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p-Chlorophenylalanine, a serotonin synthesis inhibitor, reduces the response of glial fibrillary acidic protein induced by trauma to the spinal cord

An immunohistochemical investigation in the rat

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Abstract. The possibility that serotonin may influence the early response of astrocytes around a spinal cord trauma was investigated in a rat model by making a unilateral incision into the right dorsal horn of the T10-11 segments. One group of rats received a serotonin synthesis inhibitor, *p*-chlorophenylalanine (p-CPA) before injury in doses which cause a depletion of serotonin in the cord. Another group of traumatised rats did not receive p-CPA. All animals were allowed to survive for 5 h. Samples for immunohistochemistry were taken from the T9, T10-11 and T12 segments of the cord. Paraffin sections were immunostained for glial fibrillary acidic protein (GFAP) using monoclonal antibodies and avidin-biotin complex technique. Trauma to the cord resulted in a marked increase of GFAP immunoreactivity in all the investigated segments, particularly in the ipsilateral side. Pretreatment with p-CPA markedly reduced the GFAP response. This drug did not by itself influence the GFAP immunoreactivity of the cord of untraumatised rats. Our results show that trauma to the spinal cord induces a rapid enhancement of GFAP immunoreactivity in the cord which is present even far away from the primary lesion. This response can be prevented by pretreatment with the serotonin synthesis inhibitor, p-CPA. The results indicate that serotonin influences the increase of GFAP immunoreactivity following spinal cord injury either directly or indirectly, for instance by its microvascular reactions.

Key words: Spinal cord trauma – Glial fibrillary acidic protein – Serotonin – *p*-Chlorophenylalanine – Immunohistochemistry

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Astrocytes are important constructional elements of the spinal cord and have many additional functions under normal and pathological conditions [5]. These cells play important roles for the normal barrier functions of blood vessels and for the homeostasis of the extracellular environment of the parenchyma [27]. Astrocytes are potential targets for chemical signals released from neurons and possess binding sites for neuroactive peptides, amino acids, amines and eicosanoids [21].

Many pathological processes of spinal cord are associated with swelling of astrocytes and the formation of gliosis which involves proliferation and hypertrophy of astrocytes [1, 4, 13]. During the formation of gliosis there is an activation of metabolic processes with production of cytoskeletal components including glial fibrillary acidic protein (GFAP) [5]. Using immunohistochemistry changes in the amount of GFAP antigen can be visualised in tissue sections [31, 36]. For most pathological processes of the brain and spinal cord there is a delay of one or more days before increased GFAP immunoreactivity as evident by immunohistochemistry [1, 5, 14, 19, 25, 26]. It seems quite likely that alterations in the microenvironment of the CNS by metabolic, traumatic or ischaemic insults could play an important role in activating astrocytes either directly or by an altered neurochemical metabolism [3, 6, 12, 32]. However, the mechanisms of induction of gliosis are not well understood.

We are interested in the pathophysiology of perifocal, secondary lesions to localised spinal cord injuries. Previously, in a rat model characterised by an incision into the right dorsal horn of the T10-11 segment of the cord we focused on early changes in vascular permeability, edema formation and nerve cell changes and evaluated the role of serotonin by comparing the responses between injured animals with and without depletion of endogenous stores of this amine [24, 29, 30]. Observations were made indicating that soon after an injury serotonin can be activated and that this produces secondary changes of cells and alters the fluid micro-environment.

Astrocytic reactions can be elicited by various hormones growth factors and serum constituents [3, 6, 8, 20]. The possibility therefore exists that spinal cord trauma with its release of numerous neurochemicals can induce a rapid increase in the GFAP immunostaining of astrocytes. The present investigation concerns the GFAP immunoreactivity of astrocytes in the early period after a localised minor trauma to rat spinal cord. Other animals were pretreated with *p*-chlorophenylalanine (p-CPA) to deplete the endogenous stores of serotonin [29, 30] before the production of spinal cord injury. The results of the two groups were compared to find out if serotonin somehow mediates the astrocytic response which occurs after trauma to the cord.

Materials and methods

Animals and spinal cord injury

Experiments were carried out in 20 urethane-anaesthetised inbred Wistar male rats (body wt 250–300 g) housed at controlled ambient temperature 22 ± 1 °C with 12 h light and 12 h dark schedule. Food and tap water were supplied ad libitum. This experimental condition was approved by the Ethical committee of Uppsala University.

