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Research report

Expression of nitric oxide synthase and colocalisation with Jun, Fos and Krox transcription factors in spinal cord neurons following noxious stimulation of the rat hindpaw

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Abstract

Expression of nitric oxide synthase (NOS) was investigated in neurons of lumbar spinal cord of adult rats following subcutaneous injection of formalin (FOR) in one hindpaw. NOS was visualized immunocytochemically using a specific antibody and by the NADPH-diaphorase reaction (NDP). In the untreated rat, NOS immunoreactivity (IR) and NDP were present in neurons of the superficial dorsal horn (sDH) predominantly in layers II–III, and in the deep dorsal horn (dDH) predominantly in layer X. Twenty-four hours following FOR, the numbers of neurons labelled for NOS and NDP and the density of NDP containing nerve fiber varicosities significantly increased in sDH of the ipsilateral L3–L4 segments. NOS-IR and NDP gave a rather congruent distribution of labelled neurons in the dorsal horn. In contrast, distinct NOS-IR but not NDP was visible in large diameter motoneurons and in the lateral spinal nucleus. Double labelling demonstrated that in sDH most of the NDP-reactive neurons show a close spatial relationship to fibers and varicosities immunoreactive for substance P and CGRP. These neuropeptides are considered mediators of synaptic input from nociceptive primary afferents. Colocalization of NDP with c-Jun, JunB, JunD, c-Fos, FosB and Krox-24 transcription factors was investigated in neurons of lumbar spinal cord. c-Jun, JunB, c-Fos and Krox-24 reached their maximal levels of expression 2 h after FOR and returned to basal levels after 10 h. FosB and JunD reached their maximal expression after 5 h, persisted up to 10 h and were still visible in 60%–70% of the maximal number of labelled nuclei after 24 h. This persistent expression of transcription factors might contribute to the up-regulation of NOS expression between 10 h and 24 h. In a low number of NDP neurons, suprabasal immunoreacitivity of JunB, c-Fos and Krox-24 proteins was visible up to 10 h, and of JunD and FosB up to 24 h in sDH neurons; c-Jun was not expressed in NDP labelled neurons of sDH, but, similar as JunD, showed basal colocalization in preganglionic sympathetic and parasympathetic neurons. In dDH, colocalization of Jun, Fos and Krox-24 proteins in few neurons was only observed following a second FOR stimulus given 24 h after the first one. Double-staining also demonstrated that many Jun, Fos and Krox labelled neurons are in close proximity to NDP labelled nerve fibers suggesting a functional relationship between expression of immediate-early gene encoded transcription factors and presence of nitric oxide in the rat spinal cord.

Key words: Activator protein-1; Calcitonin gene-related peptide; Immediate-early gene; Nitric oxide; Nociception; Substance P; Transcription

1. Introduction

In the mammalian central nervous system, nitric oxide (NO) represents a new class of neuronal messengers, the free radical gas which fulfills some criteria of a neurotransmitter. NO mediates actions of glutamate and is involved in lasting changes of neuronal activity such as long-term suppression and long-term potentiation [6,53]. Because of its volatility, direct measurements of NO are difficult. Therefore, the NO catalyz-

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ing enzyme nitric oxide synthase (NOS) is being investiated to evaluate NO production. Neurons containing NOS can be visualized by anti-NOS antibodies and the NADPH-diaphorase reaction (NDP) [5,13,24,32,37].

Evidence suggests that NO is also involved in hyperalgesia. Inhibition of NOS activity results in antinociception and reduces the responses of spinal neurons to electrically evoked C-fiber input [16,39–41,45]. It is a well-known observation that noxious peripheral stimulation generates lasting changes in spinal neurons including enlargement of receptive fields and hyperexcitability with consequent hyperalgesia (reviewed in refs. 11, 62). This could also be mediated by an enhanced release of NO from primary afferent neurons and spinal neurons which express NOS [5,12,13,58]. Therefore, we tested the hypothesis that peripheral noxious stimulation induces the expression of NOS in spinal neurons by studying NOS immunoreactivity and NDP labelling. As immediate-early gene (IEG) encoded transcription factors such as Jun, Fos and Krox proteins are involved in reactive alterations of protein synthesis in spinal neurons following noxious peripheral stimulation [7,10,11,13,20,21,25,42,46,48,56–58], we also studied the possible co-expression of IEG encoded transcription factors.

Formalin injection into one hindpaw of rats was used as noxious stimulus. In lumbar spinal cord, the expression of NOS and NDP was investigated by histochemistry as well as the colocalization of c-Jun, JunB, JunD, c-Fos, FosB and Krox-24 in NDP labelled neurons. Furthermore, the spatial associations of substance P and CGRP immunoreactive varicosities with NDP neurons were examined.

2. Materials and methods

2.1. Experimental procedure

In male awake Sprague–Dawley rats, 50 μl of 5% FOR (in physiological saline) was injected into the right hind paw. After 2 h, 5 h, 10 h (each $n = 3$) and 24 h ($n = 5$), the rats were deeply anaesthetized (pentobarbital 100 mg/kg b.wt., i.p.) and transcardially perfused by 4% paraformaldehyde (in phosphate buffer). In a further group of animals 24 h after the first injection of FOR, a second injection was applied into the right hindpaw and the rats were killed after a further 2 h by perfusion as described above ($n = 3$). For control, untreated rats were killed within 20 min following the

