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MAP OF SPINAL NEURONS ACTIVATED BY CHEMICAL STIMULATION IN THE NUCLEUS RAPHE MAGNUS OF THE UNANESTHETIZED RAT

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Abstract—The expression of the proto-oncogene c-*fos* was used as a cellular marker of spinal cord neurons activated by microinjection of kainic acid into the medullary nucleus raphe magnus of awake and drug-free Sprague–Dawley rats. The c-FOS protein was detected by immunocytochemistry. We found increased immunoreactivity bilaterally in laminae I–VI of the dorsal horn. The strongest c-FOS expression was observed within the inner layer of lamina II near its border with lamina III. In the ventral horn no c-FOS immunoreactivity was observed. Thus, the present results provide evidence for a descending excitation of neurons predominantly in inner lamina II, possibly mediating nucleus raphe magnus-induced inhibition of neurons in other laminae.

The nucleus raphe magnus (NRM) in the ventromedial medulla is a major source of medullo-spinal projections. Neurons originating in this area project to all levels of the spinal cord, mainly to laminae I, II, V–VII, IX and X.[5,25,34] Spinally projecting neurons in the NRM are thought to contain a variety of neurotransmitters and neuropeptides, mainly serotonin, but also leucine and methionine enkephalin or substance P.[10,11] These descending neurons are known to play a central role for the modulation of nociceptive information in the dorsal horn.[20,21,35,44,48] Their involvement in the depression of spinal nociception has attracted much attention and is now well established.[8,14,19,28,49]

Spinally projecting neurons originating in the NRM also modulate motor output from the ventral horn.[3,34] The descending inhibition of motoneurons may be monosynaptic[2] while the inhibition of dorsal horn neurons is thought to be at least partly mediated through intercalated segmental[7,35,52] or propriospinal neurons.[47] The descending inhibition, e.g. of nociceptive motor reflexes, is mediated by serotonin[25] while the neurotransmitters of the descending excitation have not yet been identified. Possibly substance P, an excitatory transmitter/neuromodulator candidate is involved in the dorsal horn.[54]

Virtually nothing is known about the number or the segmental or laminar location of spinal neurons which can be activated by descending efferents from the NRM in the unanesthetized animal. Here, we have used the expression of the proto-oncogene c-*fos* as a cellular marker of activated neurons[16] in the spinal cord following chemical stimulation in the NRM of awake, drug-free rats.

EXPERIMENTAL PROCEDURES

Experiments were performed on 13 male Sprague–Dawley rats weighing 250–280 g which were obtained from the Zentralinstitut für Versuchstierzucht (Hannover, Germany). The rats were kept in groups of two in one Makrolon type III cage in an air-conditioned room with a 12 h light–dark cycle, light on at 7 a.m. Food and water were given *ad libitum*. The animals were transported to the laboratory every day at the same time where they were handled and placed in the $30 \times 30\ cm^2$ clear plastic testing compartment.

After at least one week of handling, surgery was performed. The rats were anesthetized with an initial i.p. dose of 60 mg/kg pentobarbital. One injection of scopolamine was given i.p. at a dose of 20 mg/kg. A deep level of anesthesia was maintained during surgery by bolus i.p. injections of pentobarbital at a dose of 15 mg/kg. Body temperature was monitored by a rectal probe and kept constant at $38 \pm 0.5°C$ by a heating pad.

For the implantation of a 26-gauge guide cannula the head was fixed in a stereotaxic frame and a craniotomy was performed with a small electric drill. The cannula was lowered into the medial medulla 2 mm dorsal to the dorsal boundaries of the NRM by a stepping motor (stereotaxic coordinates AP, −10.8; L, 0.0; H, 8.5 with reference to bregma). The atlas of Paxinos and Watson[42] was used. At the top of the cannula a 4 mm long thread was attached so that a plastic cap could be screwed on for protection of the implant. Two small skull screws provided mechanical stability for the implant. A stylet was inserted into the cannula to avoid its occlusion. The small craniotomy was closed with bone wax. The implant was fixed with dental acrylic to the skull and the skin was sutured. After surgery the animals were allowed to recover for at least one week during which they were handled as described above.

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Abbreviations: IIi, inner lamina II; IIo, outer lamina II; NRM, nucleus raphe magnus.

