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Short communication

Transient functional connections between the developing corticospinal tract and cervical spinal interneurons as demonstrated by c-*fos* immunohistochemistry

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Abstract

Previous research on the rat corticospinal tract (CST) which develops mainly postnatally revealed that some CST axons grow transiently into the spinal gray matter and are subsequently eliminated. In the present study the question was addressed whether these fibres also form transient functional connections. Rats aged 14 and 60 days postnatally received unilateral injections of the potent glutamate agonist kainate into the cerebral motor cortex. After a survival period of 90 min. the rats were perfused and their brains and spinal cords processed for the immediate early gene c-*fos* by immunohistochemistry. Increased levels of c-*fos* as opposed to sham-operated animals was observed in several brain nuclei as well as in the cervical spinal cord. In the spinal gray one population of labelled interneurons in particular appeared to correlate well with the CST projection field. A decrease was noted in the number of c-*fos* positive neurons from postnatal day 14 to 60, suggesting that during development transient functional connections are formed between the CST and its target.

Keywords: Corticospinal tract; Cervical spinal cord; Interneuron; c-*fos*; Postnatal maturation; Transient connection; Rat

Immature central neurons are characterized by a high plasticity, reflected in their ability to form correct functional contacts with their target even after part of their normal trajectory has been damaged. In contrast, some time during development this capacity is lost, resulting in loss of functionality after a lesion [2,12,13,16]. Therefore, understanding of the underlying developmental events might provide insight in how functional recovery can be achieved in the adult after central pathways have been damaged.

The rodent corticospinal tract (CST) is used as a model in developmental studies since its outgrowth into the spinal cord white matter, the entrance of its constituent fibres into the spinal gray and subsequent synaptogenesis occur postnatally making this process accessible for experimental manipulations [6,8,21,22]. Previously it was shown that during the outgrowth of the CST into the spinal gray the fibres grow past their target and later during development, i.e. after postnatal day 14, aberrant fibres are eliminated [4]. In the present investigation, we analysed whether these transient fibres also form functional connections which, consequently, also disappear later during development.

Immediate early genes such as c-*fos* are expressed in the nucleus of a neuron after an excitatory stimulus to that neuron [17]. These genes are then translated into proteins which can be visualized by immunohistochemistry. This technique is now well established and can be used to demonstrate functionally connected chains of neurons. In the present study the c-*fos* technique was used to study the maturation of the CST projection on the interneuron population in the cervical spinal cord by injecting the potent glutamatergic agonist kainate into the cerebral motor cortex of rats aged 14 and 60 days postnatally. Glutamate receptors are abundant in the rat motor cortex and the CST is most likely glutamatergic [7,25]. The results described here were previously published in abstract form [5].

In the present study a total of 12 Wistar rats (Central Animal Laboratory, University of Nijmegen) were used, aged 14 days postnally (P14), approximately 30–35 g ($n = 6$) and 60 days postnatally (P60), approximately 200 g ($n = 6$). After anaesthesia with ether the animals were

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placed in a stereotaxic apparatus and the skin overlying the skull incised. In 3 rats of both age groups small holes were drilled into the skull and 3 separate injections of kainate (100 ng in 0.5 μl per injection) were made into the forelimb area of the motor cortex [19]. The wound was then sutured and the animals were allowed to recover. The other 3 rats of both age groups underwent a sham-operation consisting of an incision and subsequent suturing of the skin. During preliminary experiments using varying survival times, the optimum was assessed to be 90 min.

