

# The influence of acute and chronic open-field exposure on the hippocampal formation: an immunohistochemical study

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*The hippocampus plays a role in new learning, memory and emotion and is a component of the neuroanatomical stress circuit. The structure is involved in terminating hypothalamic-pituitary-adrenocortical (HPA) axis responses to stress and attenuates stress responses by shutting off this axis.*

*The immunoreactivity (-ir) of c-Fos, NGF and its receptor TrkA following acute and chronic open-field stress were studied in CA1-CA3 and the DG of the hippocampus. The material consisted of 21 male adult rats divided into three groups: non-stressed (control) animals and rats exposed to acute (15 min once) and chronic (15 min daily for 21 days) aversive stimulation (open-field exposure). The brains were stained with use of immunohistochemical methods for c-Fos, NGF or TrkA. In the animals exposed to acute open-field stress the number of c-Fos-, TrkA- and NGF-ir cells was higher in all the structures studied than in the control animals. However they were differentiated only in c-Fos immunoreactivity.*

*In the rats exposed to chronic open-field stress the number of c-Fos-ir cells in the structures of the hippocampal formation studied was smaller than in rats exposed to acute stress and was comparable to that in the control group. No differences were observed between the groups exposed to acute and chronic stress in the number of TrkA-ir cells in the structures under investigation. The number of NGF-ir neurons in CA1 and CA2 was lower after exposure to chronic than after exposure to acute stress but was still higher than that in the control group.*

*Our findings indicate that neurons of CA1-CA3 and the DG are engaged in the stress response after acute as well as chronic open-field exposure. This is probably related to the important role of the hippocampus in processing new spatial information as well as in the habituation processes, although these appear to have different mechanisms.*

**Key words:** NGF, TrkA, hippocampus, open field test, stress, c-Fos, neurotrophins

## INTRODUCTION

The term hippocampus is applied to the dentate gyrus (DG) and hippocampus proper [5]. The latter is divided into three sectors CA3, CA2 and CA1. The CA2 field receives a prominent innervation from the posterior hypothalamus [18], whereas the projection

from the amygdala reaches mainly the CA3 and CA1 sectors [51]. Cells of all the sectors send their axons (in differentiated quantities) to some of the subcortical (the amygdala and the hypothalamus) and cortical regions. Efferents of the DG cells reach CA3 and receive inputs from the entorhinal cortex and other

cortical structures as well as from the subcortical region (such as the hypothalamus and septum). Inputs from the subcortical regions to the hippocampus influence the behavioural state of the organism.

The hippocampus is responsible for learning, memory and emotions [1, 6, 8, 38, 41, 53]. It is involved in both short and long-term memory, mainly spatial but also emotional [1, 31, 32, 42].

The high density of glucocorticoid receptors in the hippocampus indicates their involvement in memory consolidation [6, 40, 63]. It has been found that exposure to an environment of long-lasting stress results in memory deficit [8, 15, 57].

The hippocampus is one of the main components of the stress circuit [6, 24] through its involvement in the termination of hypothalamic-pituitary-adrenocortical (HPA) axis responses to stress [39]. The hippocampus is activated during various kinds of stress. It may be especially important in assessing the stressfulness of psychological stressors such as novelty [16, 26, 44, 45, 52].

Several lines of research suggest that nerve growth factor (NGF), apart from having a trophic function, seems to be implicated in HPA axis activity [30, 54, 61]. NGF may also contribute to structural changes in the mature brain [3]. This protein is involved in neurobehavioural stress response and homeostasis. The action of NGF is mediated by neurotrophin binding to two separate receptors such as the high-affinity tyrosine kinase (TrkA) receptor and the low-affinity p75 neurotrophin receptor [3, 9, 12, 17, 33]. NGF exerts most of its biological effects on neurons through the activation of TrkA [17, 46, 48]. The expression of TrkA receptor was found within the neurons of the hippocampus [55]. NGF endogenously released during stress may promote remodelling of damaged tissues after exposure to acute or chronic stressful events [4]. Studies of both intact and injured brain structures have shown changes in the expression of NGF in the hippocampus, although its role in the function of this structure is not understood in detail [60]. There is a hypothesis that hippocampal NGF may be involved in neuroendocrine function [29]. In an adult rodent changes in NGF level (synthesis or utilisation) in the hippocampus have been reported to be related to different kinds of psychological stressors [4, 7, 58].