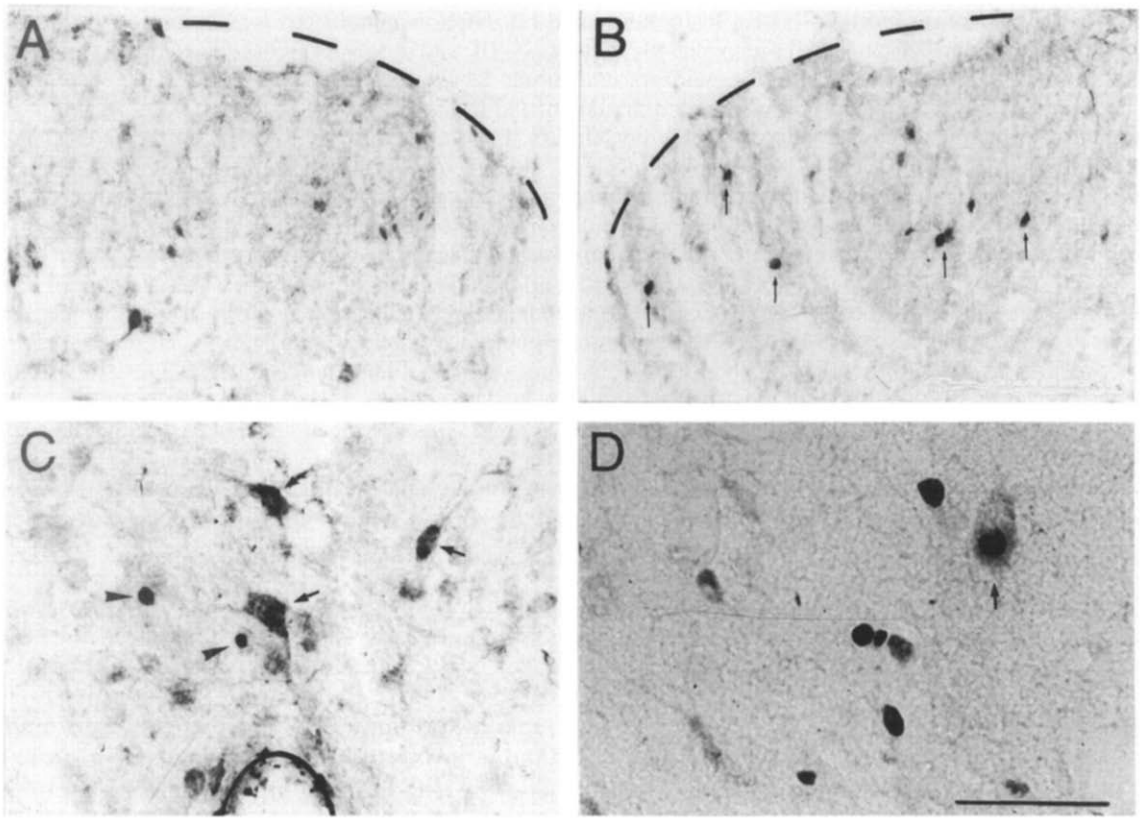


Fig. 1. NOS immunoreactive neurons in the superficial L4 dorsal horn (A) in the untreated rat and (B) 24 h following (formalin) FOR. Arrows mark neurons with suprabasal NOS-IR. The dotted lines mark the grey/white matter boundaries. C: neurons in lamina X are selectively labelled either by c-Fos (arrowheads) or by NOS (arrows) 2 h following FOR. The central canal is marked at the bottom. D: colocalization of c-Fos and NOS (arrow) in a neuron of the deep dorsal horn 2 h following second application of FOR given 24 h after the first FOR into the same hind paw. Bar (A,B) = 200 μm, (C,D) = 50 μm.

removal from the animal house ($n = 2$); furthermore, rats were injected with physiological saline and killed after 2 h and 24 h (each $n = 1$).

2.2. Immunocytochemistry

The lumbar spinal cord was removed, postfixed overnight and cryoprotected in 30% sucrose. Coronal sections of 50 μm were processed for cytochemistry as free floating sections. For NDP, sections were incubated for 45 min at 37°C with a solution containing 1 mM β-NADPH, 0.1 mM Nitro blue tetrazolium (Biomol, Hamburg) and 0.3% Triton X-100 in 0.1 M phosphate buffer (pH 7.4). Immunocytochemistry was performed by conventional avidin-biotin-complex method (Vectorstain, Vector Lab.) and visualized with diaminobenzidine as described in detail previously [17]. NOS was detected by polyclonal rabbit anti-NOS-antibody (1:8000) raised against the N-terminal part of NOS which is not shared by the cytochrome P-450 reductase [29,37,38]. Preabsorption of the anti-NOS antibody with 100 pM of purified NOS protein for 24 h abolished this immunoreactivity. Immunoblotting revealed that the anti-NOS antibody strongly reacts with the NOS peptide used as antigene, but did not recognize the cytochrome P-450 reductase [21] which shows some homology to the NOS protein.

The polyclonal antibodies against the IEG encoded proteins [17,30] were diluted as follows: c-Jun 1:1500, JunB 1:4000, JunD 1:8000, c-Fos 1:30000, FosB 1:2000 and Krox-24 1:4000. For double labelling, NDP labelled sections were processed for immuncytochemistry as described above. NDP labelled sections were also processed for substance P and calcitonin gene-related peptide (CGRP) (each 1:10000, Cambridge Biochemical Research, UK).

2.3. Calculations

The numbers of neurons labelled by NOS, NDP and IEG proteins were counted in five spinal cord sections of each animal. For each group, means (±S.D.) were calculated. The proportion of the IEG neurons labelled with NDP was given as percentage values from the total of IEG labelled neurons. Significances were determined using the Student's t-test ($P < 0.05$). The immunoreactivities were independently counted by two scientists and their results did only differ up to 15%.

3. Results

3.1. Nitric oxide synthase immunoreactivity

In untreated rats, a small number NOS labelled neurons were found in the dorsal and ventral horns of the lumbar spinal cord. In the unilateral superficial dorsal horn (sDH, laminae I–III), 11.2 ± 3.4 cells per section (c/s) labelled with NOS-IR were predominantly located in the ventral (inner) part of lamina II and lamina III (Figs. 1A and 2A). Scattered large diameter neurons were also found in the medial and lateral part of laminae IV–VI. A dense NOS immunoreactivity was found around the central canal (Fig. 1C) and also in the lateral spinal nucleus (LSN) (Fig. 5A). In the ventral horn, a distinct neuronal labelling was seen in large and medium diameter neurons presumable motoneurons (Fig. 5B). Labelling of nerve fibers and/or varicosities was rarely visible in the dorsal horn, but was distinct in LSN and ventral horn neurons (Fig. 5A,B).

Twenty-four hours, not 10 h, following the subcutaneous injection of FOR, the number of NOS labelled neurons was significantly increased in the medial part of ipsilateral sDH of L3–L4 segments (10.8 ± 2.4 cells per section, c/s) compared to the contralateral sDH (6.1 ± 1.6 c/s) (Figs. 1B and 2B). A more pronounced increase could be assessed for the intensity of neuronal labelling in this particular spinal compartment. The numbers of NOS-IR neurons of lateral sDH, dDH and lamina X did not change consistently following FOR and therefore no significant alterations in number could be seen if total numbers of NOS containing neurons were counted in the ipsilateral L3–L5 dorsal horn (Fig. 2A).

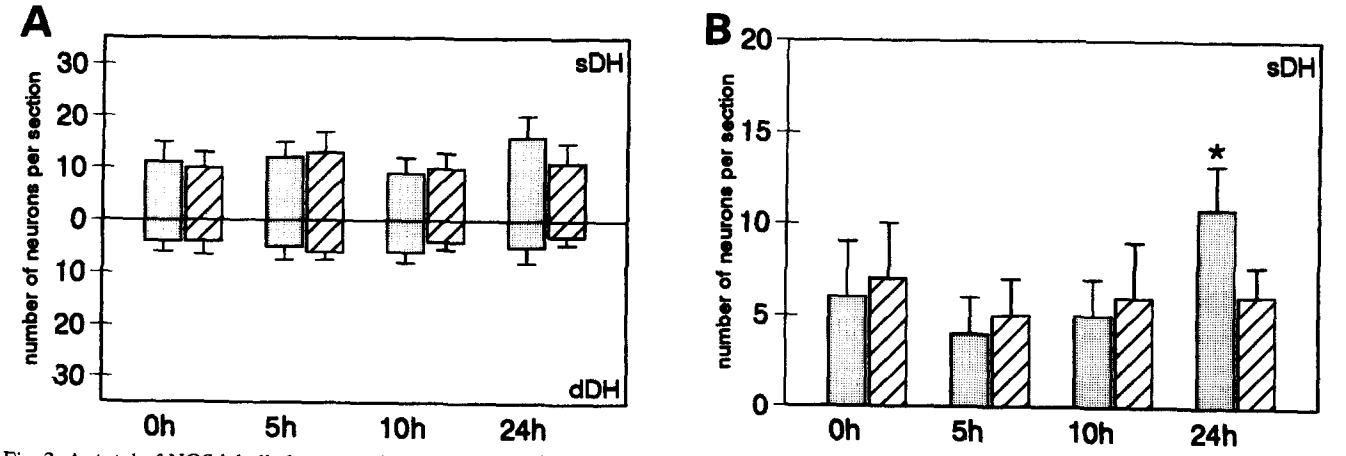


Fig. 2. A: total of NOS labelled neurons in the ipsilateral (gray bars) and contralateral (hatched bars) superficial and deep dorsal horn (sDH and dDH, upper and lower part) of L4 segment following formalin (FOR). B: NOS labelled neurons in the medial part of superficial dorsal horn (sDH) of L3 and L4 segments following FOR. *Significant ipsilateral increase ($P < 0.05$) compared to the contralateral side after 24 h.

