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Research report

Induction of *c-fos* expression in cervical spinal interneurons after kainate stimulation of the motor cortex in the rat

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Abstract

The expression of the immediate–early gene *c-fos* was used as a marker of neuronal activity to investigate the cervical spinal interneuron populations involved in the corticomotoneuronal pathway. Adult rats received unilateral kainate injections in the forelimb area of the primary motor cortex. After a survival period of 90 min, during which the animals showed vehement twitching of the contralateral forelimb, the rats were perfused and their brains and cervical spinal cords processed for Fos-like immunoreactivity. In the cervical spinal cord Fos-like immunoreactive neurons were found bilaterally in the dorsal horn and in the intermediate zone, though contralaterally significantly more labelled nuclei were encountered in two different areas. One area closely resembles the corticospinal terminal field as demonstrated with anterograde horseradish-peroxidase tract-tracing and the other reflecting primary afferent and noxious sensory neurons in the dorsal horn. Thus by monitoring the evoked expression of the immediate–early gene *c-fos*, structural components of the rat motor system can be identified.

Keywords: *c-fos*; Cervical interneuron; Corticospinal tract; Sensorimotor cortex; Kainate; Rat

1. Introduction

The corticospinal tract (CST) is a major fibre bundle originating in the cerebral cortex layer V pyramidal neurons, and projecting to the spinal cord [3,37]. It is implicated in the control of voluntary movements, and in particular those of the fingers [4,7–9,24,30,32,34,41,42]. Animals with high digital skills (such as monkeys but also rodents such as the rat and the hamster) are characterized by a direct CST innervation of the motoneurons (MNs) in the lateral motor column of the cervical spinal cord [3,12,15,19,25,26,28,32]. In previous investigations the maturation of MN dendritic fields in cervical spinal cord segments 7 and 8 (C_{7–8}) [11] and the outgrowth of the CST axons into the spinal gray of these segments [12] has been studied. From these studies it was concluded that in

the adult rat the MN dendrites, especially those of the MNs innervating the long flexors of the distal forearm, and the CST terminal field show a large area of overlap. In this area direct cortico-motoneuronal synaptic contacts [15,28] may be found (see *Note added in proof*; Section 5). However, the intermediate target of the CST, the interneuron population through which much of the CST influence upon MNs takes place remains to be established.

After a stimulus to a neuron immediate–early genes such as *c-fos* are transneuronally expressed in the nucleus, where these genes are then translated into proteins [5,14,29,35], which can be visualized by immunohistochemistry. The *c-fos* technique thus provides a powerful tool for the visualization of functionally related chains of neurons such as the corticospinal system. In the present paper the motor cortex was stimulated with kainate, a potent glutamate agonist. Glutamate receptors are abundant in layer V pyramidal neurons of the rat motor cortex and the CST is most likely glutamatergic [17,43]. Fos-like immunoreactivity (Fos-Li) then was analysed in the cervical spinal cord. Parts of these results have been reported previously in abstract form [13].

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2. Materials and methods

2.1. Animals

In the present study ten young adult (approximately 60 days old and 200 g) Wistar rats (Central Animal Laboratory, University of Nijmegen) were used, subdivided into three groups. One group of four animals was used to study the CST projection area in the cervical spinal gray with horseradish-peroxidase (HRP) anterograde tract tracing (the same material as in Curfs et al. [12]). The other six animals were used to study the interneuron population in the cervical spinal cord: three rats received kainate injections in the cerebral cortex and the other three rats were used as their sham-operated controls.

2.2. HRP labelling of the CST

The method for CST labelling with HRP is described in detail elsewhere [12]. In brief, after anaesthesia with sodium pentobarbital (60 mg per kg body weight, i.p.), HRP (Boehringer Mannheim, grade 1)-gels [18] were implanted into the cerebral cortex encompassing the entire sensorimotor cortex. After a postimplantation survival time of 48 h the rats were reanaesthetized (90 mg sodium pentobarbital per kg body weight) and transcardially perfused. After dissection from the skull and spine respectively, the brain and spinal cord were postfixed, cryoprotected and embedded in gelatin. Using a freezing microtome 30 μm sections were cut in the transverse plane. The sections were reacted for HRP-histochemistry using the 3-step procedure: tetramethylbenzidine–ammoniumheptamolybdate incubation, next diaminobenzidine–nickel (DAB–Ni) stabilization, followed by DAB–cobalt–glucose oxidase intensification, as described previously. The sections were mounted onto glass slides using a gelatin–alcohol solution, counterstained with neutral red, dehydrated and coverslipped with Depex. The cervical spinal cord segments 3 to 8 (C_3 – C_8) were examined under dark field illumination. Photomicrographs were made using an automatic Zeiss photomicroscope II.