The aims of the study were to find:

- whether c-Fos activity in the CA1-CA3 regions and the DG of the hippocampus remains under the influence of acute and chronic exposure to the open-field test in adult rats;

- whether the duration of stressful stimuli exerts any influence on the level of NGF and its receptor TrkA immunoreactivity in these structures.

## MATERIAL AND METHODS

The material consisted of adult male Wistar rats of 180 postnatal (P) days of age. The care and treatment of the rats were in accordance with the guidelines for laboratory animals established by the National Institute of Health as well as by the Local Ethical Committee of the Medical University of Gdańsk.

The rats were divided into three groups: non-stressed control rats, which remained in their home cages, an experimental group exposed to acute stress (the open-field test, once for 15 min) and an experimental group exposed to chronic stress (the open-field test, 15 min daily for 21 days). Each group consisted of seven animals.

The open-field box (100 × 100 × 40 cm) was illuminated with a 500-watt halogen light. The open-field test was applied between 9:00 a.m. and 2:00 p.m.

After 90 min of final exposure all the rats were deeply anaesthetised with lethal doses of Nembutal (80 mg/kg of body weight) and then perfused transcardially with 0.9% saline solution with heparin, followed by 4% paraformaldehyde solution in 0.1 M phosphate buffer (pH 7.4). The brains were post-fixed in 4% paraformaldehyde for 3–4 hours and then kept in 0.1 M phosphate buffer containing 10% sucrose (overnight at 4°C) and 30% sucrose (until sunk). Coronal 40- $\mu$ m-thick, serial sections of brain were cut on a JUNG 1800 cryostat (Leica, Germany).

The sections were then stained with the use of immunohistochemical methods. The free-floating sections were blocked with 5% Normal Goat Serum (NGS) containing 0.3% Triton X-100 for one hour and then incubated with primary polyclonal rabbit anti-c-Fos antibody (Santa Cruz; dilution 1:500), polyclonal rabbit anti-TrkA antibody (Santa Cruz; dilution 1:150) or polyclonal rabbit anti-NGF antibody (Chemicon; dilution 1:500) in 5% NGS for 48 hours at 4°C. After multiple rinses in PBS sections were incubated (for 2–3 h at room temperature) with appropriate secondary Cy3-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch; dilution 1:600).

The immunohistochemically stained slides were examined by a fluorescent microscope Eclipse 600 (Nikon, Japan) with confocal system Radiance 2100 (Bio-Rad, UK) equipped with a Krypton/Argon laser. The confocal microscopy images were obtained using a 40 $\times$  objective lens. The optimal iris was used for each magnification.

The number of cells in the hippocampal formation investigated was estimated semiquantitatively and classified as follows:  $\pm$  few, + moderate, ++ high, +++ very high.

## RESULTS

A moderate number of c-Fos-ir cells was observed in the control animals in the hippocampal regions examined, mainly in the pyramidal and polymorphic layers of CA1-CA3 and the granule layer of the DG. Acute exposition to the open field resulted in a higher number of c-Fos-ir neurons in the hippocampal formation in comparison to that of the control group, whereas exposition to chronic stress resulted in a decreased number of c-Fos-ir cells (Table 1, Fig. 1).

In the control rats we observed a large number of TrkA-ir cells in CA1-CA3 and the DG. The number of TrkA-ir cells in response to acute stress was very high. After chronic stress the levels of TrkA-ir cells remained high in all the hippocampal regions investigated (Table 1, Fig. 2).

The number of NGF-ir neurons observed in the region studied in the control animals was differentiated from moderate to high. Acute exposure to the open field resulted in an increased number of NGF-ir cells. This concerned mainly the pyramidal layer of

CA1-CA3 but all the layers of the DG. Following acute stress stimulation the number of NGF-ir neurons in the regions of the hippocampus studied was similar to that of the TrkA-ir neurons.