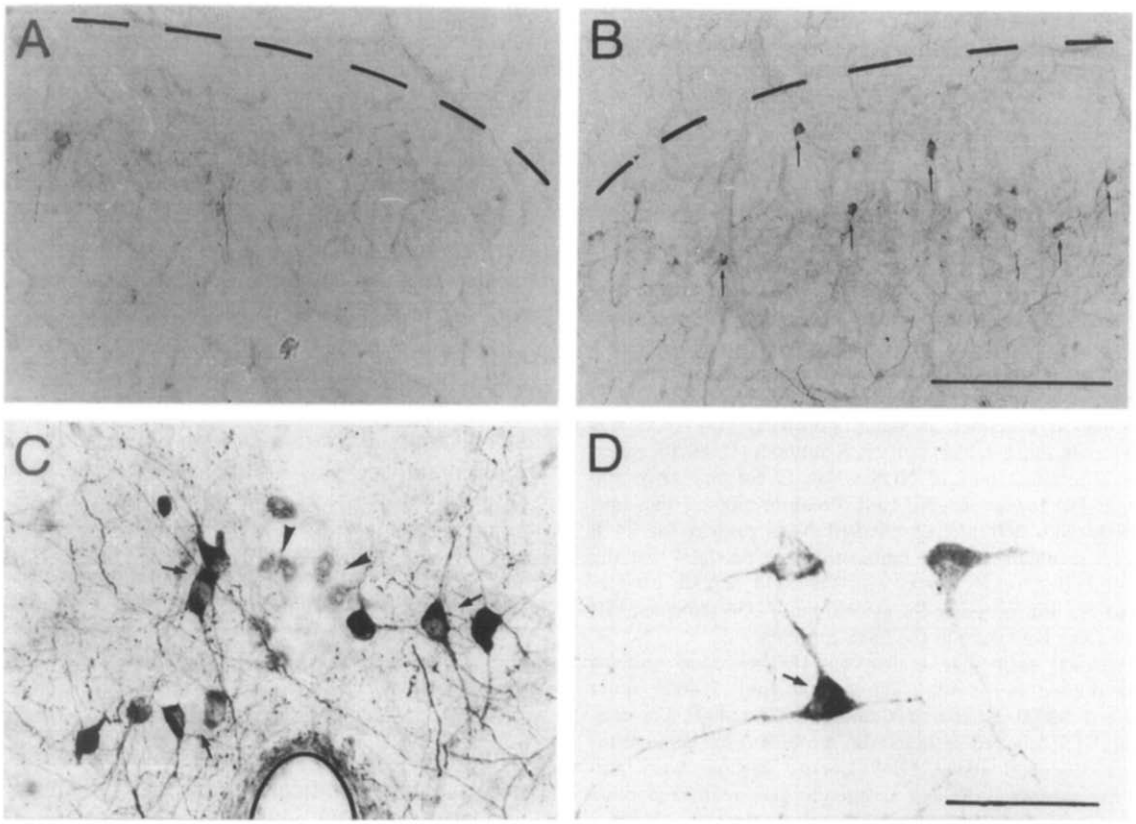


Fig. 3. NDP labelled neurons in the superficial dorsal horn (A) in the untreated rat and (B) 24 h following FOR. Arrows mark neurons with suprabasal NDP reaction. The dotted lines mark the grey/white matter boundaries. C: neurons in lamina X are selectively labelled either by c-Fos (arrowheads) or by NOS (arrows) 2 h following FOR. The central canal is marked at the bottom. D: colocalization of c-Fos in a NDP neuron (arrow) in the deep dorsal horn 2 h following second application of FOR given 24 h after the first FOR into the same hind paw. Bar (A,B) = 200 μm, (C,D) = 50 μm.

3.2. NADPH-diaphorase reaction

In the dorsal horn of untreated rats, the distribution of NADPH-diaphorase (NDP) labelled neurons was similar to that of NOS labelled neurons. NDP neurons were predominantly found in the inner layer II (Figs. 3A and 4A) and a dense cluster was located around the central canal (Fig. 3C). In contrast to NOS, NDP could not be detected in the lateral spinal nucleus and in large diameter neurons of the ventral horn (Fig. 5C,D). NDP strongly labelled fibers and varicosities particularly in sDH and lamina X. The labelling of fibers by

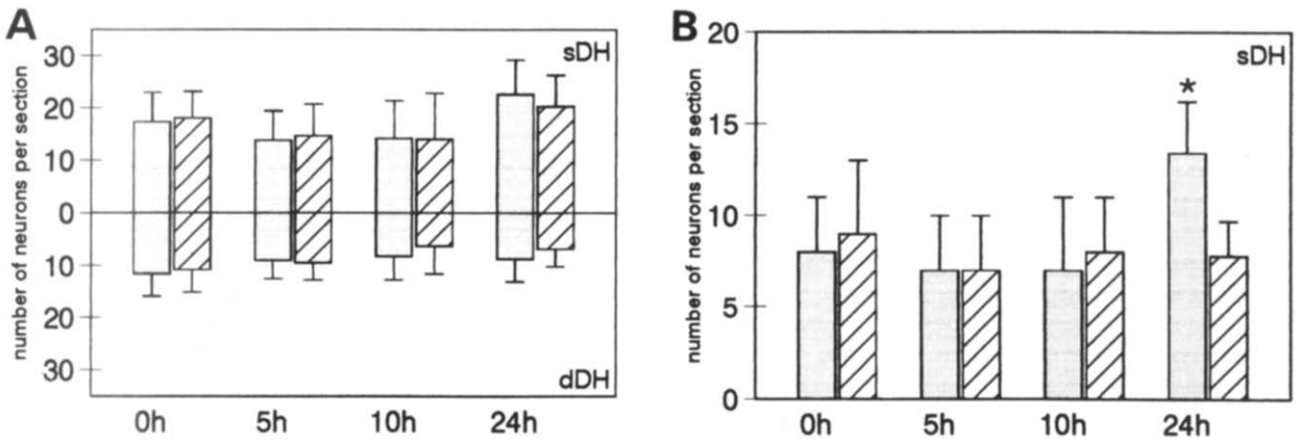


Fig. 4. A: total of NDP labelled neurons in the ipsilateral (gray bars) and contralateral (hatched bars) suprficial and deep dorsal horn (sDH and dDH, upper and lower part) of L4 segment following FOR. B: NDP labelled neurons in the medial part of superficial dorsal horn (sDH) of L3 and L4 segments following FOR. *Significant ipsilateral increase ($P < 0.05$) compared to the contralateral side after 24 h.