2.3. Fos labelling of the cervical interneuron population

In the *c-fos* experiments the anaesthesia was initiated by placing the rats in a glass box containing tissue paper saturated with ether. During the operation the anaesthesia was maintained with a tube filled with a gauze saturated with ether. The animals were then transferred to a stereotaxic apparatus and the skin overlying the skull was incised. In one group of three rats small holes were drilled into the skull and three separate injections of kainate (100 ng in 0.5 μl per injection) were made into the forelimb area motor cortex (stereotaxic coordinates: 3.5 mm anterior of bregma and 1 mm lateral of the midline, 2 mm anterior/3 mm lateral, and 0.5 mm anterior/1 mm lateral

[31]). The wound was then sutured and the animals were allowed to recover. The other group of three rats received a sham-operation consisting of only an incision and subsequent suturing of the skin. After a 90-min survival period (which after preliminary experiments appeared to be the optimum) during which the animals showed vehement twitching of the contralateral forepaw, the rats were transcardially perfused under deep ether anaesthesia with ice-cold 0.1 M phosphate buffered saline (PBS, pH 7.5) followed by Samboni's fixative (1.8% paraformaldehyde and 7.5% pikric acid in PBS, pH 7.5). After dissection from the skull and spine, the brain and spinal cord respectively were postfixed by immersion in the above mentioned fixative for 24 h and stored in PBS. As soon as possible 50 μm vibratome sections of the brain and cervical spinal cord were cut and collected in a one in two series and processed for Fos-Li. All incubations mentioned were performed at room temperature. Sections were first pretreated against endogenous peroxidase with 0.3% H_2O_2 in aqua dest, and after rinses in 0.05 M Tris buffered saline (TBS, pH 7.6), pre-incubated in 5% normal horse serum, 0.1% Triton and 0.1% BSA in TBS (TBS–BT–NHS) for 1 h. Then the sections were incubated overnight in sheep IgGs against Fos (dilution 1:2000 for brain and 1:4000 for spinal cord sections; Cambridge Research Biochemicals, batch: OA 11-824) in TBS–BT–NHS. After rinses in TBS the sections were incubated for 90 min in horse anti sheep antibodies (1:100; Nordic Immunology, Tilburg) in TBS–BT, again rinsed in TBS and incubated for 90 min in sheep peroxidase–anti-peroxidase complex (1:600; Nordic Immunology, Tilburg) in TBS. The sections were rinsed again in TBS and the presence of Fos-Li was visualized using a nickel-intensified DAB procedure (20 mg DAB, 300 mg ammonium nickel sulphate and 10 μl 30% H_2O_2 in 100 ml 0.05 M Tris buffer, pH 7.6) for 3 min. The sections were then mounted onto glass slides using a gelatin chrome–alum solution, air-dried and embedded in Depex. The labelled cell nuclei in one out of four randomly selected sections were drawn under bright field illumination using a Zeiss microscope equipped with a drawing tube. The spinal cord was subdivided into three parts: cervical spinal cord segment 3 and 4 (C_{3-4}), C_{5-6} , and C_{7-8} . The drawings of two consecutive spinal cord segments were pooled and plotted onto a representative section. The number of labelled nuclei in these pooled sections were counted, and subdivisions were made for the dorsal horn, the intermediate zone, and the ventral horn. Differences were tested for statistical significance by means of an ANOVA.

3. Results

3.1. CST projection area after labelling with HRP

The CST projection area as demonstrated by HRP-gel implantation in the cerebral cortex after 48 h is shown in