Under urethane anaesthesia (1.5 g/kg, i.p.) a laminectomy was done over the T10-11 segments. Using a sterile scalpel blade, a unilateral incision into the right dorsal horn was made by hand [24, 29]. The dimensions of the injury were approximately 2.5 mm deep, 3–5 mm long and it was located about 1.5 mm to the right of the midline. The deepest part of the lesion involved Rexed's laminae VII. Animals were allowed to survive for 5 h ($n = 5$). One group of animals ($n = 5$) anaesthetised with urethane for 5 h (without laminectomy) served as controls.

Another group of animals ($n = 10$) were treated with p-CPA (100 mg/kg, Sigma Chemical Co., USA) i.p. daily for 3 days. On the 4th day, one group of animals ($n = 5$) were operated and exoposed to the same kind of spinal cord injury as in the rats not given the drug. The remaining five animals served as drug-treated controls. It is known from many previous studies that such a treatment will deplete the stores of endogeneous serotonin in the cord [24, 29].

GFAP immunohistochemistry

The animals were perfused transcardially with 100 ml Somogyi fixative (2.5 % glutaraldehyde, 2 % paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 containing 2.5 % picric acid) preceded by a brief saline rinse (0.9 % NaCl, about 50 ml) [31]. The animals were wrapped in aluminium foil and kept in a refrigerator at 4 °C overnight [31, 36]. The next day, the spinal cord was removed and kept in the same fixative at 4 °C for 2–3 days.

Samples originating from T9, T10-11 and T12 segments of the spinal cord were removed and embedded in paraffin. Multiple 3- to 5-μm-thick sections were cut and processed for GFAP immunostaining. Sections from controls and experimental rats were run at the same time. In brief, after deparaffination, sections were exposed for 20 min to a solution of 0.3 % hydrogen peroxide to which 1 % non-immuno horse serum in phosphate-buffered saline (PBS, pH 7.4) was added. A primary monoclonal antibody to GFAP (Dakopatts, Hamburg, Germany) diluted 1:500 in PBS was then applied for 8 h. After incubation with biotinylated horse anti-mouse IgG at a 1:50 dilution and avidin-biotin complex (ABC; Vector, Burlingame) for 45 min, the reaction product was developed with 3,3′ diaminobenzidine tetra hydrochloride and hydrogen peroxide in 0.05 M TRIS-HCl buffer (pH 7.4) for 4 min. The sections were counter-stained with haematoxylin. Some sections were incubated with pre-immuno horse serum at a 1:50 dilution as the primary antiserum. Such control sections did not show any immunoreactive product [31, 36].

Physiological variables

The mean arterial blood pressure (MABP) was recorded in controls ($n = 5$) and in rats with spinal cord injury ($n = 5$) through a cannula (PE 25) placed in the common carotid artery. The arterial catheter was connected to a pressure transducer (Statham P 23, USA) attached to a chart recorder. In addition, the arterial pH, PaO_2 and $PaCO_2$ were analysed using a blood gas analyser (Radiometer, Copenhagen) [24].

Results

Physiological variables

The results are shown in Table 1. At 5 h after the spinal cord injury there was a mild hypotension of about 20 ± 4 torr. The PaO_2 was significantly increased, whereas the $PaCO_2$ values declined from the control group. The arterial pH was unaffected. Pretreatment with p-CPA did not affect these physiological variables significantly.

GFAP immunoreactivity

Normal animals. The distribution of GFAP immunoreactivity of normal rats was in accordance with earlier observations [15]. A representative example of the normal GFAP staining is presented in Fig. 1a. The sections of the normal cord showed few scattered GFAP-immunoreactive cells of astrocytic configuration with brown reaction product extending into the cytoplasmic processes. Such cells were present in the glia limitans externa and around the central canal. The grey matter contained few immunopositive astrocytes (Fig. 1a).