NDP depended on the segmental level being strong in the L5–L6 segments, moderate in the L2–L3 segments and weak in L4 segment.

Twenty-four hours following FOR, a significant increase in number of NDP neurons could be determined in the medial part of sDH of ipsilateral L3–L4 segments compared to the contralateral side (13.4 ± 2.8 versus 7.8 ± 1.9 c/s) and compared to the numbers after 5 h and 10 h (Figs. 3B and 4B). In this area an increase of NDP labelled fibers and varicosities was also clearly visible. These significant changes could not be detected in the L5–L6 segments. In contrast, in the ipsilateral sDH of L6, there was a tendency for a decrease in the number of neurons and fibers labelled by NDP compared to contralateral NDP reaction.

NOS-IR and NDP labelled neurons were arranged in clusters thus the variability was substantial between consecutive sections from a defined spinal segment as well as between the ipsi- and contralateral side.

3.3. Spatial association of substance P and CGRP labelled fibers with NDP neurons

The neuropeptides substance P (SP) and calcitonin gene-related peptide (CGRP) are considered mediators of the peripheral nociceptive information transfer to spinal neurons, and therefore we have searched for spatial associations of SP and CGRP immunoreactive nerve fibers with NDP neurons. At the light microscopic level, SP- and CGRP immunoreactive fibers formed extended networks in sDH including NDP neurons (Fig. 6A,B). In laminae IV–VIII, these spatial proximities were sparse but distinct. In lamina X, CGRP labelled fibers formed a dense network around the large diameter NDP neurons.

3.4. IEG encoded proteins

In contrast to the delayed and minor changes of NOS-IR and NDP, the expressions of c-Jun, JunB, JunD, c-Fos, FosB and Krox-24 showed an early and strong increase following the application of FOR. The immunoreactivities of IEG encoded proteins showed an exclusive nuclear labelling. Time courses and distributions of labelled nuclei in sDH and dDH (superficial and deep dorsal horn) are summarized in Fig. 7; the numbers given for JunD and Krox-24 refer only to those neurons with an enhanced intensity of labelling compared to that of the contralateral side and to that of untreated rats.

3.4.1. Jun proteins

c-Jun showed a weak basal expression in numerous neurons of sDH, dDH and ventral horn. The maximal

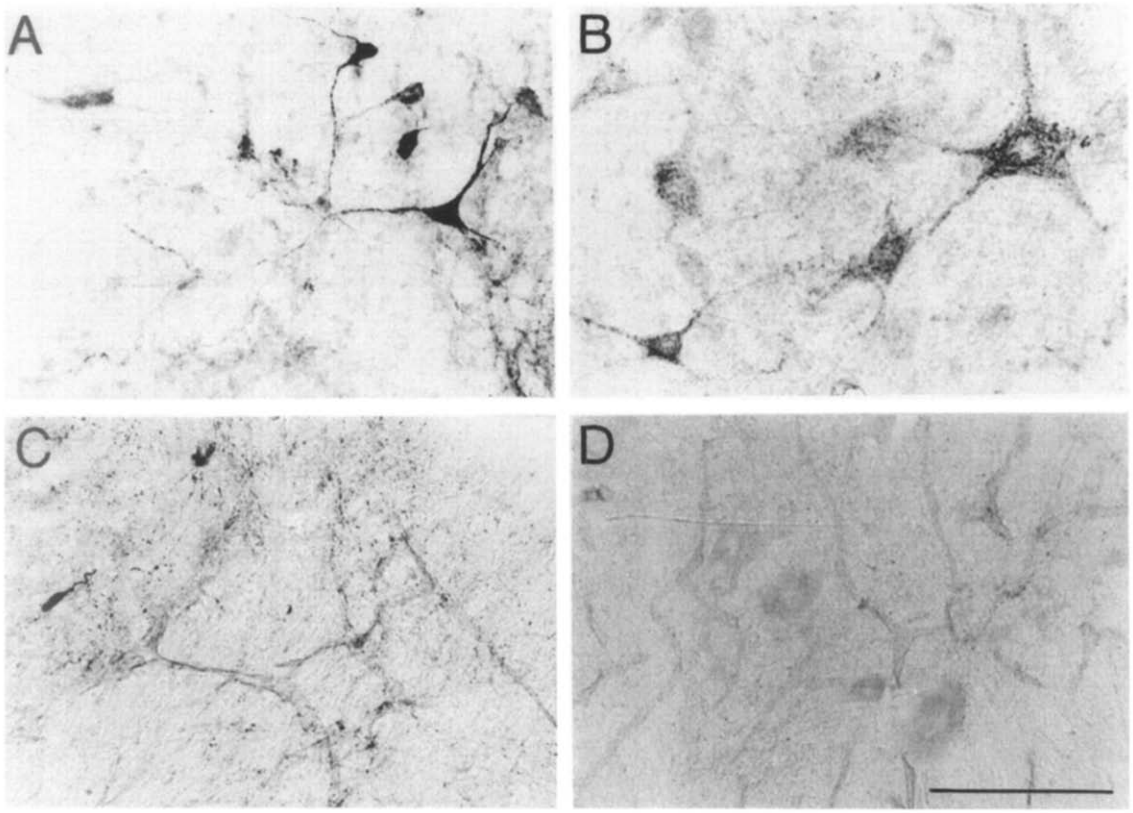
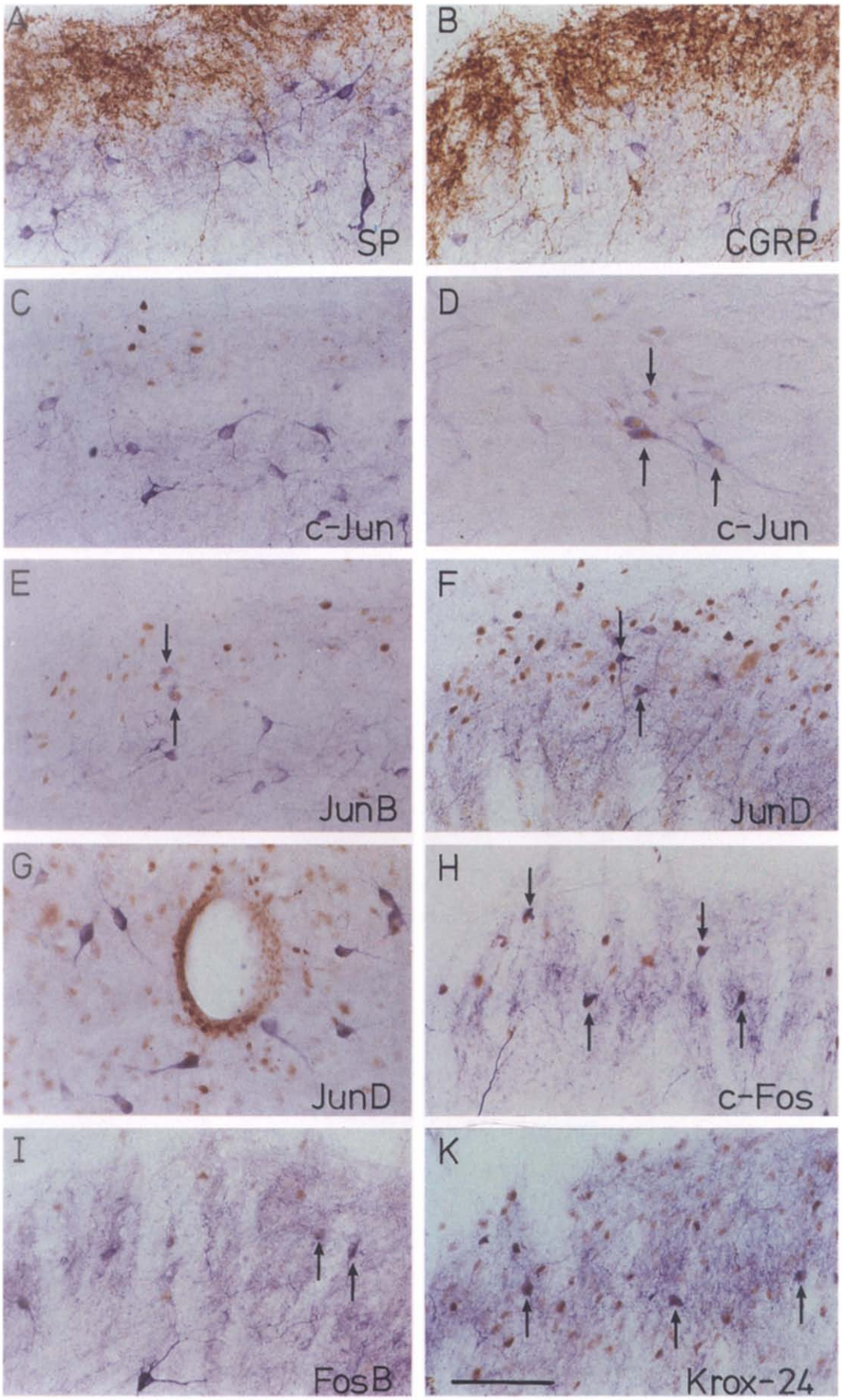