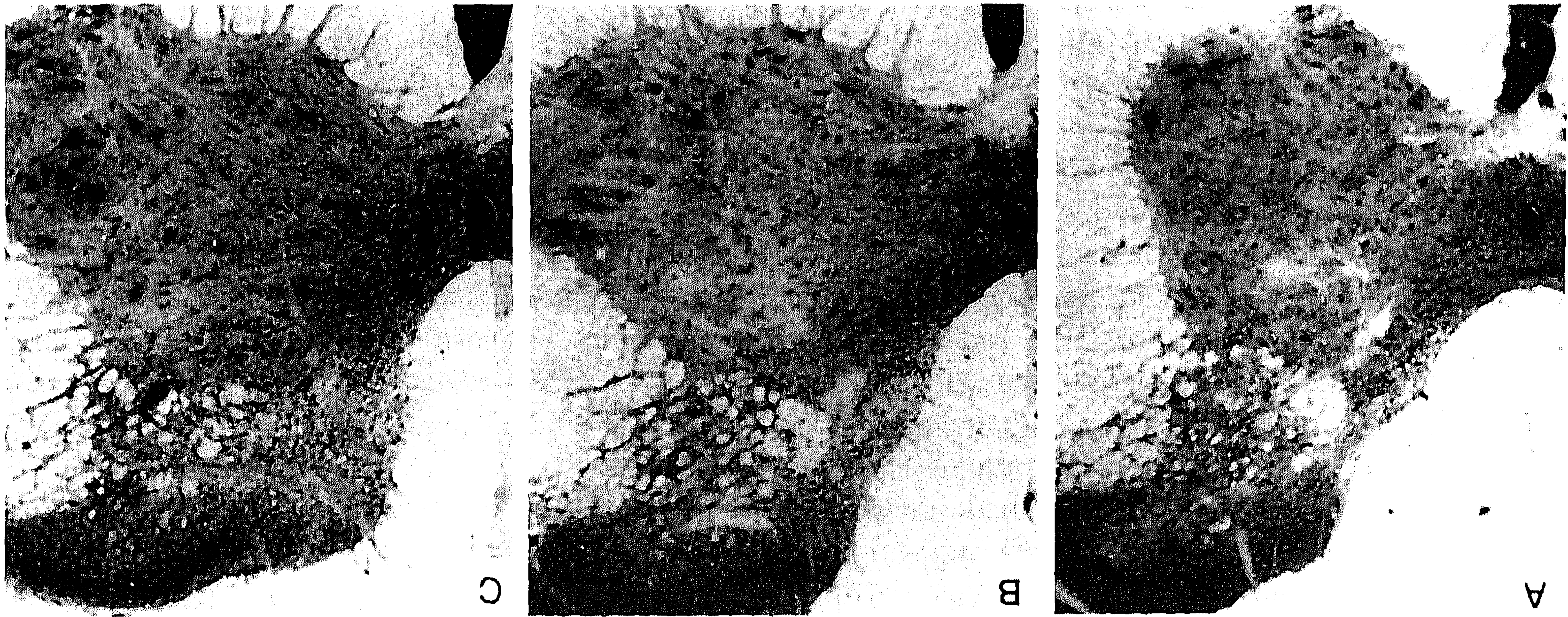


Fig. 1. Photomicrographs of the contralateral corticospinal tract and spinal gray matter in cervical spinal cord segment 3 (A), 5 (B), and 7 (C) 48 h after HRP-gel implantation into the cerebral motor cortex under dark field illumination. Labelled corticospinal fibres appear light and the background dark. Scale bar = 200 μm .

From the photographs it can be deduced that labelled CST axons cover large parts of the contralateral spinal gray matter, in particular the medial portion of the dorsal horn, the entire intermediate zone and the ventral

horn with exception of the lateral part of the lateral motor column. The number of fibres was highest in the direct vicinity of the CST and decreased progressively further distally. It can also be noted that the CST projection area

