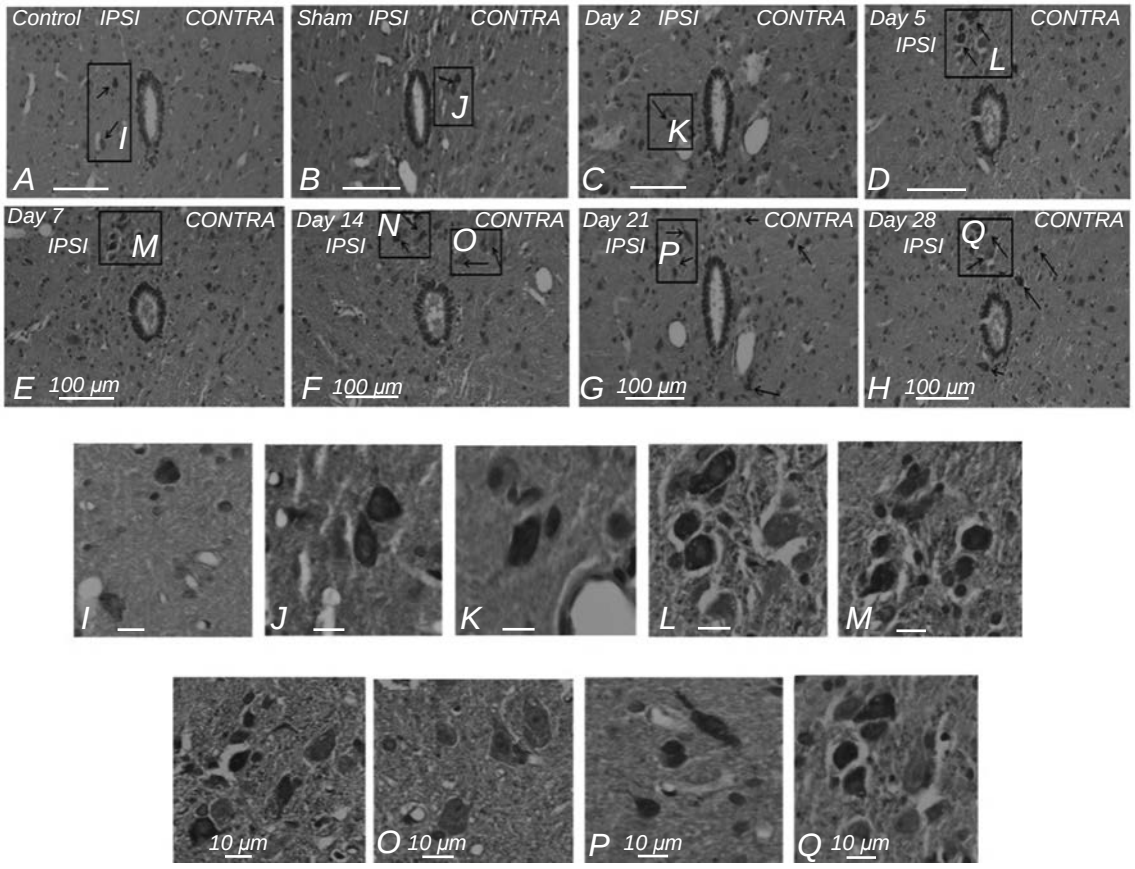
F i g. 2. Numbers of nNOS-ir neurons in laminae I-II at the contralateral and ipsilateral sides of the L_5 spinal segment in neuropathic rats. Means ± s.e.m. are shown (calculations were performed for five rats per group). Data are expressed as percentage of the control. $^{**}P < 0.01$ and $^{***}P < 0.001$ indicate significant differences with respect to control rats.

Р и с. 2. Кількість nNOS-імунореактивних нейронів у пластинах I та II дорсального рога спінального сегмента L_5 контра- та іпсилатерально перерізці дорсального корінця.



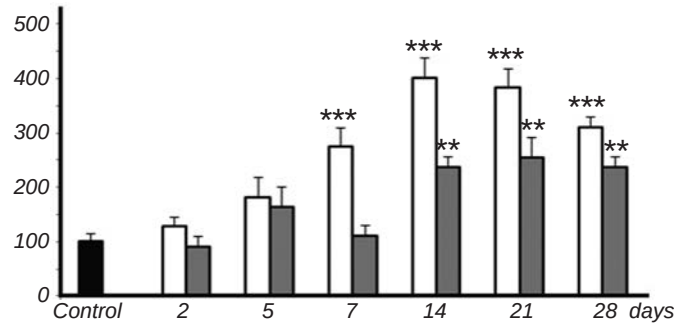
F i g. 3. Numbers of nNOS-ir neurons in laminae III-V. $^{*}P < 0.05$; other indications are similar to those in Fig. 2.