Fig. 5. (A,B) Distinct NOS immunoreactivity and (C,D) absent NDP reaction in (A,C) the lateral spinal nucleus and (B,D) presumable motoneurons of untreated rats. Bar (A,C) = 300 μm, (B,D) = 100 μm.


A
SP
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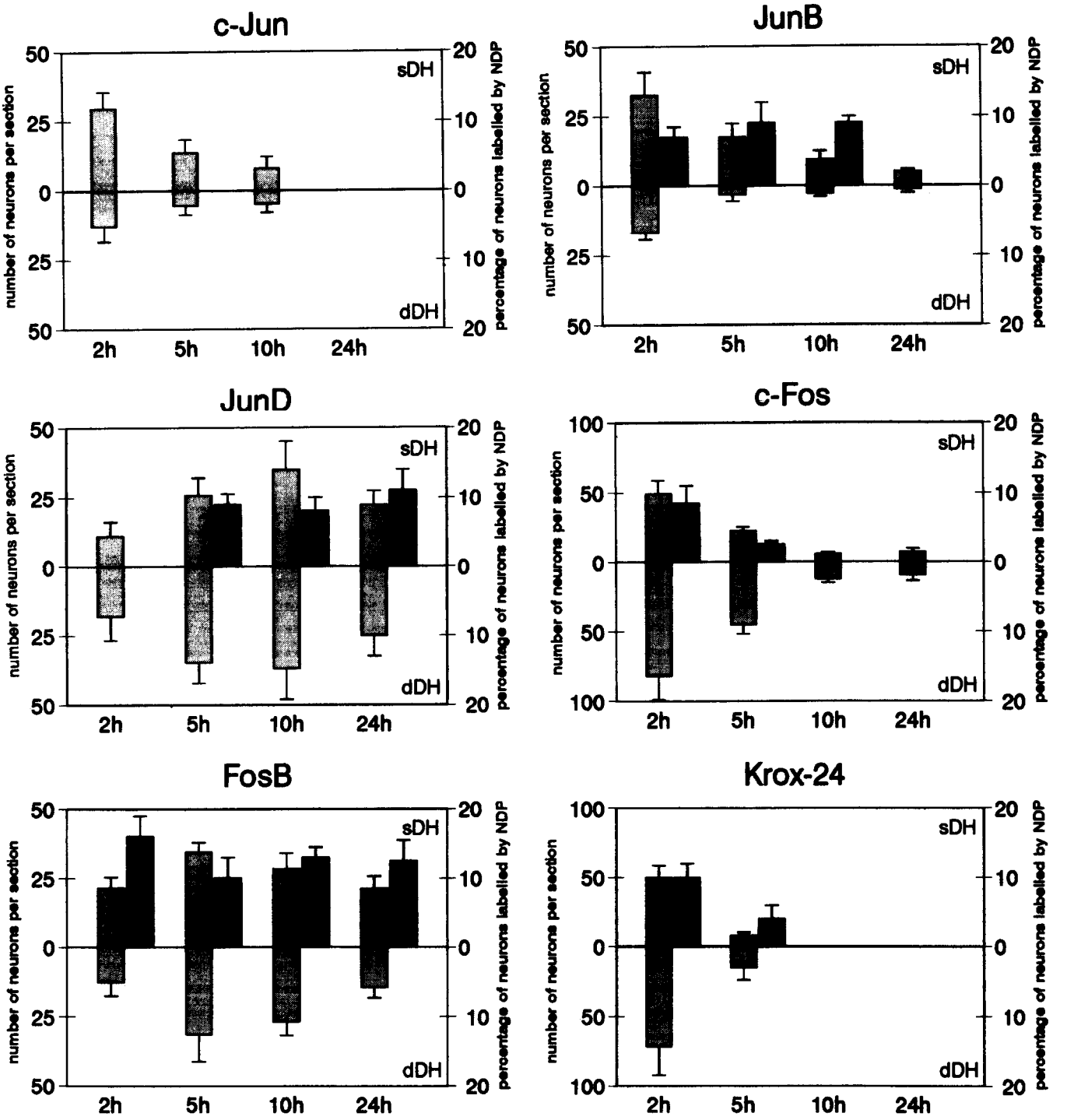

Fig. 7. Time courses of numbers of neurons labelled by Jun, Fos and Krox-24 proteins (gray bars and left hand scale) in superficial dorsal horn (sDH, upper part) and deep dorsal horn (dDH, lower part) following subcutaneous injection of formalin. Only the numbers of those JunD and Krox-24 neurons are given which are labelled with suprabasal intensity. The black bars and the right hand scale give the percentage of neurons double labelled by NDP and IEG proteins from the total number of neurons labelled by IEG proteins (see gray bars).