Chronic stress exposure caused a decrease in the number of NGF-ir cells in CA1 and CA2 but not in CA3 and the DG. The number in CA3 and the DG was similar to that of the TrkA-ir neurons but was lower in CA1-CA2. (Table 1, Fig. 3).

## DISCUSSION

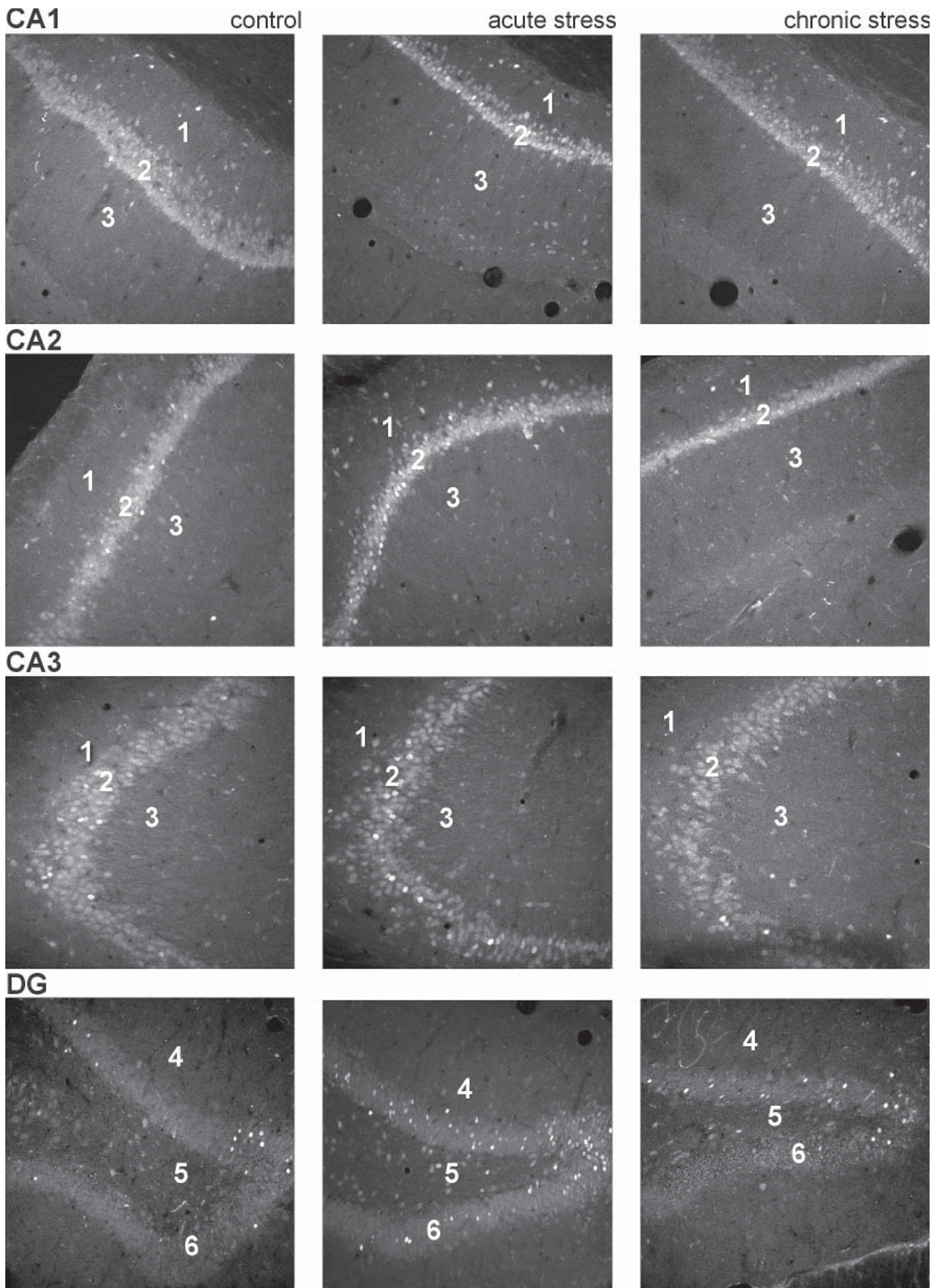
In home-cage control rats a moderate number of c-Fos-immunoreactive cells were observed in the CA1-CA3 and DG regions of the hippocampus. Such expression was also observed by Jenkins et al. [25] and Lee and their colleagues [34].