Table 1. Physiological variables in normal rats and in animals with spinal cord injury

Parameters	Control ($n = 5$)	Spinal cord injury 5 h ($n = 5$)	p-CPA + Spinal cord injury 5 h ($n = 5$)
MABP torr	110 ± 8	80 ± 6*	84 ± 4*
Arterial pH	7.37 ± 0.04	7.37 ± 0.06	7.37 ± 0.08
PaO_2 torr	80.56 ± 0.45	81.64 ± 0.37	81.34 ± 1.25
$PaCO_2$ torr	34.76 ± 0.43	33.23 ± 0.38	33.68 ± 0.84

The physiological variable were measured 5 h after injury (for details see text). Values are mean ± SD p-CPA, *p*-Chlorophenylalanine; MABP, mean arterial blood pressure
* = $P < 0.05$ Unpaired Student's *t*-test. (Compared from control group)

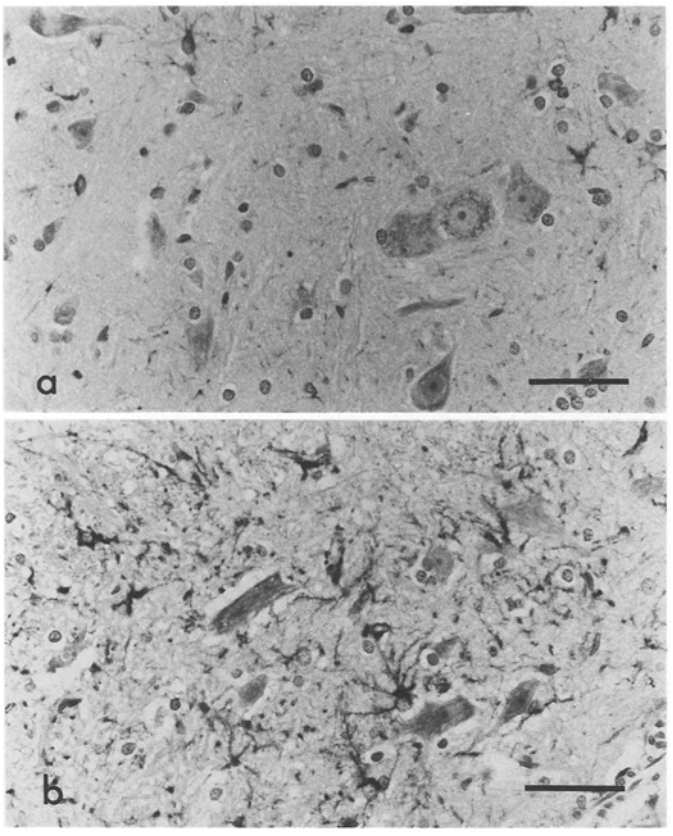

Fig. 1. GFAP immunoreactivity in (**a**) control and (**b**) 5 h after trauma to the cord in the T10-11 segments. The sample was taken from the right ventral horn of the T9 segment. A large number of reactive astrocytes can be seen in the traumatised animal as compared with the control. Bar = 50 μm

Trauma to the spinal cord. At the site of the incision of the T10-11 segments there were tissue destruction and haemorrhages extending into Rexed lamina VIII. The grey matter particularly of the ipsilateral side of the spinal cord in T9 and T12 segments was expanded. The ventral horns were spongy in appearance and the neurons had distorted cell bodies; some were slightly swollen and others were shrunken. The white matter was also expanded.

Compared with the controls there were marked changes in the injured spinal cords at 5 h, both with regard to the frequency of immunopositive astrocytes and the intensity of immunostaining in individual cells (Fig. 1b). These responses were most marked in the vicinity of the trauma (see Fig. 5). The reactions occurred in astrocytes both of the grey and the white matter. However, the GFAP-positive cells were particulary abundant in the grey matter (Fig. 1b, 2).

The perifocal segments (T9 and T12) showed a moderate increase in GFAP immunoreactivity (Figs. 1b, 2a, b) as compared to the controls (see Fig. 5). Immunostained astrocytes were somewhat more abundant and the staining often more intense. Such reactions were seen in the both grey and white matter and in dorsal and ventral horns of both sides of the cord. Representative illustrations of the changes of the ventral horn of T9 and T12 segment are provided in Figs. 1 and 2.

Influence of serotonin depletion with p-CPA

Pretreatment with the serotonin synthesis inhibitor, p-CPA markedly diminished the trauma-induced expansion of the T9 and T12 segments. The cell changes were much less pronounced compared with those of the untreated group.

The increased immunostaining of GFAP seen in the traumatised rats was not evident in animals with the same type of spinal cord trauma if p-CPA was given before the injury to deplete the stores of endogenous serotonin. The pattern of GFAP staining was in fact quite similar to that of normal animals (Fig. 3). The drug by itself given in untraumatised animals did not influence the GFAP response (Fig. 4).