Fig. 6. Colocalisation of blue NDP reaction in L3–L4 segments with (A,B) neuropeptides and (C–K) IEG encoded proteins after various time points following injection of formalin (FOR). (A) Substance P and (B) CGRP in the superficial dorsal horn (sDH) of the untreated rat; (C) c-Jun in sDH after 2 h; (D) c-Jun in preganglionic parasympathetic neurons of S1 segment of untreated rat; (E) JunB in sDH after 2 h; (F) JunD in sDH after 5 h; (G) JunD in lumbar lamina X of untreated rat; (H) c-Fos in sDH after 2 h; (I) FosB in sDH after 5 h; (K) Krox-24 in sDH after 2 h. Arrows mark the suprabasal expression of IEG encoded proteins in NDP neurons. Bar (A–K) = 100 μm.

number of 43.4 ± 8.4 c/s with suprabasal c-Jun-IR was reached 2 h following FOR. After 10 h, c-Jun-IR had almost declined to basal levels. The expression of c-Jun was predominantly visible in sDH (Figs. 6C and 7).

JunB was absent in untreated rats. Two hours following FOR, JunB-IR was maximal in 51.7 ± 9.3 c/s and thereafter rapidly declined (Figs. 6E and 7). Similar to c-Jun, the majority of JunB-positive neurons were present in sDH.

JunD showed a constitutive expression in many nuclei of white and gray matter. Two hours following FOR, a substantial number of labelled nuclei were visible with an intensity slightly exceeding that of basal immunoreactivity. JunD had a maximal suprabasal IR between 5 and 10 h in 71.1 ± 16.4 c/s (Figs. 6F and 7). In contrast to c-Jun and JunB, neurons with suprabasal JunD-IR were equally distributed in sDH and dDH.

3.4.2. Fos proteins

c-Fos was rapidly expressed and reached its maximum 2 h following FOR in 128.7 ± 21.8 labelled nuclei. The number of labelled neurons rapidly declined by about 50% after 5 h (Figs. 6H and 7). c-Fos was almost absent in sDH and dDH after 10 h. Similar to Krox-24, the distribution of c-Fos immunoreactive nuclei showed a preference for the dDH.

The increase of FosB was delayed and reached its maximal number in 63.2 ± 12.4 c/s after 5 h (Figs. 6I and 7). FosB persisted at a submaximal level up to 10 h, and after 24 h, a significant number of nuclei still exhibited FosB-IR. The distribution of FosB-IR showed a slight preference for the sDH.

3.4.3. Krox-24 protein

In untreated rats, the zinc-finger protein Krox-24 was present in numerous neuronal nuclei of sDH, mainly lamina III. Two hours following FOR, Krox-24 showed a maximal suprabasal labelling in 118.8 ± 24.9 c/s with a slight preponderance in the dDH (Figs. 6K and 7). After 10 h, the number of Krox-24 labelled neurons had returned to that of untreated rats. Interestingly, 24 h following FOR, the number of Krox-24 labelled nuclei was reduced in the ipsilateral sDH compared to that of the contralateral side and to that of untreated rats.

In the contralateral dorsal horn, we could not detect substantial changes of immunoreactivities of Jun, Fos and Krox-24 proteins.

3.5. Colocalization of IEG encoded proteins and NDP following a single injection of formalin

As the double labelling of IEG proteins and NOS by immunochemistry was difficult to assess, we investigated the colocalization of IEG encoded proteins and NDP. Fig. 7 summarizes the percentages of those Jun, Fos and Krox-24 immunoreactive neurons that were co-labelled with NDP. Except from c-Jun, up to 17% of all Jun, Fos and Krox-24 labelled nuclei were visible in NDP neurons following FOR. Importantly, this colocalization was exclusively found in sDH but not in dDH (Fig. 7). In dDH and lamina X, Jun, Fos and Krox-24 protein containing neurons were found very close to NDP neurons but colocalization could not be detected (Figs. 1C and 3C).

3.5.1. Jun proteins

High colocalization of c-Jun and NDP was present in preganglionic sympathetic and parasympathetic neurons in the untreated rat. Following FOR, c-JUN-IR remained absent in NDP neurons of sDH and dDH (Figs. 6C,D and 7). JunB was co-localized with NDP in 2.3 ± 0.7 c/s in sDH after 2 h (Figs. 6E and 7), between 10 h and 24 h only one NDP neuron/section showed JunB-IR. In untreated rats, 6.4 ± 1.6 c/s and 3.4 ± 1.3 c/s were unilaterally labelled for NDP and JunD in sDH and dDH, respectively. Five hours following FOR, suprabasal intensity of JunD-IR was visible in further 3.1 ± 1.3 NDP c/s (Figs. 6F and 7) and the same percentage of colocalization persisted in sDH up to 24 h. In contrast, suprabasal JunD-IR could not be determined in NDP neurons of dDH and lamina X (Fig. 6G) and in neurons of contralateral spinal cord.

3.5.2. Fos proteins

After 2 h, c-Fos was expressed in 4.1 ± 1.3 NDP neurons/section of sDH (Fig. 6H) and was almost completely absent in these neurons after 5 h. FosB was expressed in 2.8 ± 0.5 to 3.8 ± 1.2 NDP neurons/section between 2 h and 24 h (Fig. 6I).

3.5.3. Krox-24

In lamina III, basal Krox-24-IR was unilaterally present in 2.6 ± 1.4 c/s labelled by NDP. Two hours following FOR, additional 2.9 ± 1.0 c/s showed a suprabasal intensity of Krox-24-IR and NDP (Fig. 6K) which had rapidly disappeared within 5 h.

The percentage values of Fig. 7 demonstrate that c-Fos and Krox-24 declined faster in NDP neurons compared to non-NDP neurons. After 24 h, when the labelling by NOS and NDP has significantly increased in the medial sDH, there was no concomitant increase in the colocalization values of the IEG encoded proteins. However, the colocalization values of FosB and JunD in NDP neurons did not decline and remained on a plateau level (Fig. 7).

3.6. Colocalization of IEG encoded proteins and NDP following a second injection of formalin

We wanted to know whether the IEG expression in NDP neurons of dDH could be triggered by increase of

stimulation intensity. Therefore, 24 h following a first injection of FOR, a second injection was applied into the same hind paw. The colocalization of IEG proteins and NDP was investigated 2 h following this second stimulus in neurons of lumbar spinal cord. In sDH, the pattern of colocalization did not substantially differ from that following a single application of FOR. However, JunD, c-Fos, FosB and KROX-24 appeared in some NDP neurons of lamina X and dDH (Figs. 1D and 3D) 2 h following the second stimulus.

3.7. Spatial associations between neurons labelled by NDP- and IEG encoded proteins

At the level of light microscopy, many neurons labelled for Jun, Fos and Krox-24 proteins were contacted by NDP labelled fibers. In the sDH, the high density of NDP fibers raises the possibility for the association with many neurons, and therefore, we could not recognize a particular close relationship between NDP fibers and IEG labelled neurons. However, in the dDH, we observed that numerous neurons labelled by IEG were in close association to NDP fibers. Because both, NDP fibers and IEG expression were sparse in the dDH compartment, this close association could be distinctly and reproducibly discriminated. Fig. 8 illustrates the net of NDP fibers in dDH which pass along IEG labelled neurons and contact further NDP neurons. In some sections, more than 50% of IEG labelled neurons in laminae IV–VII were at close apposition to NDP fibers at the level of light microscopy.