Before any stimulation the rats were put into the testing compartment for 5 min during which their behavior was watched and recorded on video tape. Neurons originating from the NRM were stimulated by a microinjection of kainic acid (40 pmol in 100 nl) through an injection cannula (0.23 mm o.d., 0.13 mm i.d.). The injection cannula was connected to a 5 μl Hamilton syringe by a polyethylene tube (PE 10). The injection time took about 5 s and the cannula was left in place for an additional 25 s. After the injection the animals were put into the testing compartment for 5 min to monitor their behavior. The rats showed no signs of distress before, during or after the stimulation with the exception of two rats which showed some struggling during the insertion of the injection cannula.

Ninety minutes after the injections the rats were killed by an overdose of pentobarbital. The stimulation site was marked by an injection of 100 nl of a saturated solution of Fast Green and the animals were perfused transcardially with phosphate-buffered saline followed by 4% paraformaldehyde. The brains and spinal cords were removed and fixed in paraformaldehyde overnight and stored in 30% sucrose for 48 h. The spinal cord was cut in 50 μm coronal sections in a cryostat. Sites of Fast Green deposition were localized with reference to the atlas of Paxinos and Watson.[42] For immunocytochemical studies the sections were incubated with goat serum (2% in phosphate-buffered saline and 0.2% Triton X-100) for 1 h followed by the primary antiserum at 1:40,000 for 48 h. The sections were washed and incubated in biotinylated goat anti-rabbit antiserum followed by avidin–peroxidase complex (Vectastain, Vector Laboratories) for 1 h. Finally, they were developed in 0.02% diaminobenzidine with 0.02% hydrogen peroxidase followed by 0.02% cobalt chloride and nickel ammonium sulfate. The polyclonal rabbit antibody was generously provided by Dr R. Bravo, The Squibb Institute Medical Research (Princeton, U.S.A.). For each of the spinal cord segments investigated the number of c-FOS positive cells was counted in five 50 μm thick transverse sections. Significance of differences between the sham-treated and the kainic acid stimulated animals was tested by the use of the Mann–Whitney test.

RESULTS

Eight rats received a microinjection of kainic acid (40 pmol in 100 nl) into the NRM and five sham-treated rats received an injection of 100 nl of 0.9% NaCl solution. In seven of the eight kainic acid stimulated rats the injection sites were histologically verified to lay within the boundaries of the NRM. One injection site was located right of the midline at the lateral part of the right pyramid (Fig. 1). In three of the five sham-treated animals the injection site was histologically recovered and shown to be in the NRM (Fig. 1). In the two remaining animals only the tracks of the pipette were identified. These tracks were consistent with an injection site within the NRM as can be seen in Fig. 1.

The sham-treated animals showed no change in their behavior during the 5 min observation period after the injection as compared with the observation period prior to the injection. In all these animals the number of c-FOS positive cells was low in laminae I, II, III and IV–VI of the cervical, thoracic and lumbar spinal cord (Fig. 2) and virtually absent in laminae VII–XII.

Five of the seven rats which received an injection of kainic acid into the NRM showed evident changes in their behavior within about 1 min after the injection. The behavior was characterized by a significant slowdown of locomotion and explorative behavior and the occurrence of intermittent periods of immobility lasting about 3–6 s on average. One rat showed a very long period of immobility lasting 240 s. Two rats showed no change in their behavior following the stimulation. The animal which received an injection of kainic acid outside the NRM also showed intermittent periods of immobility lasting about 3–6 s.

All seven rats which received a kainic acid injection into the NRM showed a significantly enhanced expression of c-FOS bilaterally throughout the rostrocaudal extent of the spinal cord. The most prominent increase was observed in the inner layer of lamina II at the border with lamina III of the cervical cord