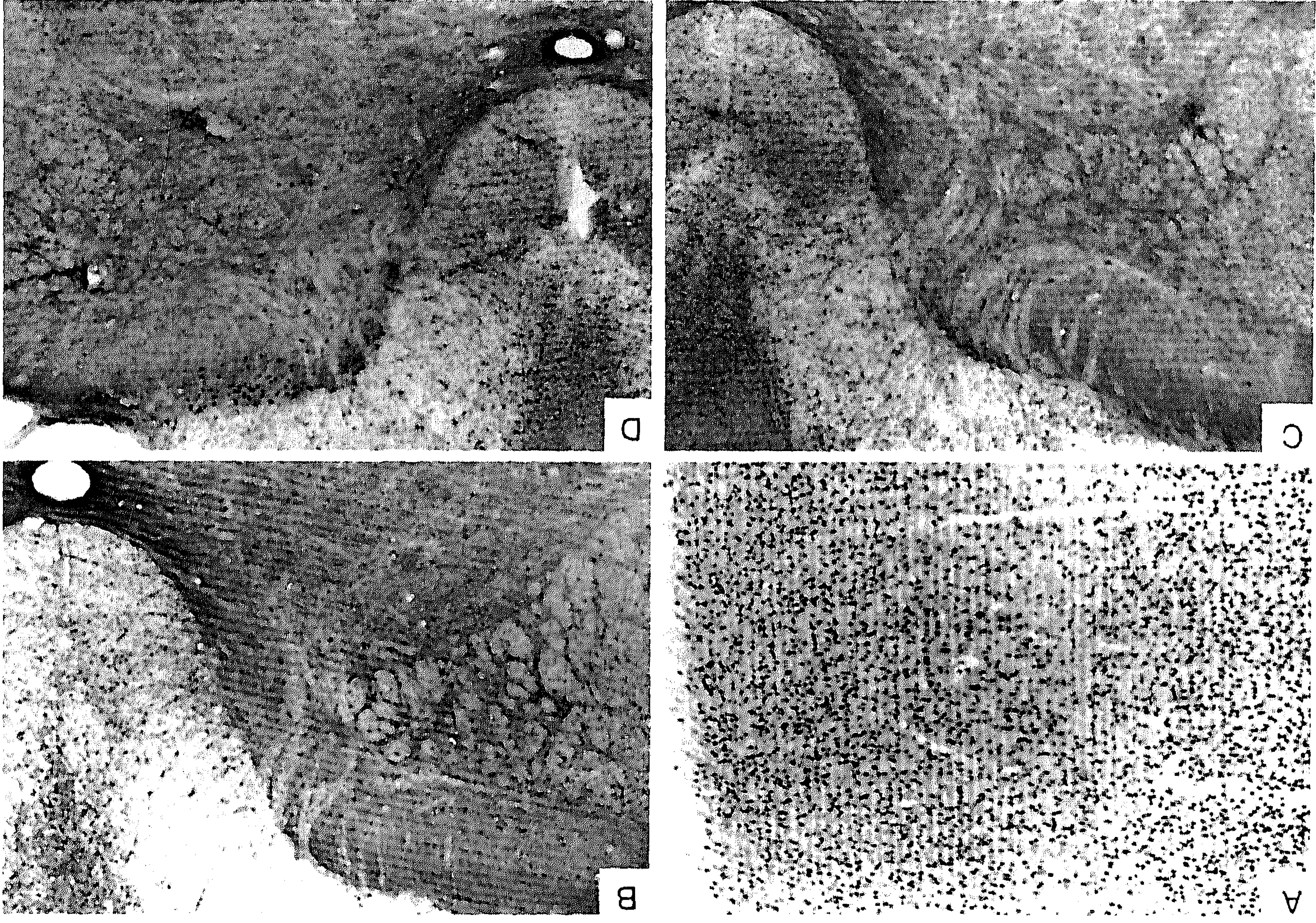


Fig. 2. (A) Photomicrograph of the Fos-Li in the ipsilateral cerebral cortex 90 min after kainate injections into the cerebral motor cortex. Pia mater is to the right. Note that *c-fos* is expressed in nearly all neurons. (B) Photomicrograph of the Fos-Li in the dorsal horn and intermediate zone in cervical spinal cord segments 5 to 6 (C_{5-6}) of a sham-operated rat. Virtually no neurons are labelled. (C) Photomicrograph of the Fos-Li in the ipsilateral dorsal horn and intermediate zone in C_{5-6} 90 min after kainate injections into the cerebral motor cortex. Several Fos-positive nuclei can be found in especially the dorsal horn. (D) Photomicrograph of the Fos-Li in the contralateral dorsal horn and intermediate zone in C_{5-6} 90 min after kainate injections into the cerebral motor cortex. A substantial increase in the number of Fos-positive neurons as opposed to both sham-operated animals (B) and the ipsilateral half of the spinal cord of kainate-injected rats (C) is found. Scale bar = 200 μm .

increased progressively from C₃₋₄, and C₅₋₆ to a maximum in C₇₋₈ with regard to both the number of labelled fibres and the size of the projection area.

3.2. Fos induction after kainate injections

Approximately 20 min after the injections, the cortex-stimulated animals started to display typical kainate-induced behaviour, consisting of nearly constant locomotion, and misplacement and twitch-like movements of the contralateral forelimb, which was not observed in their sham-operated counterparts. Examination of sections taken from their brains after a 90-min survival period revealed an increased Fos-Li in the ipsilateral cerebral cortex, including the forelimb representation area (Fig. 2A). Increased Fos-Li as opposed to sham-operated animals was also observed in the ipsilateral caudate nucleus, putamen, and red nucleus, and bilaterally in the thalamus (reticular,