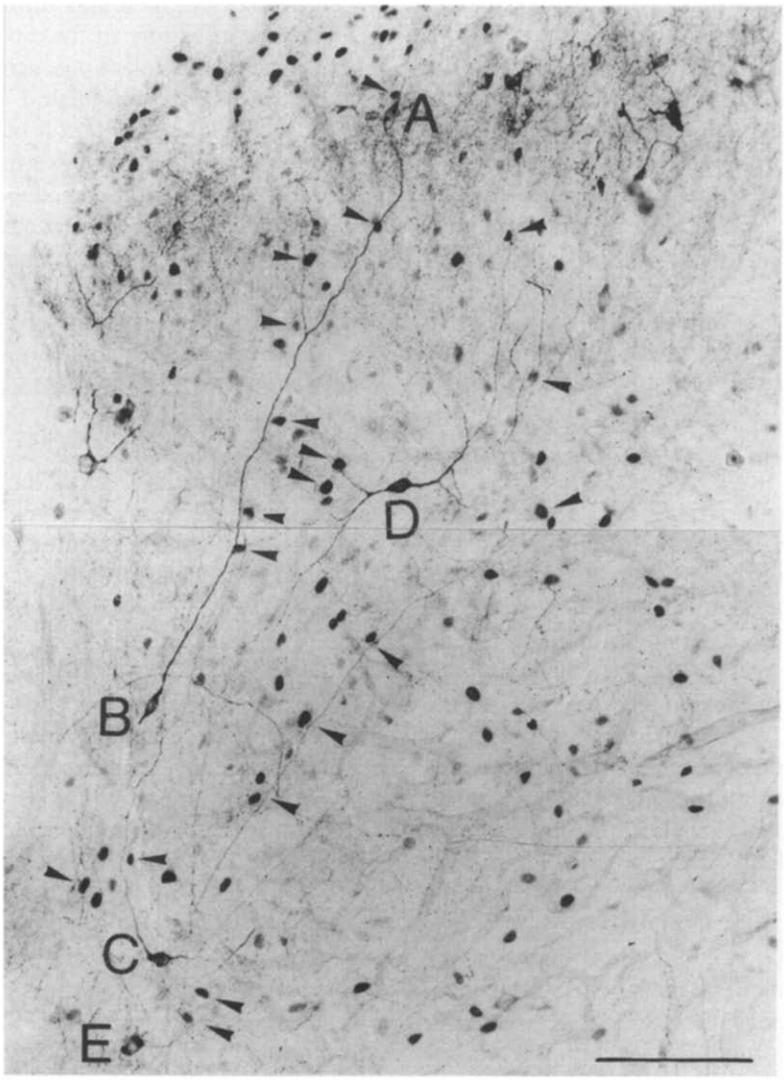

Fig. 8. NDP neurons and fibers and c-Fos positive nuclei in the ipsilateral superficial and deep dorsal horn (sDH and dDH, respectively) 2 h following injection of formalin. The neurons A–E show numerous spatial appositions forming a net between sDH and dDH. In dDH, a major part of c-Fos protein (arrows) is expressed in neurons of which the cell membrane is contacted by NDP fibers araising from the NDP neurons B–E. Bar = 200 μm.

4. Discussion

The present study has investigated the expression of nitric oxide synthase (NOS) and the NADPH-diaphorase reaction (NDP) as well as the associations of NOS-IR and NDP to the expression of IEG encoded transcription factor proteins in spinal neurons following subcutaneous injection of formalin (FOR) into one hindpaw. The main results are summarized as follows:
(1) Spatial patterns of NOS-IR and NDP are congruent but not identical in lumbar spinal cord.
(2) There is a significant increase in number above basal values of neurons labelled by NOS-IR and NDP in the sDH of L3–L4 segments 24 h following FOR.
(3) Differential temporo-spatial expressions of c-Jun, JunB, JunD, c-Fos, FosB and Krox-24 in ipsilateral spinal neurons between 2 h and 24 h following FOR.
(4) Co-expression of JunB, JunD, c-Fos, FosB and Krox-24 proteins in small proportions of NOS and NDP neurons of sDH, and in neurons of dDH only following a second injection of FOR. c-Jun was not induced in NDP neurons following FOR, but similar to JunD, it was colocalized in preganglionic sympathetic and parasympathetic neurons of untreated rats.
(5) Many neurons expressing Jun, Fos and Krox-24 proteins following FOR are in close spatial proximity to NDP labelled fibers and varicosities at level of light microscopy.
(6) Varicosities labelled by substance P and CGRP form close spatial associations with NDP and NOS neurons.

4.1. NOS immunoreactivity and labelling by NDP

The NOS immunoreactivity (NOS-IR) was evoked by a specific anti-NOS antibody raised against the N-terminal part of the NOS protein [29,38]. Immunoblotting demonstrated a strong reaction of the antibody with NOS isolated from brain synaptosoms but no reaction at all against the cytochrome P-450 reductase which is highly homologous to the C-terminal part of NOS [21]. The pattern of NOS-IR in lumbar spinal cord was very similar to that recently published [12] and paralleled the NDP labelling in this compartment. Our protocol for NDP reaction evoked a reproducible pattern which confirms previous reports [2,54]. The lower number of NOS-IR neurons and the weak NOS-IR of varicosities in sDH compared to the corresponding NDP reaction could be explained by the presence of small and moderately labelled NDP neurons which might not be detected by the NOS antibody. However, NOS-IR and NDP reaction were dissociated in motoneurons and neurons of the lateral spinal nucleus which exhibited a distinct NOS-IR in absence of NDP reaction. NOS-IR in the lateral spinal nucleus has also been described elsewhere [58] whereas our finding of NOS-IR in motoneurons is at variance with the reported absence of NOS-IR in motoneurons [12]. Taken together, our data contribute to recent observations [12] that NOS-IR and NDP may not always label identical sets of neurons as it has previously been claimed [5,8].

Both NOS-IR and NDP marked an increase of labelled neurons in the ipsilateral sDH of the L3–L4 levels 24 h following FOR. This area represents the main region of termination of sciatic nerve fibers [36,55]. The increase in the numbers of NOS-IR and NDP in sDH following FOR is about the same order of magnitude as the increase of NDP labelled neurons following injection of carrageenan [54]. However, in contrast to this report [54] we could not determine changes in numbers of NDP labelled neurons in dDH and contralateral DH. This difference may be due to the higher inflammatory potency of carrageenan compared to FOR. A similar upregulation has been found for the expression of enkephalin and dynorphin in spinal neurons following acute stimulation of cutaneous nociceptors projecting through the sciatic nerve [10,46–48]. Thus, excitation of cutaneous chemical nociceptors results in increase in NOS expression in DH neurons which could prolong the pain status via the hyperalgesic action of nitric oxide [16,39,40,45].

Apart from neuronal staining, NOS immunoreactive and NDP labelled fibers had an increased incidence in the medial sDH of L3–L4 following FOR. The origin of these fibers and varicosities remains to be defined. Evidence has been provided that the major pool for spinal NOS-IR is formed by spinal rather than by primary afferent neurons [58]. On the other hand, peripheral noxious stimulation increases NDP labelling in primary afferent neurons (R. Traub, unpublished observation) which could also contribute to the enhanced spinal NDP reaction.