A semiquantitative data of GFAP immunoreactivity in control, untreated and p-CPA-treated 5-h spinal cord traumatised animals are presented in Fig. 5. The number of GFAP astrocytes were significantly increased after trauma to the spinal cord in untreated animals as compared to the control group ($P < 0.001$, Student's unpaired t-test). This effect was more pronounced in the

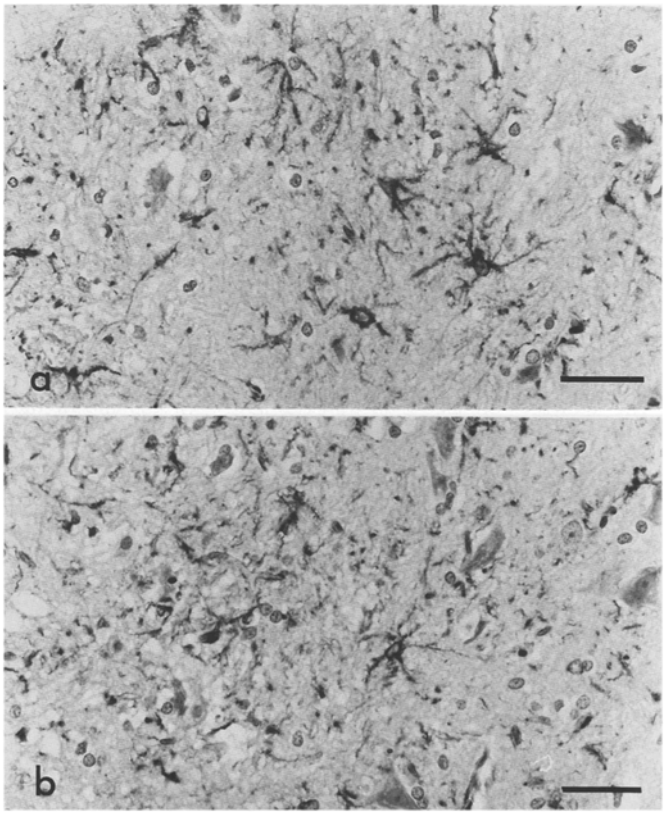

Fig. 2a,b. Reactive astrocytes in a rat with trauma to the spinal cord and 5-h survival. Samples were taken from (**a**) the right ventral horn and (**b**) lateral horn of a T12 segment. Bar = 50 μm

ipsilateral side. In p-CPA-pretreated and injured animals, the number of GFAP-positive astrocytes were not increased significantly from the control group (Fig. 5).

Discussion

The essential new findings of this study is that pretreatment with p-CPA (a serotonin synthesis inhibitor) markedly prevented the increase of immunostaining with GFAP antiserum in astrocytes 5 h after a localised mild injury to the spinal cord. This indicates that serotonin may either directly or indirectly influence the activation of astrocytes.

The possibility that p-CPA may have a direct effect on astrocytes, causing inhibition of protein synthesis, appears unlikely [17]. This view is supported by the fact that we did not get any difference in the GFAP response between normal and p-CPA-treated untraumtised animals.

Which are the signals inducing an increased expression of GFAP far away from the site of primary traumatic injury of the spinal cord? Recent reports indicate that the edema and spread of humoral factors derived from the blood or from the region of injury are of major importance [2, 7, 22, 23]. Other possible signals could be a variety of hormones, growth factors and chemicals which may initiate the expression of GFAP [16, 33–35]. It is known that astrocytes in tissue culture increase their content of GFAP when exposed to substances like dibutyryl cyclic AMP, hydrocortisone, putrescine, prostaglandin F-2α and pituitary fibroblast growth factor [10, 11, 13, 28]. Some of these chemicals are available in nervous tissue following injury.