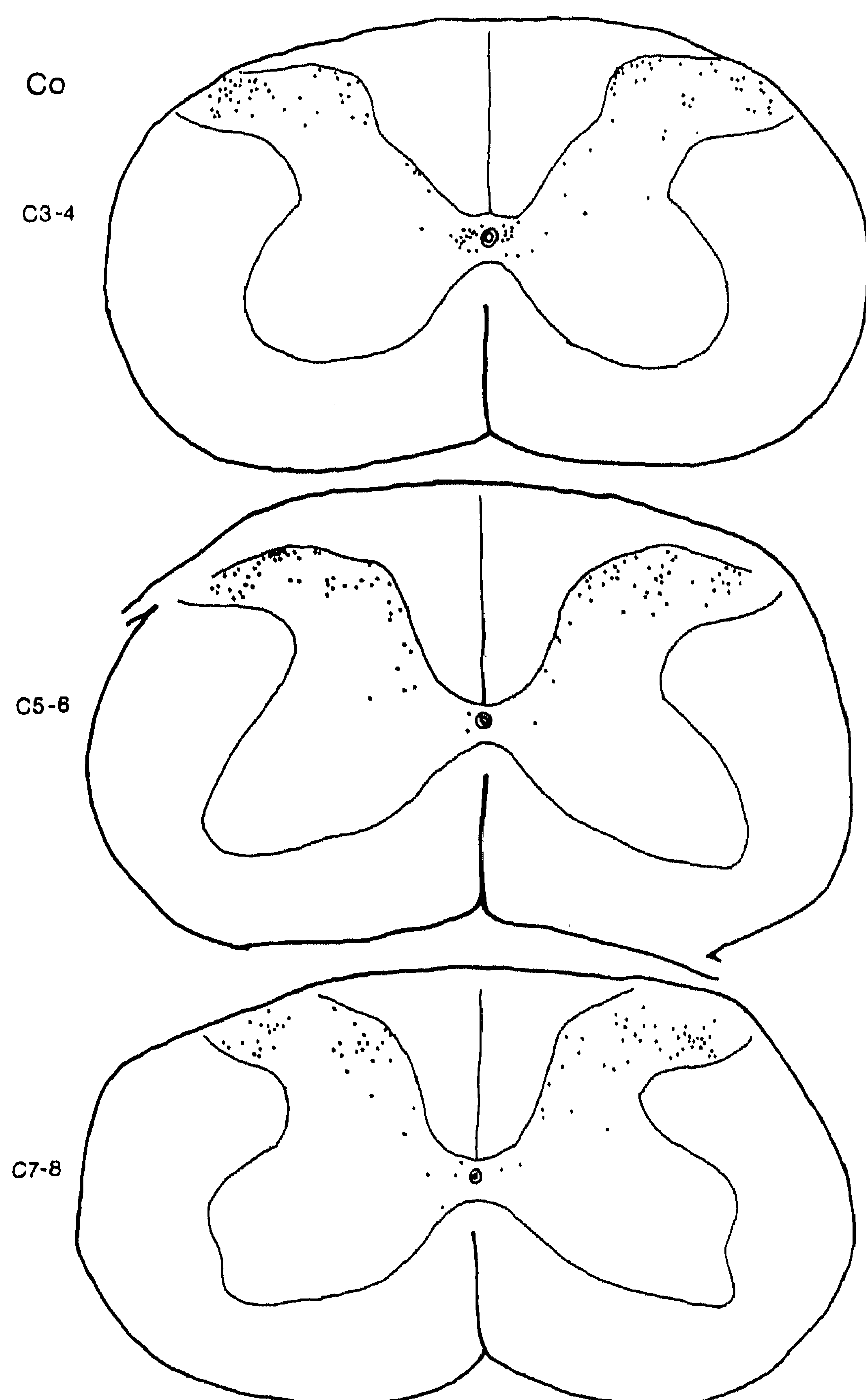


Fig. 3. Composite illustration of schematized drawings of the Fos-Li in the combined cervical spinal cord segments 3 and 4 (C₃₋₄), C₅₋₆, and C₇₋₈ of a sham-operated rat. One in four sections were pooled and plotted onto its respective representative drawing. A few Fos-positive neurons are found scattered throughout especially the dorsal horn, and no difference is noted between the left and right half of the spinal cord.