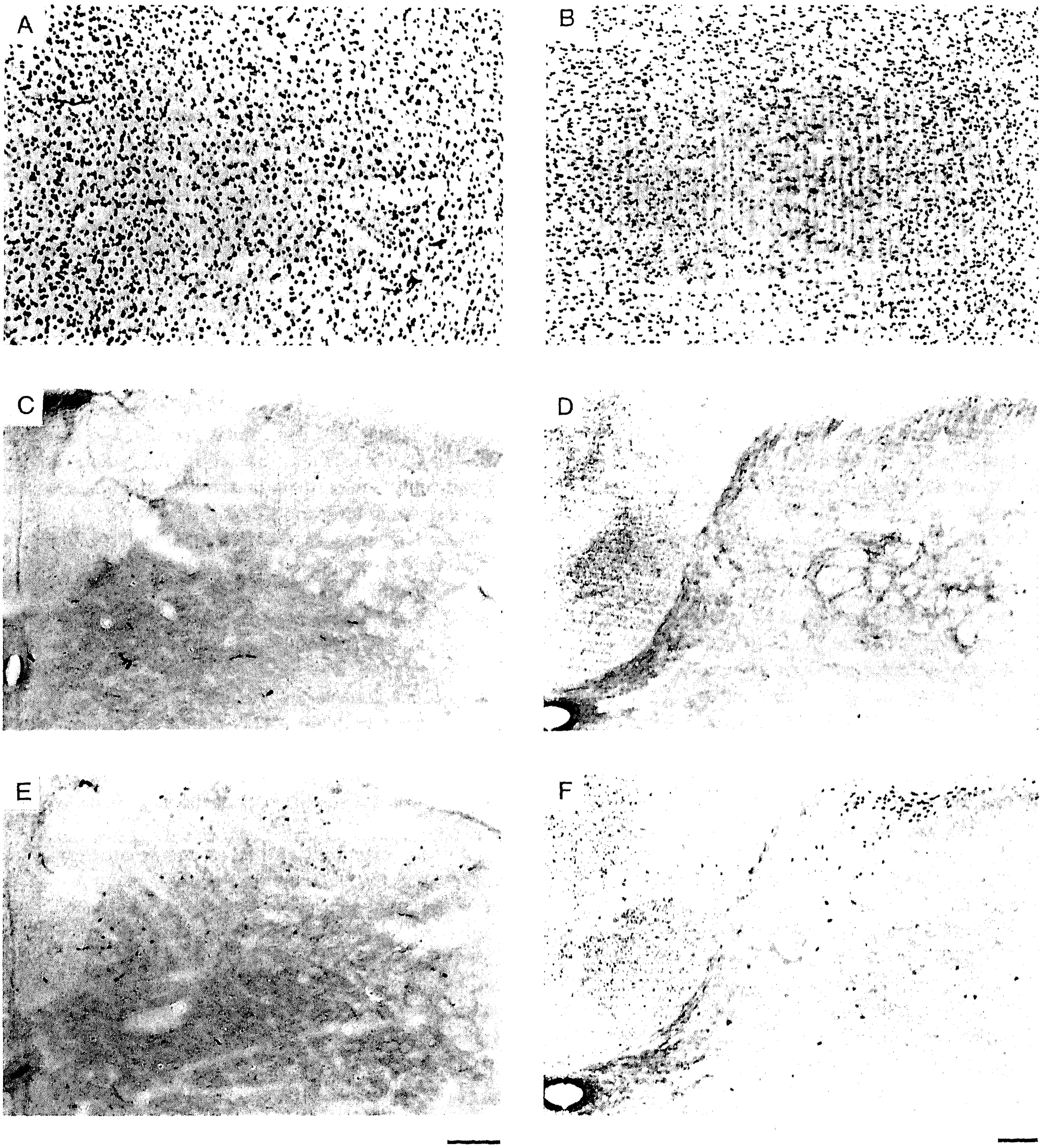


Fig. 1. Photomicrographs of 50 μm transverse sections from the ipsilateral cerebral motor cortex (A, B) and cervical spinal cord (C–F) at postnatal day 14 (A,C,E) and postnatal day 60 (B,D,F). After kainate injections into the cerebral motor cortex nearly all nuclei in the ipsilateral motor cortex stain positively for the immediate early gene c-*fos*. No changes were observed between P14 (A) and P60 (B). Pia mater is to the left. In the cervical spinal cord of sham-operated animals only few nuclei stained positively for c-*fos* at both P14 (C) and P60 (D). After kainate stimulation the number of Fos-Li neurons was significantly increased in the contralateral cervical spinal cord. This number was significantly higher at P14 (E) than at P60 (F). Scale bar = 100 μm for both P14 (left bar) and P60 (right bar).