4.2. Expression of Jun, Fos and Krox proteins

This is the first study comprising the changes of six immediate-early gene encoded proteins following acute inflammatory stimulation of cutaneous nociceptors. The present work confirms our previous study about JunB, JunD and FosB expression [19] and includes also c-Jun, c-Fos and Krox-24 proteins. c-Jun, JunB, c-Fos and Krox-24 had their maximal expression in the ipsilateral neurons of sDH and dDH within 2 h and declined thereafter, whereas expression of FosB and JunD reached their maxima not until 5 h and were still present in a considerable amount of neurons after 24 h. Similar time courses in the immunoreactivities of Jun, Fos and Krox proteins have been observed in the spinal cord following electrical stimulation of sciatic nerve fibers [17] and noxious heat [19,56] as well as in

the brain following epileptic seizures [14], ischemia [15] and cortical spreading depression [23].

The differential temporo-spatial expression of Jun, Fos and Krox-24 proteins presumably has strong impact on transcriptional activities. Thus, co-expression of all transcription factors seems to be restricted to neurons of lamina I–II representing neurons of second order of primary afferent Aδ- and C-fibers [36]. This co-expression of Jun and Fos proteins might underly the formation of variable transcription complexes, the so-called AP-1 (activator protein-1) complexes. The absence of c-Jun in NDP neurons indicates that NDP neurons might exert transcriptional operations different from non-NDP neurons with c-Jun. Because of differential effects of JunB on c-Jun and c-Fos mediated transcription, the presence or absence of c-Jun could result in a more suppressive or more transcriptive effect, respectively, of JunB [9,51,52].

In dDH, substantial formation of AP-1 transcription complexes is restricted to the contribution by c-Fos, FosB and JunD proteins. Recently, we have reported about congruent patterns of c-Fos and JunD expression in dDH neurons following electrical stimulation of sciatic nerve fibers [17], and the lasting presence of JunD might contribute to the stability of c-Fos expression as was shown by in vitro experiments [30,31]. The persistence of FosB could also contribute to the termination of gene transcription because FosB can act as suppressor of gene expression [34,61]. The putative molecular genetic consequences of differential formation of AP-1 complexes have been discussed elsewhere [17,19,30,31,50].

4.3. Co-expression of NDP with IEG encoded proteins

Except for c-Jun, 8%–17% of the total number of Jun, Fos and Krox-24 labelled neurons following FOR are also labelled by NDP. The immunoreactivities of c-Fos and Krox-24 declined faster in NDP neurons compared to the non-NDP neurons as it is indicated by the rapid decrease of the colocalization values (Fig. 7). This suggests a restricted inducibility of IEG encoded transcription factors and/or a restricted de novo synthesis of IEG encoded proteins in NDP neurons. Moreover, IEG are not expressed in NDP neurons of dDH following single application of FOR and became only visible following a second noxious stimulus. In the dDH and around the central canal, IEG labelled neurons are in very close proximity to NDP neurons (Figs. 1C and 2C) suggesting that NOS/NDP neurons have intrinsic properties with high threshold for IEG expression rather than to separated transynaptic input of neurons labelled by IEG and NOS/NDP. We conclude from these findings that NOS neurons have a restricted potency for IEG expression which underlies the *stability* of neuronal programs in relation to changes in protein synthesis. The low probability of IEG expression in NOS neurons could be based on the presence of calcium-binding proteins which buffer the stimulation-associated calcium influx thereby blocking the calcium mediated transmembranous signal transfer to the nucleus. In this context it is noteworthy that c-Fos was only induced in cortical neurons labelled by parvalbumin, a calcium binding protein, following acoustic stimulation at high stimulation intensity whereas stimulation at low intensity is not effective for c-Fos expression in neurons with parvalbumin immunoreactivity [63].

c-Jun is not expressed in NDP labelled neurons. This failure might be related to the relatively low inducibility of c-Jun by *transmembranous* stimulation as it has been demonstrated following cortical spreading depression and ischemia in the brain [15,23] as well as following membrane depolarisation *in vitro* [3]. In contrast, *transection of nerve axons* is highly effective for induction of c-Jun but not of c-Fos in the axotomized neuron [18,20,21,26,27]. The absence of c-Jun in NDP labelled neurons demonstrates that at least in these neurons there is no parallel expression of c-Fos and c-Jun as it has been suggested normally to occur.

The persistence of JunD and FosB during the complete observation period could contribute to the increase of NOS-IR by activation of NOS gene between 10 h and 24 h. The delayed onset of NOS expression might indicate a particular property of the NOS promotor because delayed expression of NOS protein was also found between 5 and 10 days following transection of peripheral and central nerve fibers in the axotomized neurons [13,21].

Summarizing, neurons can co-express NOS and inducible transcription factors but this co-expression is very restricted to neuronal subpopulations such as sDH neurons and involves formation of specific AP-1 complexes e.g. without participation of c-Jun.

4.4. Neuronal network between NDP and IEG labelled neurons

NDP labelled fibers and varicosities form a dense net within the sDH. The localization of Jun, Fos and Krox-24 immunoreactive neurons did not indicate a particular spatial relation to these NDP labelled fibers. However, preferential spatial associations between NDP neurons and/or fibers and IEG neurons became apparent in the dDH. As judged at the light microscopy level, individual NDP neurons apparently contact up to 15 neurons labelled by IEG proteins per section. Because both NDP fibers and IEG proteins are fairly rare in the dDH, this close spatial association of IEG labelled neurons and NDP fibers indicates the possibility of nitric oxide mediated induction of IEG. However, the effects of NO on IEG expression re-

mained to be elucidated. On the one hand, NO mediates the phosphorylation of the transcription factor CREB with consecutive transcription of c-fos and c-jun genes [49]; and inhibition of NO synthesis prevents the induction of c-Fos in spinal neurons following noxious stimulation [35]. These findings indicate the induction of IEG by NO. On the other hand, NOS is colocalized with GABA in terminals of intrinsic spinal neurons [47], and the release of GABA could counteract the NO mediated excitation of spinal neurons thus preventing IEG expression.

Our findings about temporo-spatial patterns of IEG and NOS expression following FOR point out a sequence of events starting with the release of NO, substance P and CGRP from primary afferents followed by expression of IEG encoded transcription factors in second and third order neurons. The enhanced IEG levels could activate the expression of the NOS gene in sDH resulting in enhanced production of NO. By this chain of transmitter release and gene expressions, transient and acute noxious stimulations can be transduced into more lasting states of hyperalgesia and pain.

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5. Abbreviations

AP-1	activator protein-1
CGRP	calcitonin gene-related peptide
c/s	cells per section
dDH	deep dorsal horn (laminae IV-VII,X)
FOR	injection of formalin
IEG	immediate-early genes
IR	immunoreactivity
LSN	lateral spinal nucleus
NDP	NADPH-diaphorase reaction
NO	nitric oxide
NOS	nitric oxide synthase
sDH	superficial dorsal horn (laminae I–III)

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