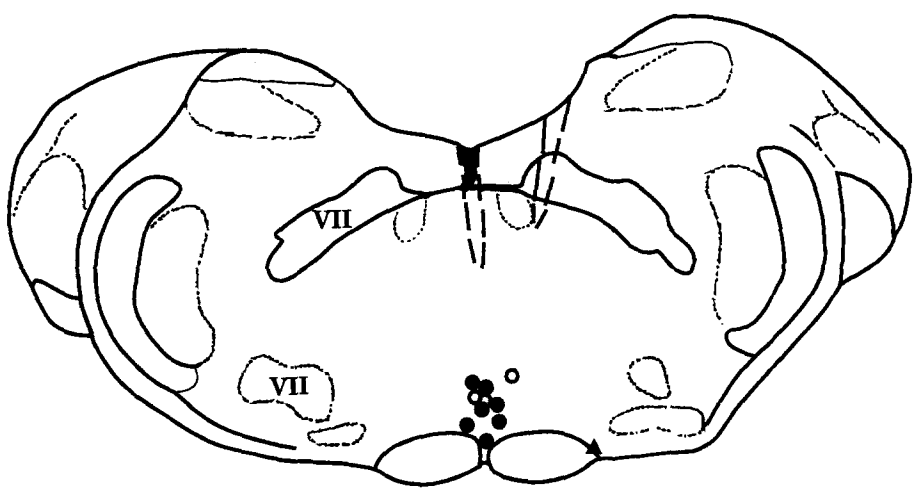

Fig. 1. Histologically verified injection sites superimposed on a representative frontal section through the medulla at the level of the facial nuclei (VII) at AP −10.3 to −11.3 according to the atlas of Paxinos and Watson.[40] Filled circles indicate sites of kainic acid microinjection, open circles represent sites of saline injection. One injection of kainic acid (triangle) was located outside the boundaries of the NRM. The tracks of the two saline-injection cannula are demonstrated by the dashed lines.

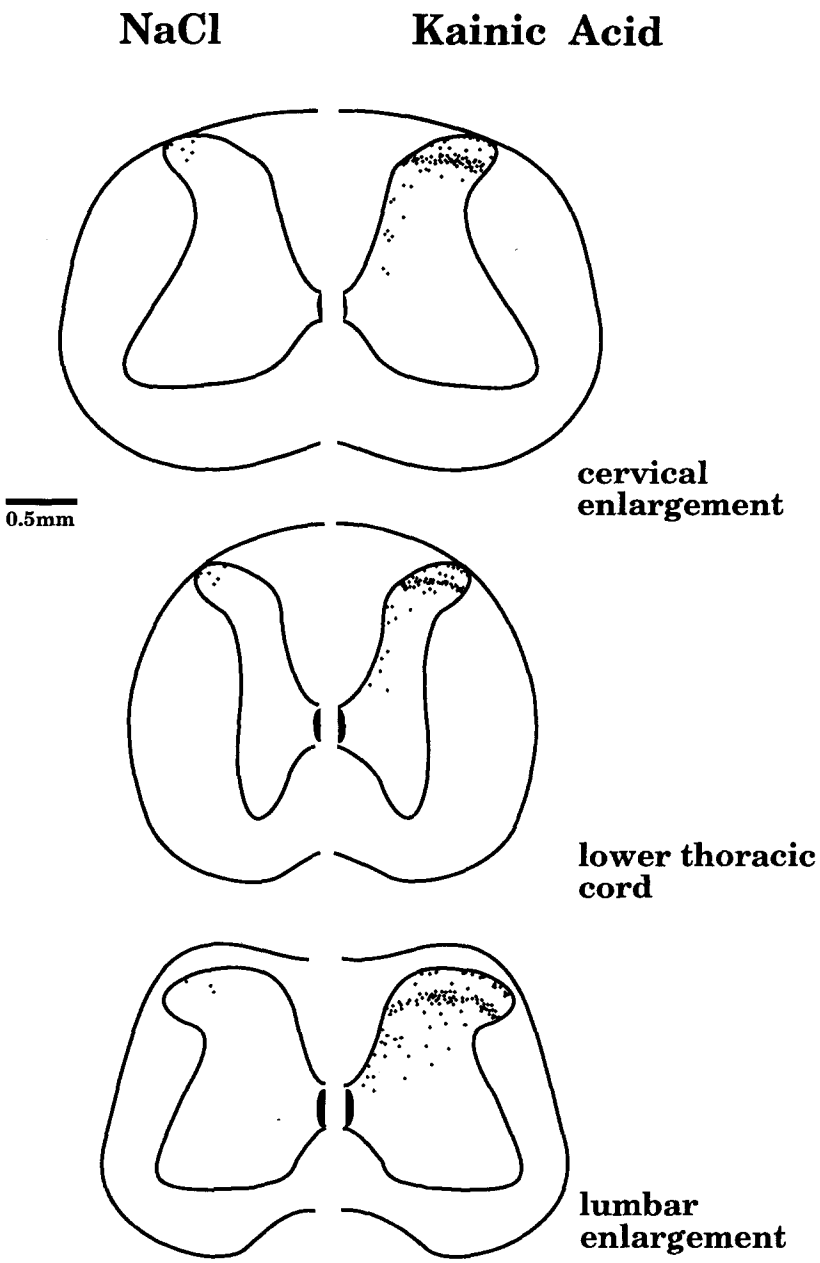

Fig. 2. The locations of c-FOS positive cells in the cervical, thoracic and lumbar spinal cord of a characteristic sham-treated animal (NaCl, left-hand column) and a typical kainic acid stimulated rat (kainic acid, right-hand column) are indicated on representative sections. The detected immunoreactivity was similar on both sides of the cord. Each dot represents one c-FOS positive cell. Results are pooled from five 50 μm thick sections through each of the spinal segments.