In our study acute exposition to the open field caused increased c-Fos expression in all sectors of the hippocampus investigated. Similar results were observed by Wirtshafter [62], who placed rats into a number of new and varied environments. However, it is thought that the degree of expression of c-Fos-ir varies between the regions of hippocampus [34, 36, 52]; it also depends upon the level of exploration of a new environment [45]. According to Nagahara and Handa [44] a strong increase of c-Fos and its mRNA following acute open-field exposure involved mainly the granule cell layer of the DG and the pyramidal cell layer of CA3 and CA1. Other authors have noticed that exposure to restraint, immobilisation or treadmill exercise caused intense expression of both c-fos mRNA and c-Fos in the DG and CA1-CA3 regions of the hippocampus [10, 14, 34, 59]. However, Pace et al. [45] also found the increase in the number of c-Fos-ir in CA1-CA3 but not in the DG after exposure to a novel experience (tub, arena, pedestal or restraint).

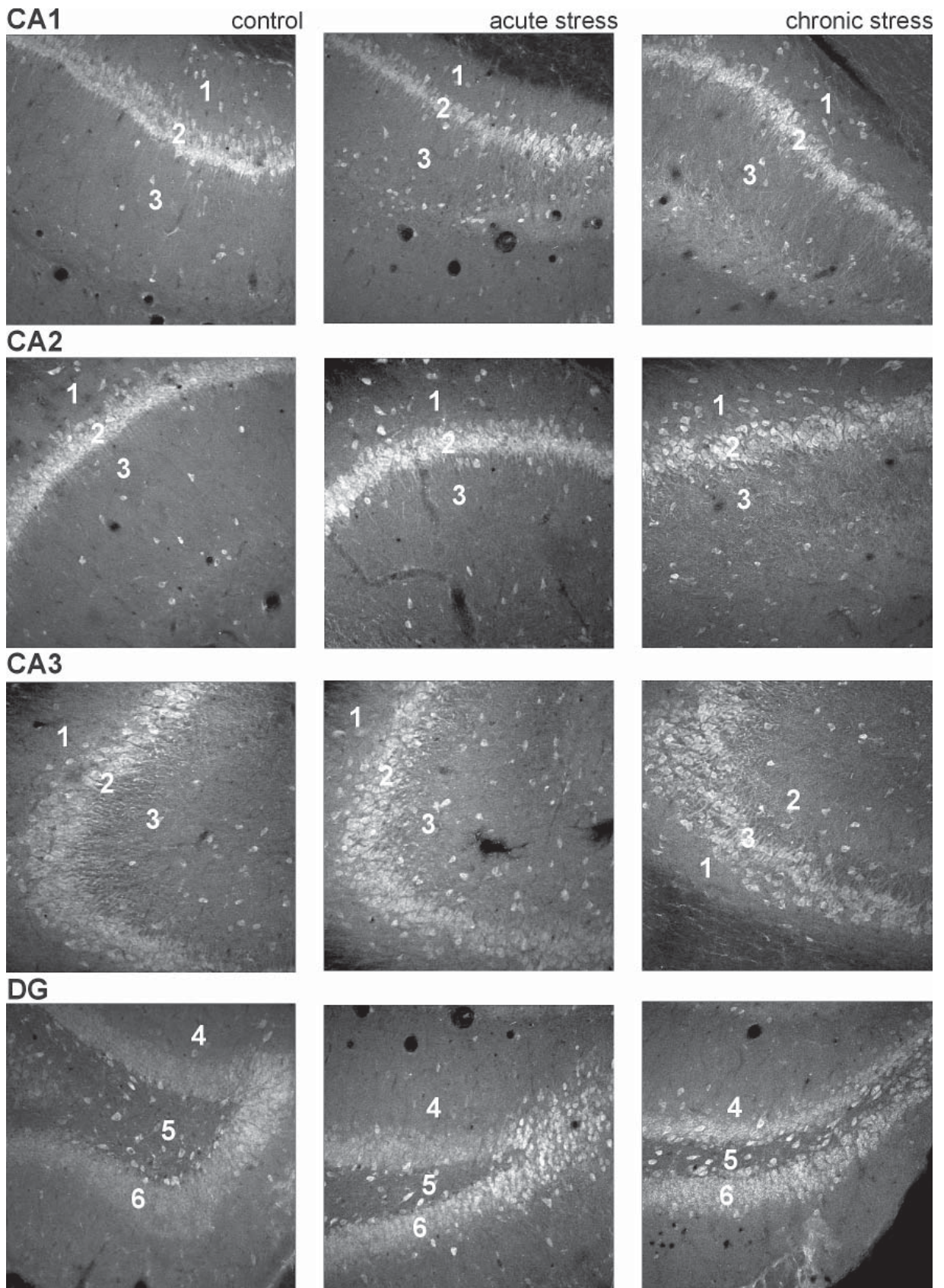
We noted that chronic open-field exposure caused a decrease in the number of c-Fos-ir cells in comparison to that following acute stress in CA1-CA3 and in the DG. The number of c-Fos-ir neurons after chronic stress was comparable to that of the control group. Most authors have agreed that different kinds of chronic stress strain (social defeat, restraint, immobilisation or treadmill) cause a decrease in both c-fos mRNA and c-Fos-immunoreactivity within the hippocampus. This effect is also duration-dependent [10, 28, 34]. It is thought that a decrease in c-Fos in the hippocampus is a result of the adaptive response to stress [10, 43]. He et al. [21] showed that inhibition of c-Fos expression in the hippocampus (especially in CA3) by antisense oligonucleotide treatment