Р и с. 3. Кількість nNOS-імунореактивних нейронів у пластинах III–V.



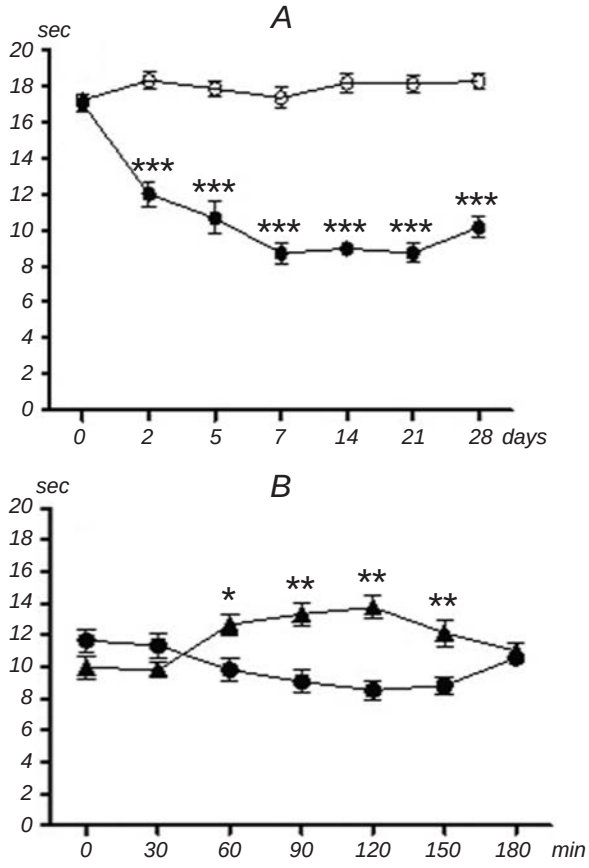
F i g. 4. Representative photomicrographs showing nNOS-ir neurons (arrows) in lamina X of the L_5 segment in neuropathic rats. *A* and *B*) In control and sham-operated animals. *C-H*) Images at different terms after root transection (2, 5, 7, 14, 21, and 28 days, respectively). *I-Q*) Images at higher magnification.

Р и с. 4. Мікрофотографії розподілу nNOS-імунореактивних нейронів (вказані стрілками) в пластині X сегмента L_5.



F i g. 5. Numbers of nNOS-ir in neurons lamina X of the L_5 segment in control and neuropathic rats. Indications are similar to those in Figs. 2 and 3.

Р и с. 5. Кількість nNOS-імунореактивних нейронів у пластині X сегмента L_5.



F i g. 6. Thermal hyperalgesia induced by L_5 root transection. *A*) Time profile of thermal hyperalgesia measured by the plantar test. Thermal thresholds were measured on day 0 (one day before nerve injury) up to day 28. *B*) Time profile ot the effect of interathecal injection of the nNOS inhibitor 7-NI on thermal hyperalgesia (day 14). Designations are similar to those in Figs. 2, 3, and 5.

Р и с. 6. Термічна гіпералгезія, індукована перерізкою корінця L_5.

the development of transection-induced neuropathy. Thermal hyperalgesia was induced from day 2 to day 28, similarly to what was observed in a previous study [15]. To study the involvement of spinal nNOS in the development of neuropathic pain, we used the nNOS inhibitor 7-NI. Our results showed that 14 days after nerve transection, intrathecal administration of 7-NI significantly attenuated thermal hyperalgesia 60 min after injection, and such a shift persisted up to 120-150 min. We did not observe any significant behavioral effect of administration of the nNOS inhibitor on days 2 and 5 (data not shown). Our pharmacological results are consistent with reports that inhibition of nNOS attenuated neuropathic pain-related behaviors [7]. However, there is some evidence that nNOS inhibition did not reduce the pain sensitivity in neuropathic rats [4, 6, 12]. Our quantitative analyses demonstrated that a significant increase in the number of nNOS-ir neurons was observed within both ipsilateral and contralateral sides of laminae I-V and even in a region around the central canal (lamina X) of the spinal cord on day 7 after L_5 nerve transection. The respective changes reached a maximum level on day 14 and remained elevated up to 28 days.