Thereafter, the rats were transcardially perfused under deep ether anaesthesia with ice-cold 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by Samboni's fixative (1.8% paraformaldehyde and 7.5% pikrinic acid in PBS, pH 7.5). The brain and spinal cord were dissected from the skull and spine respectively, postfixed by immersion in the above mentioned fixative for 24 h and stored in PBS.

Vibratome sections of 50 μm of the brain and cervical spinal cord were cut and collected in an one out of two sections series and processed for c-*fos* like immunoreactivity (Fos-Li). In brief, sections were pretreated with 0.3% H_2O_2 in aqua dest, rinsed in 0.05 M Tris buffered saline (TBS, pH 7.6), and pre-incubated in 5% normal horse serum, 0.1% Triton and 0.1% BSA in TBS (TBS-BT-NHS). After overnight incubation in sheep IgG's against c-*fos* (1:2000 for brain and 1:4000 for spinal cord sections; Cambridge Research Biochemicals) in TBS-BT-NHS, the sections were rinsed in TBS, incubated for 90 min. in horse anti sheep antibodies (1:100; Nordic Immunology, Tilburg) in TBS-BT, rinsed in TBS and incubated for 90 min. in sheep peroxidase-anti-peroxidase complex (1:600; Nordic Immunology, Tilburg) in TBS. After rinses in TBS the presence of Fos-Li was visualized by incubation for 3 min. 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 7.4). After final rinses in TBS, the sections were mounted onto glass slides using a gelatin chrome-alum solution, air-dried and embedded in Depex. The cervical spinal cord was subdivided into two parts: comprising segments 5 and 6 (C5–6), and C7–8, respectively. The labelled cell nuclei in one out of four randomly selected sections were plotted onto the respective drawing using a Zeiss microscope equipped with a drawing tube. In order to visualize the results in a convenient way, the results of each of the two parts of the spinal cord were then pooled and plotted onto a representative section and the number of labelled nuclei was counted. Differences in the numbers found were tested for their statistical significance by means of an ANOVA.