(control animals: 7 ± 3 S.E.M. c-FOS positive cells in five 50 μm sections versus stimulated animals: 101 ± 9 S.E.M., $P = 0.0025$), the thoracic cord (control animals: 6 ± 4 S.E.M. versus stimulated animals: 73 ± 6 S.E.M., $P = 0.0061$) and the lumbar spinal cord (control animals: 2.0 ± 1 S.E.M. versus stimulated animals: 124 ± 15 S.E.M., $P = 0.0061$). Significant increases in the number of c-FOS positive cells were also found in laminae I, II outer (IIo), III and IV–VI (see Table 1).

The predominance of c-FOS like immunoreactivity in laminae II inner (IIi) and the smaller but significant increase in laminae I, IIo, III and IV–VI was verified for all segmental levels investigated. Thus, the pattern of c-FOS like immunoreactivity was similar in the cervical, thoracic and lumbar spinal cord with the exception of the lumbar spinal cord which in addition showed also a relatively strong increase of c-FOS positive cells in laminae IV–VI (70 ± 17) as compared with the cervical cord (17 ± 5) or the thoracic cord (16 ± 4). In the rat which received a kainic acid injection outside the NRM close to the pyramid, the c-FOS expression was not elevated above levels seen in the sham-treated animals. In the spinal cord of this

animal only very few cells were found in laminae I and II of the cervical and thoracic spinal cord. Within the lumbar spinal cord more c-FOS positive cells were detected compared with the latter segments which were scattered throughout lamina I–VII (results are not shown).

DISCUSSION

The present study has identified the pattern of c-FOS expression in the spinal cord following selective activation of neurons originating from the medullary NRM in the unanesthetized, drug-free rat.

Use of kainic acid for chemical stimulation

Kainic acid was used as a general excitant of neurons of origin but not fibers of passage with an intermediate duration of action.[4,13] Diffusion characteristics of kainic acid after intracerebral injections have been analysed in detail. Butcher and Rogers[12] who evaluated the effect of kainic acid after microinjection in the brainstem on the base of histopathological parameters found that after application of 2 μg of kainic acid given in a volume of 1 μl an effect can be seen up to 1.2 mm from the injection site. In the present study a 10 times smaller volume and a more than 200 times smaller amount of kainic acid was used suggesting a more circumscribed effect of kainic acid. Furthermore, after an injection of 40 pmol of kainic acid in a volume of 200 nl, kainic acid was found at the tip of the injection cannula diffusing into the surrounding tissue <400 μm from the injection site.[13] Thus, in our experiments with an even smaller volume of 100 nl a local effect of kainic acid on NRM neurons and neurons in close vicinity of the NRM, i.e. in the ventromedial medulla is most likely. The possibility of diffusion of kainic acid into remote structures of the brain can be neglected. This conclusion is consistent with our finding that a kainic acid injection 0.5 mm lateral to the NRM failed to induce a comparable expression of c-FOS in the spinal cord.

Table 1. Number of c-FOS positive cells

		Sham, $n = 5$	Kainic acid, $n = 7$
Cervical laminae	I	8 ± 2.3	35 ± 1.2
	IIo	2 ± 0.5	12 ± 2.9
	IIi	7 ± 2.8	101 ± 8.5
	III	1 ± 1	10 ± 2.8
	IV–VI	0 ± 0	17 ± 5.0
Thoracic laminae	I	4 ± 1.2	24 ± 8.3
	IIo	2 ± 0.3	8 ± 2.6
	IIi	6 ± 3.6	73 ± 5.7
	III	1 ± 0.5	10 ± 2.7
	IV–VI	0 ± 0	16 ± 4.5
Lumbar laminae	I	1 ± 0.5	34 ± 10.2
	IIo	1 ± 1	12 ± 3.3
	IIi	2 ± 1.3	124 ± 15.3
	III	0 ± 0	15 ± 3.4
	IV–VI	0 ± 0	61 ± 17.0

Number of c-FOS positive cells ± S.E.M. as found in the dorsal horn after kainic acid and after saline microinjections into the nucleus raphe magnus. The total number of c-FOS positive cells in five 50 μm thick sections through the cervical (at the top), thoracic (at the middle) and lumbar (at the bottom) spinal cord were counted separately for laminae I, IIo, IIi, III and IV–VI in each animal. Significant differences between sham-treated and kainic acid stimulated animals were verified by Mann–Whitney test, for all laminae in all segments.