Our immunohistochemical results to a certain extent contradict those of our behavioral studies. We observed significant appearance of thermal hyperalgesia on day 2 after spinal nerve transection, but immunohistochemical results showed that the maximum number of nNOS-ir neurons was found on day 14. Furthermore, as was mentioned, our pharmacological data did not reveal any analgesic effect of application of the nNOS inhibitor on postoperation days 2 and 5. Therefore, it should be suggested that nNOS may be involved within relatively late stages (days 7-28) more than within early stages (days 2-5) in our neuropathy model. So, it seems that nNOS overexpression is more involved in the development than in the initiation of hyperalgesia.

Some studies showed that nerve injury increased the number of nNOS-positive neurons in the spinal cord [17-19]. However, there is controversial evidence concerning nNOS expression during the development of neuropathy. For example, Zhang et al. [5] showed that spinal axotomy in the rat reduced the number of nNOS-positive neurons in lamina II of the ipsilateral dorsal horn but increased the respective indices in DRG neurons at the L4-L6 level [5]. Several pathological conditions occur after nerve injuries that may induce nNOS overexpression in the spinal cord. Among those, there are: (i) abnormal spontaneous ectopic action potentials (APs) in injured nerve fibers, which come to the spinal cord, and (ii) different signaling

molecules that are up- or down-regulated in the injured axons [20-21]. So, these conditions may have either a negative or a positive influence on spinal nNOS expression. It seems that, under normal conditions, primary afferent firing and retrogradely transported signaling molecules regulate spinal nNOS expression mostly by a negative feedback mechanism. Peripheral nerve injury disrupts this feedback mechanism and leads to nNOS overexpression. Our results showed that there is no significant increase in the number of nNOS-ir neurons within early stages of the development of neuropathy (between days 2-5). It is suggested that relatively slow nNOS expression after nerve injury is not directly controlled by the arrival of initially evoked ectopic APs. However, it is possible that ongoing ectopic discharges could indirectly intensify *de novo* synthesis of the nNOS protein. Such a supposition is supported by the findings of Herdegen et al. [22]; these authors demonstrated that transcription factors (such as c-Jun and c-Fos) are overexpressed and colocalized with NOS overexpression in spinal cord neurons after noxious stimulation. Our results confirm that nNOS plays an important role in the development of thermal hyperalgesia within the late stages of our neuropathy model.

In conclusion, our data suggest that nNOS overexpression in the dorsal horn, as well as around the central canal, is more involved in the development than in the initiation of thermal hyperalgesia in our neuropathy model (spinal root transection in rats).

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Animal experiments were performed according to the Guidelines for the Care and Use of Laboratory Animals by the National Institutes of Health (NIH) and were approved by the Animal Ethics Committee of the Shahid Beheshti University of Medical Sciences, Tehran, Iran. (194/-90/3/18; 2011).

The authors of this communication, Z. Bahari, H. Manaheji, L. Dargahi, S. Daniali, M. Norozian, G. H. Meftahi, and M. Sadeghi, confirm the absense of any conflict related to comercial or financial interests, to interrelations with organizations or persons in any way involved in the research, and to interrelations of the co-authors.

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ЧАСОВИЙ ПРОФІЛЬ ЕКСПРЕСІЇ nNOS У ДОРСАЛЬНОМУ РОЗІ СПИННОГО МОЗКУ ЩУРІВ ПІСЛЯ ПЕРЕРІЗАННЯ СПІНАЛЬНОГО КОРІНЦЯ L_5

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Р е з ю м е

Ми досліджували часовий профіль експресії нейронної NO-синтази (nNOS) у люмбальному відділі спинного мозку щурів протягом 28 діб після перерізання спінального корінця L_5, використовуючи імуногістохімічну методику. Ми також оцінювали впливи інтратекальних аплікацій 7-нітроіндазолу (7-NI) – селективного інгібітора nNOS (8.15 мкг у 5 мкл) на термічну гіпералгезію через 14 діб після ушкодження. В результаті секції корінця кількість nNOS-імунореактивних клітин у поверхневих та глибоких пластинах дорсального рога зростала на відносно пізніх етапах (із сьомої по 28-му добу) використаної моделі нейропатії. Аплікації 7-NI зменшували термічну гіперсенситивність, викликану пошкодженням нервових волокон, на 14-ту добу, але не впливали на цей феномен протягом другої–п'ятої діб після індукції нейропатії. Подібні дані вказують на те, що після перетину корінця L_5 у щурів підвищена експресія nNOS більшою мірою залучена в процес розвитку, ніж в ініціацію термічної гіпералгезії.

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