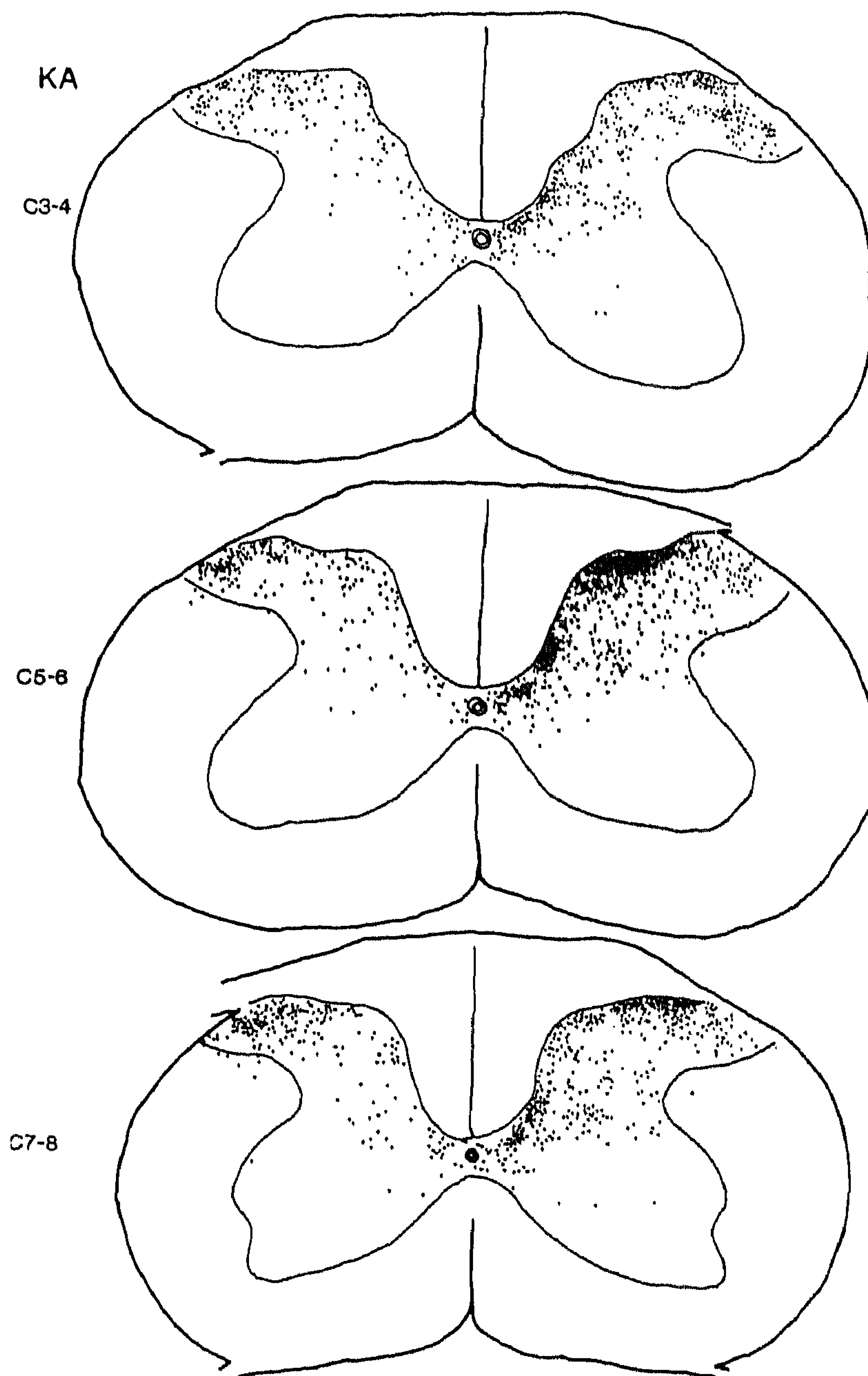


Fig. 4. Composite illustration of schematized drawings of the Fos-Li in the combined cervical spinal cord segments 3 and 4 (C₃₋₄), C₅₋₆, and C₇₋₈ 90 min after kainate injections into the cerebral motor cortex. One in four sections were pooled and plotted onto its respective representative drawing. A large increase is noted in the number of Fos-positive neurons both on the ipsilateral (left) and contralateral (right) half as opposed to sham-operated animals. It should further be noted that substantially more Fos-positive neurons are found contralaterally than ipsilaterally condensed in two regions: in the vicinity of the corticospinal tract and in the dorsal horn, especially in the medial part of the superficial layers.

ventromedial and posterior nucleus), globus pallidus, subthalamic nuclei, tectum, and brainstem nuclei such as substantia nigra, pontine and raphe nuclei. In the cervical spinal cord an increased number of Fos-Li cells was noted bilaterally after kainate injection in all segments examined (Fig. 2B–D, Figs. 3 and 4). The majority of these neurons (62%) was found in the dorsal horn, the minority was located in the intermediate zone (15%) and in the ventral horn (23%). This distribution was the same in all cervical spinal cord segments studied, although the number of Fos-Li nuclei varied considerably rostrocaudally (Fig. 5). In addition to the distinct regional distribution of Fos-Li neurons, significantly more Fos-positive nuclei were found in the contralateral half of the spinal cord as was noted in every spinal cord segment and in every region analysed.