*The proto-oncogene c-*fos *as a marker for cellular activation*

To identify the number and the location of neurons in the spinal cord which can be activated by stimulation in the NRM, we used the expression of the proto-oncogene c-*fos* as a cellular marker for activated neurons in the CNS.[41,46] The neuronal expression of c-FOS protein can be effectively induced by strong mono- and polysynaptic activation[45] probably via modification of the open time of voltage-dependent calcium channels.[41] Inhibitory agents or other conditions have not been reported to induce c-*fos*. Glial cells as well as neurons may show c-FOS immunoreactivity, in response to heat-shock[15] or after cortical trauma[17] but not following depolarization.[24]

In the spinal cord c-FOS immunoreactivity can be induced in all laminae of the dorsal horn if tonic noxious stimulation is used in awake animals. Following the induction of subcutaneous or periarticular inflammation in awake rats c-FOS immunoreactivity can be observed throughout laminae I–X of the spinal cord.[38] A study in which mustard oil was applicated subcutaneously and intramuscularly in rats c-FOS immunoreactivity was detected within spinal laminae I, II IV–VII and X.[26] Since primary afferent nociceptors terminate predominantly in the superficial dorsal horn, c-FOS expression in deep laminae could be induced by an extrasynaptic spread of neuropeptides.[50] However, Beck and Sandkühler[6] have shown that substance P superfusion of the cord dorsum induce c-FOS in the superficial dorsal horn only. Thus, polysynaptic pathways involving segmental interneurons or a supraspinal loop including raphe–spinal neurons[9,23] may cause c-FOS expression in the deep dorsal horn following noxious stimulation.[56] These indirect pathways are probably less potent than the monosynaptic induction, as lamina III neurons were not as readily induced by primary afferent stimulation as lamina I or II neurons.[30,53]

*False negative results obtained by the c-*fos *technique*

Some neurons show no c-FOS expression although excitatory anatomical afferents exist. In the present study the absence of c-FOS immunoreactivity in the intermediolateral cell column represents an example for possibly false negative results obtained by the use of c-*fos*. Stimulation in the ventromedial medulla may excite descending serotonergic pathways to the intermediolateral cell column[39] and may change arterial blood pressure in both directions.[1,55] This suggests

that in the present study preganglionic sympathetic neurons in the intermediolateral cell column could have been activated. However, no c-FOS was detected by immunocytochemistry. Thus, false negative results can not be excluded when c-FOS immunoreactivity is used as a marker for activated neurons.

Furthermore, in the spinal cord c-FOS expression may produce false negative results if used as a marker for activated neurons, e.g. if non-noxious stimuli are applied, exciting low-threshold mechanoreceptors.[26] However, Jasmin *et al.*[27] have demonstrated that walking can induce c-FOS expression in the spinal cord. Thus, the presently identified pattern of a c-FOS immunoreactivity may not represent all spinal neurons which were activated by descending projections originating from the NRM.

The false negative results could indicate that some neurons never express c-FOS no matter which stimulus is applied[16] or that the stimulus occurred during a "refractory period" for the induction of c-FOS.[40] False negative results may also be due to the fact that the amount of c-FOS synthesized by some neurons is too low to be detected by immunocytochemistry. Another cause is the use of anesthetics in some studies which can powerfully depress the expression of c-FOS.[38,40] For example in pentobarbital anesthetized rats electrical stimulation in the NRM for 20 s depressed c-FOS expression in laminae I and II following noxious skin stimulation and failed to induce any c-FOS expression in the lumbar spinal cord in the absence of heat stimulation.[29] These findings are contrary to the results of the present study in awake animals which show increased c-FOS immunoreactivity in laminae I and II following chemical stimulation of neurons originating in the NRM. The failure to detect c-FOS expression may also be due to the relatively short stimulation duration as it is known that brief stimuli are less effective to induce c-FOS as compared with longer lasting stimuli. Further descending excitation from the NRM strongly depends on the location of the stimulation electrode and the stimulation parameters,[37] thus, an insufficient descending excitation may have been present in the study of Jones and Light[29] to induce c-FOS in the spinal cord.