Our present results indicate that serotonin may also play an important role in augmenting GFAP expression in spinal cord injury. This is evident from the results obtained with p-CPA treatment before injury. The GFAP response was markedly reduced in such animals which have a depletion of their stores of serotonin in the cord. At this moment we do not know if the expression of GFAP in spinal cord injury is directly influenced by serotonergic mechanism or indirectly mediated by breakdown of microvascular permeability, spread of edema and cell damage caused by the amine [24, 25]. Since serotonergic receptors are present on astrocytes [21] a direct effect may be involved. However, Le-Prince et al. [18] found an inhibitory effect of serotonin on GFAP expression in rat brain stem astrocytes in primary

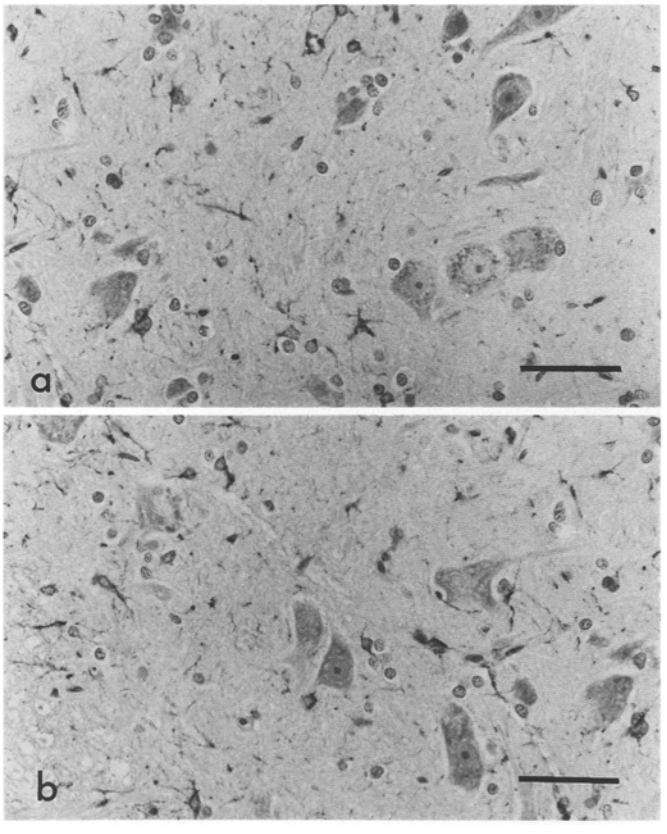

Fig. 3a,b. GFAP immunoreactivity in one *p*-chlorophenylalanine (p-CPA)-pretreated rat killed 5 h after a trauma to the spinal cord. The drug was given before injury to deplete the stores of serotonin. Samples were taken from (**a**) the right ventral horns of T9 and (**b**) T12 segments. Only a few GFAP-positive astrocytes are visible. Bar = 50 μm

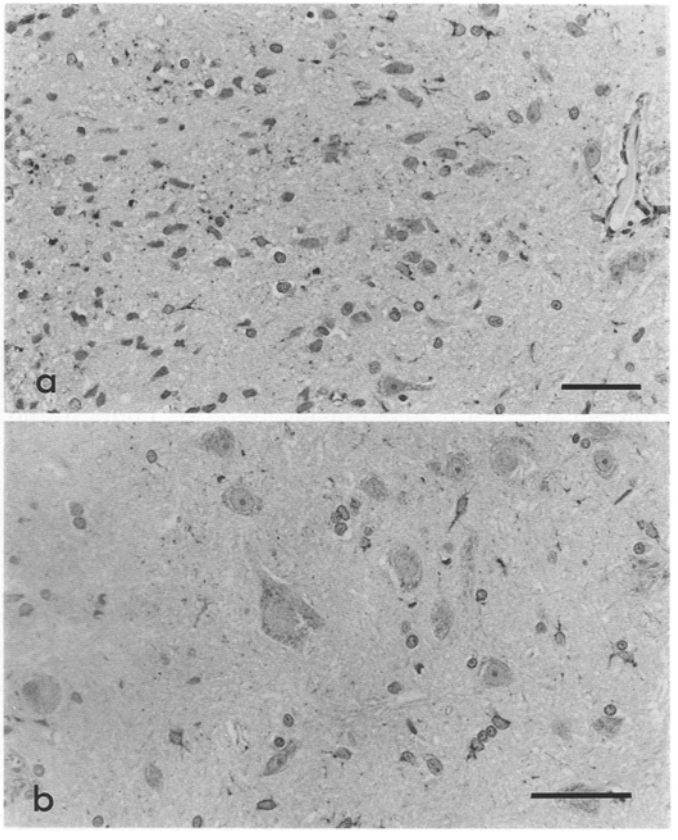

Fig. 4a,b. GFAP-immunoreactive astrocytes in one p-CPA-pretreated normal animal. Only a few GFAP-positive astrocytes can be seen in the right dorsal horn (**a**) and ventral horn (**b**) of T9 segment. Bar = 50 μm