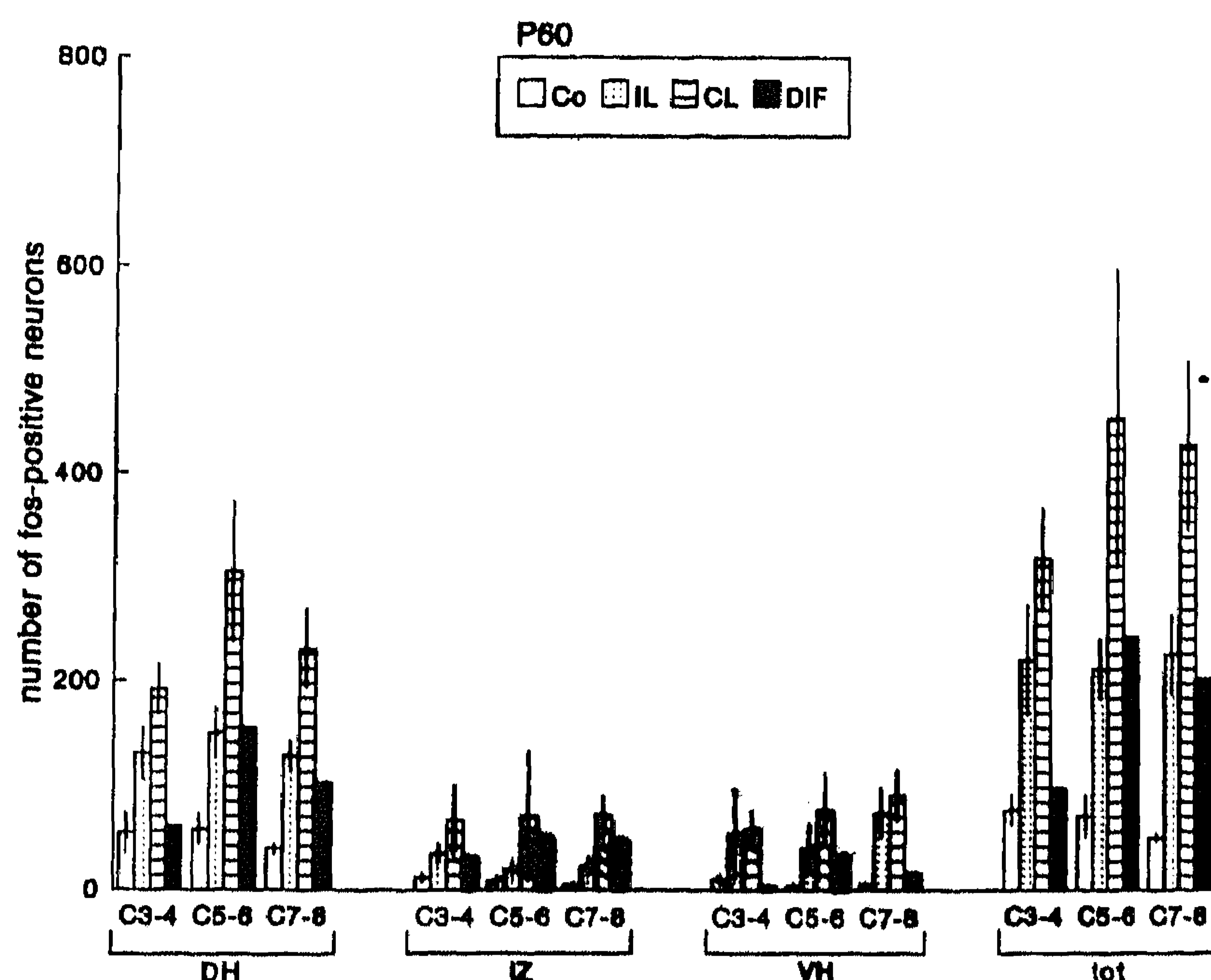


Fig. 5. Histogram showing the mean numbers and standard deviation of Fos-Li neurons in sham-operated (Co) and motor cortex kainate injected adult (P60) rats in the combined cervical spinal cord segments 3 and 4 (C_{3-4}), C_{5-6} , and C_{7-8} . For kainate-injected animals a further differentiation is made for the ipsilateral (IL) and contralateral (CL) side and the difference between these two sides is represented by the filled bars (DIF). The spinal cord is subdivided in dorsal horn (DH), intermediate zone (IZ), and ventral horn (VH), total numbers (tot) are also shown. From this figure it can be concluded that kainate injections result in more neurons being labelled for *c-fos*, and that the increase is largest in the dorsal horn, in the combined C_{5-6} , and on the contralateral side.

The difference was however most prominent in the dorsal horn and the largest in the combined C_{5-6} segment and, slightly smaller, in C_{7-8} (Figs. 4 and 5). Fos-Li nuclei on the contralateral side are mainly found in two areas, one located in the vicinity of the dorsal funiculus and one located in especially the medial portions of the superficial layers of the dorsal horn (Fig. 4).