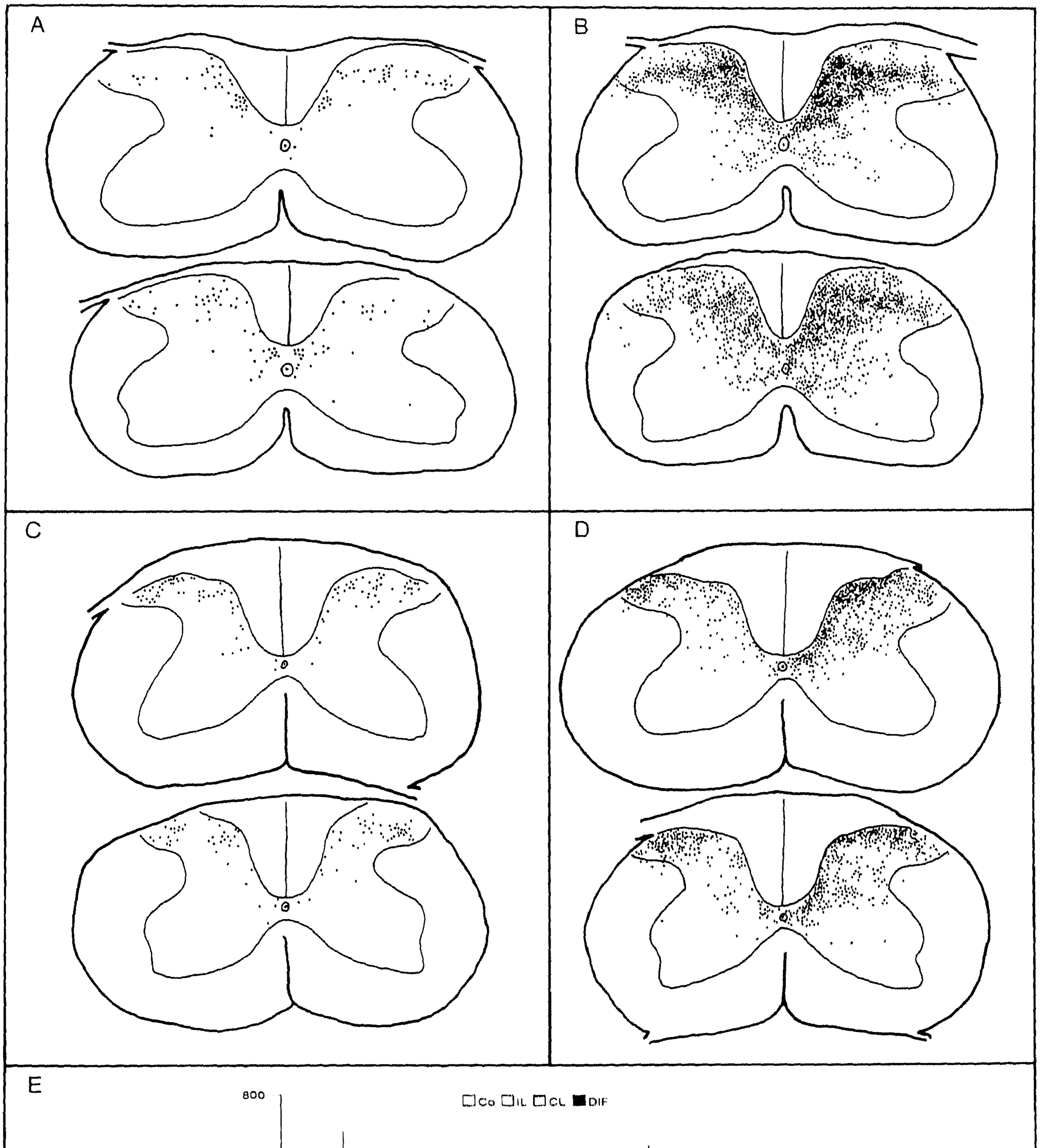
After recovering from anaesthesia (after approximately 20 min) all rats receiving kainate injections into the cerebral motor cortex started to display a specific motor behaviour, at both ages. In particular, nearly constant locomotion, and twitch-like movements and misplacement of the contralateral forelimb were observed. When compared to sham-operated rats, virtually all neurons stained positively for c-*fos* in the forelimb representation of the motor cortex (Fig. 1A,B). An increased number of Fos-Li nuclei was also observed in the ipsilateral caudate nucleus, putamen, and red nucleus, and bilaterally in the thalamus (reticular, ventromedial and posterior nucleus), globus pallidus, subthalamic nuclei, tectum, and brainstem nuclei such as substantia nigra, pontine and raphe nuclei. Quantification of the number of Fos-Li neurons in brain structures was beyond the scope of the present investigation.

Sections taken from the cervical spinal cord of sham-operated animals revealed a low number of Fos-Li neurons. The majority of these nuclei was located in the dorsal horn and the rest was equally distributed between the intermediate zone and the ventral horn (Figs. 1C,D, 2). No significant difference was noted between the sham-operated P14 and P60 animals. Kainate injections in the cerebral motor cortex resulted in a marked increase in the number of Fos-Li neurons at the two postnatal ages examined (Figs. 1E–F, 2). This increase was noted both in the ipsilateral and in the contralateral half of the cervical spinal cord, although the increase was largest in the latter. Most Fos-Li nuclei were found in the dorsal horn and the rest dispersed in the intermediate zone and the ventral horn, and along the rostrocaudal axis in C5–6 at both ages. The number of Fos-Li nuclei in the young adult was significantly lower than that found at P14, the relative decrease being larger in C5–6 (Fig. 2).

Fos-Li neurons in the ipsilateral half of the cervical spinal cord were located more or less scattered throughout the spinal gray (Fig. 2), whereas those in the contralateral half of the cervical cord could be subdivided in broad outline into two separate populations, with some overlap. One population was located in the dorsal horn and the intermediate zone in the vicinity of the corticospinal tract in the dorsal funiculus. This population in particular decreased in size during postnatal maturation. The other population was found further dorsally in the dorsal horn, especially in the medial part of the superficial layers (Fig. 2).