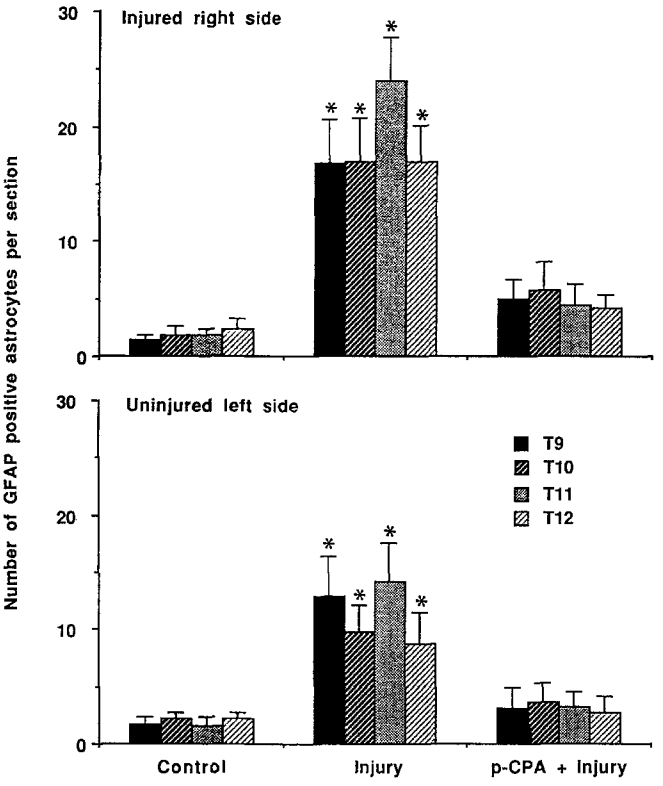

Fig. 5. Semiquantitative data on number of GFAP-positive astrocytes per section in traumatised animals and its modification with p-CPA. The GFAP-positive astrocytes were counted under light microscope in each side of the spinal cord at T9, T10, T11, and T12 levels from rats taken 5 h after injury. Normal animals served as controls. Each *column* denotes mean and *bar* represents standard deviation. *Asterisk*, $P < 0.001$, Student's unpaired *t*-test

culture. The difference between our findings and those of Le-Prince et al. [18] may be due to heterogenity of astrocytes in terms of neurochemical responses and their number of receptors. Further studies using serotonin receptor antagonists are needed to clarify this point.

The mechanism causing an increased GFAP immunostaining in untreated animals following injury to the spinal cord is not clear. Obviously, there must be an increase in the number of antigenic sites available for binding with the GFAP antibodies in pre-existing astrocytes resulting in an increased immunostaining. The brief survival period excludes the possibility for cell division as a major cause of increased number of GFAP-positive astrocytes.

Theoretically, an increase in antigenic sites may be caused by some change rendering the epitopes more easily accessible to the antibodies or by synthesis of new epitopes. However, a rapid increase in GFAP mRNA could also be possible. A rapid expression of GFAP mRNA occurs within 6 h of cerebral injury in mice which reaches its peak by 5–7 days after injury [7]. Condorelli et al. [9] reported in rats a similar rise in the GFAP mRNA at 6 h after an injury with a peak at 1.3 days. These authors found that the astrocytic activation occurred in the cortex at a distance away from the lesion and in the contralateral hemisphere. Our observation with GFAP changes in segments away from the primary injury is in line with the observations derived from experiments on the traumatised rat brain [9].

Thus, it appears that a signal which may induce GFAP mRNA response is initiated very soon after an injury, leading to a rapid expression of the message which can be recognised within a few hours after injury. This effect may be related to increased protein synthesis, increased transcription of the GFAP gene or stabilisation of the GFAP mRNA. Since the temporal expression of the GFAP immunoreactivity usually follows the expression of GFAP mRNA [7, 9], it is reasonable to assume that in spinal cord injury this expression can be induced at 5 h and be recognised if highly sensitive immunohistochemical methods are applied. A better understanding of the mechanisms leading to increased GFAP immunoreactivity following trauma to the spinal cord and its modification with p-CPA may emerge from studies on GFAP mRNA expression using Northern blot or in situ hybridisation techniques.

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