4. Discussion

In the present study it is clearly shown that stimulating the rat motor cortex with the powerful glutamate agonist kainate results in an increased number of Fos-Li neurons in the cervical spinal cord as opposed to that found in sham-operated rats. This finding is contrary to previous findings in literature [40]. Although these authors noted similar labelling in forebrain and brain stem structures, they found no Fos labelling in the spinal cord. Probably, the kainate stimulation as used in the present investigation is much more potent than the intracortical microstimulation of the motor cortex as applied in their experiments. That the Fos labelling is principally found in the dorsal horn is in agreement with the data in literature. After an inflammatory stimulus to the spinal cord, neurons expressing immediate-early genes belonging to the *fos*-family are mainly located in the dorsal horn and intermediate zone, whereas *jun* (another family of immediate-early genes) -positive neurons are mainly found in the ventral horn plus

the superficial layer of the dorsal horn [27]. Since the main component of the CST projects through the dorsal funiculus to the contralateral spinal cord gray matter [3], it was to be expected that most Fos-Li neurons were present in the contralateral gray matter as was exactly found in the present study.

On closer investigation of the numbers of Fos-Li neurons and their respective location, it became apparent that contralaterally two distinct populations could be discerned. One population was located adjacent to the dorsal funiculus in the dorsal horn and in the intermediate zone. This area shows great resemblance to the corticospinal projection area as was demonstrated by anterograde HRP tract-tracing. We have shown that the CST projection area increases progressively in the caudal direction and reaches its maximum in C_{7-8} with regard to both the number and extension of labelled CST fibres. This distribution highly corresponds to the number and location of the Fos-Li neurons. The other Fos-Li neuron population is principally located in the dorsal horn, i.e. the area that receives primary afferent input [6,20,36]. Obviously, the kainate stimulation of the CST activates the premotor interneurons and in turn the motoneurons, causing locomotion and twitch-like movements of the forelimb and thereby results in the activation of primary afferents. The evoked *c-fos* expression pattern found closely resembles that of walking rats as was recently described [22]. In addition, a second component can be discerned in the Fos-Li neuron population encountered in the dorsal horn, namely those located in the superficial layers of the dorsal horn, especially their medial parts. These neurons represent a subpopulation which is activated by noxious stimulation [1,22,33,38,39]. Apparently, the kainate stimulation of the sensorimotor cortex also raises sensory sensation, either directly or indirectly.

The increased number of Fos-IR neurons in the ipsilateral half of the spinal cord can be attributed to several factors. Firstly, this merely can be a side-effect from the increased locomotion in the contralateral forelimb which can be expressed in bilateral activity such as walking. Ipsilateral interneurons are then stimulated by primary afferent axons from the ipsilateral forelimb. Secondly, *c-fos* expression in spinal interneurons can be induced by bilateral descending tracts other than the CST (such as the rubrospinal, vestibulospinal, and reticulospinal tracts) originating in areas which are stimulated bilaterally by the cerebral cortex (eg. the thalamus and brainstem nuclei). And thirdly, CST fibres in the ipsilateral spinal cord might add to the increased number of Fos-Li neurons. These might be the contralateral CST fibres which return to the ipsilateral side in the spinal cord or the minor uncrossed CST component located in the ventral funiculus [23]. Evidence for the latter hypothesis was found in one animal in which significant increased numbers of Fos-Li neurons were found located near the ipsilateral ventral funiculus.

In conclusion, in the present study we have clearly

shown, that kainate injections into the cerebral motor cortex result in many spinal interneurons being labelled for the immediate-early gene *c-fos*. At least part of these neurons are under the direct influence of the CST while others could be activated by other descending tracts or primary afferents. It is also conceivable that neurons, and especially interneurons, are multiply innervated by several systems. On the other hand, only a certain subpopulation of interneurons can be labelled with the *c-fos* technique, since excitation is a prerequisite for the induction of immediate-early genes [5,14,29,35]. It is known from the literature that the influence of the CST upon spinal neurons is not only excitatory, but also inhibitory [2,10,16,21]. Nevertheless, the induction of *c-fos* after kainate stimulation of the cerebral cortex provides a powerful tool in the study of the influence of the CST upon the spinal cord and future research should be aimed at the elucidation of the influence of other structures upon the corticospinal system, for instance by lesion of other descending tracts and/or the primary afferents.

5. Note added in proof

We recently provided electronmicroscopic evidence that such direct cortico-motoneuronal synaptic contacts are present in the adult rat cervical spinal cord and are first established at postnatal day 7 (Curfs, M.H.J.M., Gribnau, A.A.M. and Dederen, P.J.W.C., *Neurosci. Lett.*, 205 (1996) 123–126).

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