**Table 1.** Semiquantitative data concerning the c-Fos-, TrkA- and NGF-immunoreactivity in the amygdala nuclei of the groups studied. The number of immunoreactive cells:  $\pm$  few; + moderate; ++ high; +++ very high

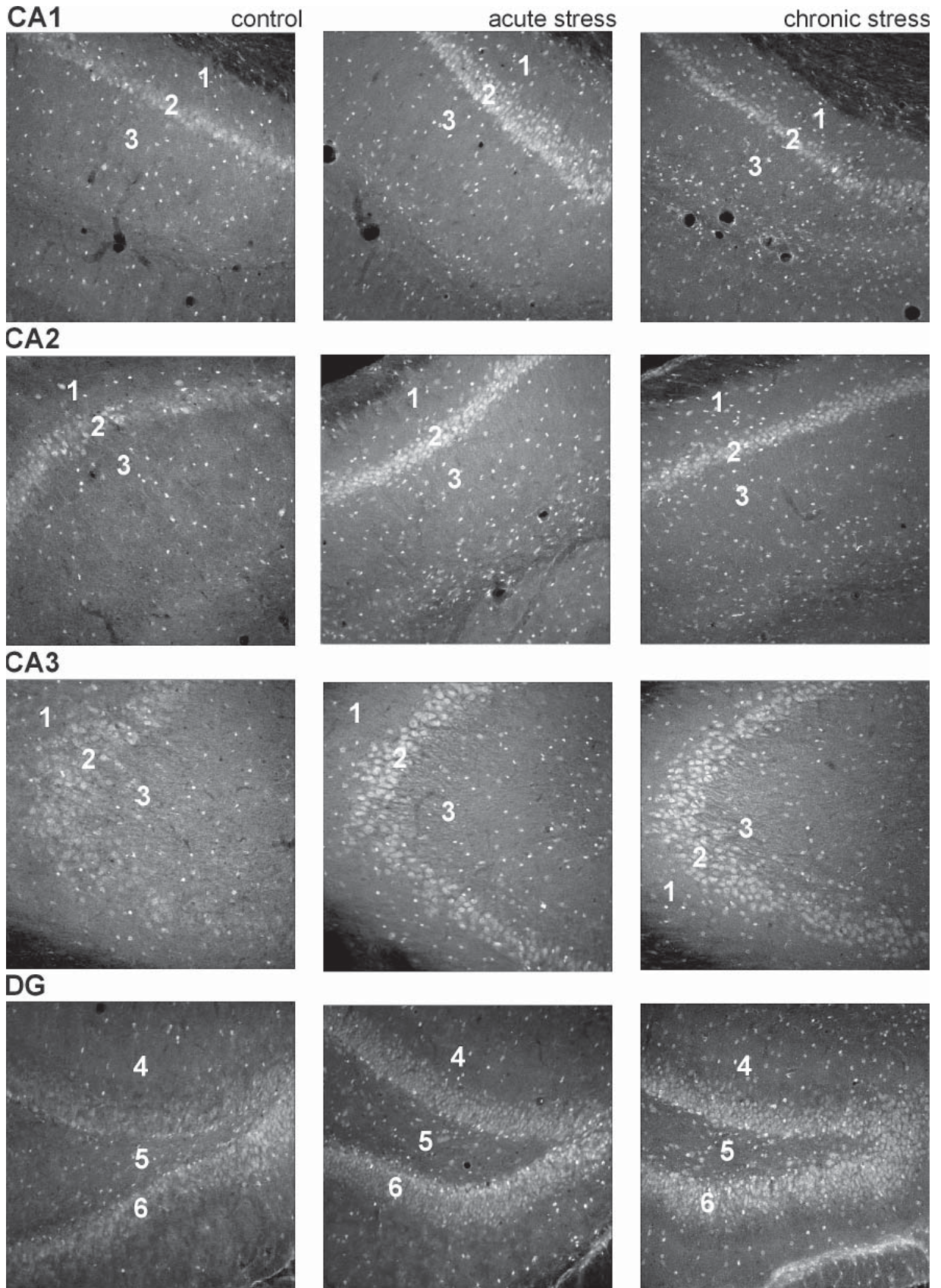
Region	Non-stressed (control group)	Acute stress	Chronic stress
<b>c-Fos-immunoreactivity</b>			
CA1	+	++	+
CA2	+	+++	$\pm$
CA3	+	++	+
Dentate gyrus	$\pm$	++	+
<b>TrkA-immunoreactivity</b>			
CA1	++	+++	+++
CA2	++	+++	+++
CA3	++	+++	+++
Dentate gyrus	++	+++	+++
<b>NGF-immunoreactivity</b>			
CA1	+	+++	++
CA2	+	+++	++
CA3	+	+++	+++
Dentate gyrus	++	+++	+++



**Figure 1.** c-Fos-immunoreactivity in the CA1, CA2, CA3 and DG of the hippocampus in rats exposed to acute or chronic open-field tests as well as in the control group. 1 — polymorphic layer; 2 — pyramidal layer; 3 — radial layer; 4 — molecular layer; 5 — polymorphic layer; 6 — granule layer. Scale bar: 100  $\mu$ m.



**Figure 2.** TrkA-immunoreactivity in the CA1, CA2, CA3 and DG of the hippocampus in rats exposed to acute or chronic open-field tests as well as in the control group. 1 — polymorphic layer; 2 — pyramidal layer; 3 — radial layer; 4 — molecular layer; 5 — polymorphic layer; 6 — granule layer. Scale bar: 100  $\mu$ m.



**Figure 3.** NGF-immunoreactivity in the CA1, CA2, CA3 and DG of the hippocampus in rats exposed to acute or chronic open-field tests as well as in the control group. 1 — polymorphic layer; 2 — pyramidal layer; 3 — radial layer; 4 — molecular layer; 5 — polymorphic layer; 6 — granule layer. Scale bar: 100  $\mu$ m.

resulted in an impairment of spatial memory formation in a maze test and they put forward the hypothesis that c-Fos is essential for encoding this memory.