Effects of animal behavior on the expression of c-FOS

It seems unlikely that the c-FOS expression observed in the present study was related to changes in the behavior of the animals following kainic acid stimulation as the two kainic acid stimulated rats which showed no behavioral changes after microinjection presented the same spinal pattern of c-FOS immunoreactivity as compared with the five rats which showed significant changes in their behavior. Finally, in the animal which received a microinjection of kainic acid outside the NRM c-FOS expression was not enhanced in the spinal cord despite the fact that similar changes in the behavior were induced. The results of the sham-treated group show that the microinjection and the handling itself did not induce c-FOS immunoreactivity.

Absence of c-FOS immunoreactivity in the ventral horn

We did not observe c-FOS immunoreactivity in the ventral horn although anatomical studies have shown that spinally projecting neurons in the NRM not only project to laminae I, II and IV–VI but also to motoneurons.[34] The latter projections would not be expected to directly induce c-FOS expression as electrophysiological data have demonstrated that the projections to motoneurons are mainly inhibitory either pre-[43] or postsynaptic.[7,22,51] This is consistent with the hypothesis that hyperpolarization does not induce c-*fos*. In all but two animals which were chemically stimulated, we observed intermittent short periods of total inhibition of spontaneous motor behavior. This could be due to a direct monosynaptic inhibition of motoneurons.[2]

Pattern of c-FOS expression in the dorsal horn

Here the strongest c-FOS expression was observed within lamina IIi and clearly less in lamina IIo. This supports the notion that significant functional differences exist between both layers of lamina II.[31] It is known that nociceptive and wide dynamic range neurons are located in laminae I and IIo, while neurons in laminae IIi and III may integrate information from low-threshold mechanoreceptors of skin and muscle. Neurons within lamina II project mainly to laminae II–IV and to a lesser extent to lamina V.[36]

Nucleus raphe magnus and descending inhibition

It is not clear whether NRM-induced descending inhibition of nociceptive spinal dorsal horn neurons is monosynaptic or is mediated via spinal interneurons. Inhibitory interneurons are thought to be located in lamina II.[31] Our results which show a strong c-FOS expression mainly in lamina IIi near its border with lamina III but also in laminae I, IIo, III and IV–VI are consistent with the hypothesis that at least part of the descending inhibition could be mediated by the excitation of spinal inhibitory interneurons. Evidence has been provided that descending inhibition of nociceptive spinal dorsal horn neurons induced from the midbrain periaqueductal gray which is believed to be mediated in part through raphe-spinal projections,[19,49] is not only conducted through long descending fibers in the lateral funiculus but also by propriospinal interneurons in the lower thoracic cord.[48] Some of the presently identified c-FOS positive neurons in the dorsal horn of the thoracic cord could represent these relay neurons.

Nucleus raphe magnus and descending excitation

The predominance of activated neurons in laminae I and II in the present study is in good agreement with electrophysiological reports showing that electrical stimulation in the dorsolateral funiculus can excite neurons in these laminae.[18] In cats it could be

demonstrated that electrical stimulation in the NRM can evoke excitation of spinal neurons in the dorsal horn depending on the location of the electrode and the intensity and the frequency of the stimulation.[37] In this study it was also shown that bilateral lesions in the dorsolateral funiculus can eliminate the descending excitation of spinal dorsal horn neurons, suggesting that this funiculus besides mediating descending inhibition is also an important pathway for descending excitation from the NRM. Furthermore, it has been shown that at least 50% of the neurons with low-threshold input in laminae IIi and III were excited after electrical stimulation in the NRM.[32] Zhou and Gebhart[57] reported that descending facilitation of spinal neurons after electrical brainstem stimulation in nucleus reticularis gigantocellularis and gigantocellularis pars alpha may be mediated by spinal 5-hydroxytryptamine receptors. These serotonin receptor subtypes are found at highest densities in laminae I and II and to a lesser degree in laminae III–V of the dorsal horn.[33] This distribution overlaps with the pattern of c-FOS expression observed in the present experiments.

CONCLUSIONS

In summary, the present results provide evidence for a descending excitation of neurons in laminae I–VI of the dorsal horn induced by chemical stimulation of neurons originating from the NRM. The strong increase of c-FOS immunoreactivity in lamina IIi at the border with lamina III indicates a prominent function of these interneurons for descending spinal effects from the NRM.

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