Kainate injections into the rat motor cortex induces the expression of the immediate early gene c-*fos* in many cervical spinal neurons at both ages investigated. Surprisingly, no spinal neurons were labelled for c-*fos* after microstimulation of the motor cortex [24], especially since the labelling of the forebrain and brainstem nuclei shows great resemblance in the two investigations. Probably, kainate produces a more massive excitation of the cerebral

Fig. 2. A–D: composite drawings of the Fos-Li nuclei in one in four pooled sections from the combined cervical spinal cord segments 5 and 6 (C5–6, top drawing), and C7–8 (bottom drawing) at P14 of a sham-operated rat (A) and after kainate injections into the cerebral motor cortex (B) and at P60 of a sham-operated rat (C) and after kainate injections (D). When compared to sham-operated rats, a large increase of Fos-Li neurons was noted in the kainate stimulated rat, both ipsilaterally (left side of the spinal cord) and contralaterally (right side) although the increase was largest in the latter. The increase was less high at P60 when compared to P14. E. Histogram displaying the mean numbers and standard deviation of Fos-Li neurons in sham-operated (Co) and motor cortex kainate injected rats in the combined cervical spinal cord segments 5 and 6 (C5–6), and C7–8 at P14 and at P60. For kainate injected animals a further differentiation is made for the ipsilateral (IL) and contralateral (CL) side. DIF is the difference between IL and CL.


A
B
C
D
E
Co IL CL DIF
total number of fos-positive neurons
800
600
400
200
0
P14
VW
C5-6
P14
VW
C7-8

motor cortex and, as a consequence, more cortical neurons are stimulated.

Most Fos-Li neurons were encountered in the dorsal horn and the intermediate zone. Recently, it was shown that after an inflammatory stimulus of the foot, c-*fos* is mainly expressed in the dorsal horn and the intermediate zone of the lumbar spinal cord whereas c-*jun* is principally found in the ventral horn and the superficial layer of the dorsal horn [14]. Since the CST also projects to the ventral horn [4], it is obvious that in the present study an underestimation is made of the number of spinal neurons involved in the CST pathway. On the other hand, not all Fos-Li neurons can be accounted for by direct corticospinal functional contacts. The population of Fos-Li neurons located contralaterally in the more superficial layers of the dorsal horn and especially in their medial parts are probably labelled by two different mechanisms. Firstly, this population receives input from primary afferents [3], which might be activated by motoneurons involved in the CST pathway, either directly or indirectly via interneurons. The resemblance with the c-*fos* expression in walking rats is striking [9]. Secondly, the population of Fos-Li neurons in the medial parts of the dorsal horn closely resembles that encountered after noxious stimulation [1,20,23].

Fos-Li neurons in the ipsilateral spinal gray can be attributed to several mechanisms. Firstly, since part of the kainate behaviour is expressed in bilateral activity, ipsilateral primary afferents are also stimulated. Secondly, other bilateral descending tracts originating in areas which are stimulated bilaterally by the cerebral cortex (e.g. the thalamus and brainstem nuclei) also project upon spinal interneurons. And thirdly, contralateral CST fibres which return to the ipsilateral side in the spinal cord or the minor uncrossed CST component located in the ventral funiculus [10] might add to the increased number of Fos-Li neurons.

A statistical significant overall decrease in the number of Fos-Li nuclei was noted from P14 to P60, both ipsi- and contralaterally. In the latter, this decrease was striking especially in the area of the corticospinal projection, ie. in the direct vicinity of the CST in the dorsal funiculus and laterally in the ventral part of the dorsal horn and intermediate zone. This correlates well with the previously described decreasing CST projection pattern and number of CST axons in the spinal gray from P14 onwards [4]. From these results it can be concluded that developing corticospinal fibres form transient functional contacts with interneurons. The decrease found in the number of Fos-Li neurons, however, can not be ascribed entirely to the developing CST. Most likely, transient aberrant projections in higher brain nuclei are also responsible for the decrease found, including the decrease in the ipsilateral cord.

In conclusion, we have provided strong evidence that at least part of the developing corticospinal axons form transient functional connections with interneurons in the cervical spinal cord. It is generally agreed upon that during development several peripheral motor axons form synapses upon one motor endplate. Eventually all aberrant axons are eliminated by competition for a trophic substance, which is secreted by the target in an activity-dependent manner [11,15,18]. Future in vitro or quantitative ultrastructural research may reveal whether the same mechanisms also apply to the developing CST.

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