A decreased number of c-Fos-ir cells in the hippocampus after chronic stress in comparison with that after acute stress may indicate the phenomenon of habituation to open-field exposure and may reflect a state of molecular plasticity within this structure [47]. Structural plasticity in response to repeated stress starts out as an adaptive response [41, 63]. Most probably neuroendocrine changes in HPA axis activity underlie the habituation process [28, 43, 45].

In the regions of the hippocampal formation studied in the control group we observed a large number of TrkA-ir neurons in CA1-CA3 and the DG. This has also been reported by other authors [3, 56].

We noted a very large number of TrkA-ir neurons in all the hippocampal regions studied in response to acute stress. This mainly concerned the polymorphic and granule cell layers of the DG and the pyramidal and polymorphic layers of CA1-CA3. The strong expression of TrkA suggests that this receptor may be involved in activity-dependent neuronal plasticity and may play a role in signal transduction mechanisms linked to NGF [20, 55].

We have shown that after chronic stress the number of TrkA-ir cells was high and similar to that in rats exposed to acute stress. An increased number of TrkA cells in the hippocampus has also been reported after exploration of an enriched environment [49, 50]. However, our results are contrary to those of Ueyama et al. [60], who noted a reduced level of NGF high-affinity receptors in the brain after exposure to long-lasting immobilisation stress. The effects of chronic stress on the expression of the TrkA receptor probably depend on stress paradigm experience. A persistently large number of TrkA-ir neurons following chronic open-field exposure may be involved in the cognitive function of hippocampal formation in this kind of stress.

In our study we have found that in the control rats the number of NGF and TrkA-ir neurons was similar in the DG, whereas in CA1-CA3 the former were less numerous. NGF (and its receptor TrkA) was present in the neurons of the adult hippocampal formation of the control animals, indicating the engagement of trophic factors in the normal functioning of cells and regulation of synaptic activity and neurotransmitter synthesis [35, 37, 61, 64].

We noted that in the animals exposed to acute stress the number of NGF-ir neurons was higher than

that in animals of the control group. NGF was localised in the granule cells of the DG and pyramidal neurons of the CA1-CA3, which are also targets for glucocorticoid action [13, 60]. Several studies have reported changes in neurotrophin levels in the hippocampus in relation to the stress model [2-4, 11, 54]. Our results are in accordance with those of some authors who have shown that NGF levels in the hippocampus are enhanced by emotional engagement [7]. However, others have reported a contrary effect [54, 60].

We found an increased number of NGF-ir cells in the structures studied in the rats who had undergone chronic open-field exposure in comparison with the control group. This phenomenon has also been observed by other authors [19, 60, 64]. However, the number of NGF-ir cells after chronic stimuli in CA1 and CA2 was smaller compared to that after acute stimuli, but was not changed in CA3 and DG.

Neurotrophins elicit numerous brain neuroprotective effects during stress [7, 22, 30]. Our findings suggest that a role is played by NGF as a factor mediating both the short and long-term effect of experience on brain structure and function [7] and indicate brain plasticity in adult rats.

It is known that the hippocampus is implicated in processing fear responses, cognitive function and phenomena related to the activity of the HPA axis [23, 54]. Although the activity-dependent release of NGF from hippocampal neurons has been reported [27], a change in neuronal activation during stress does not always correspond to a change in NGF concentration [54]. While the level of c-Fos-immunoreactivity indicates adaptation after chronic open field exposure, the increase in TrkA-ir and NGF-ir observed was similar following both acute and chronic stress.

We noted that the molecular layer of the DG was characterised by an absence of TrkA-ir cells, suggesting that not only the TrkA receptor but also the other receptor (p75) takes part in NGF binding. The growing significance of the p-75 receptor to the biological effects of NGF has recently been described [11, 17].

Our findings indicate that neurons of CA1-CA3 and the DG are engaged in the stress response after acute as well as chronic open-field exposure. This is probably related to the important role of the hippocampus in processing new spatial information as well as in habituation processes, although these mechanisms appear to be different.

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