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# The immunoreactivity of c-Fos, NGF and its receptor TrkA after open-field exposure in the central and medial nuclei of the rat amygdala

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*The amygdala is a critical component of the neuroanatomical stress circuit. It plays a role in the generation of responses to emotional stimuli. The central (CeA) and medial (MeA) amygdaloid nuclei are implicated in activation of the hypothalamic-pituitary-adrenocortical (HPA) axis.*

*The immunoreactivity (-ir) of c-Fos, NGF and its receptor, TrkA, following acute and chronic open-field stress were studied in the CeA and MeA nuclei of the amygdala. The material consisted of 21 male adult rats divided into three groups: non-stressed (control) animals, rats exposed to acute (once only lasting 15 min) and chronic (15 min daily over 21 days) aversive stimulation (open-field exposure). The brains were stained with the use of immunohistochemical methods for c-Fos, NGF or TrkA.*

*In the control rats c-Fos-, TrkA- and NGF-ir cells were observed in the nuclei studied, but the quantity varied, being moderate or high (immunoreactive to TrkA and NGF) or low (immunoreactive to c-Fos).*

*In the animals exposed to acute open-field stress the number of c-Fos-ir, NGF-ir and TrkA-ir cells in the nuclei under examination was differentiated but higher than that in the control animals.*

*In the animals exposed to chronic open-field stress the number of c-Fos-ir cells in the nuclei studied was similar and was smaller than those in animals exposed to acute stress. The number of TrkA-ir neurons was also lower in comparison to that in animals exposed to acute stress. However, no significant differences in the number of NGF-ir cells were observed between the groups exposed to acute and chronic stress.*

*Diverse expression of c-Fos protein following both acute and chronic stress stimulation may prove the functional heterogeneity of the amygdaloid nuclei investigated. The decrease observed in both c-Fos- and TrkA-ir in MeA (only TrkA in CeA) of animals exposed to chronic stress may indicate the phenomenon of habituation.*

**Key words: amygdaloid complex, open-field test, stress, neurotrophins**

## INTRODUCTION

The amygdala plays an important role in the generation of appropriate responses to emotional stressors [12] and takes part in the perception of stress severity [7]. The central (CeA) and medial (MeA) amygdaloid nuclei are especially involved in the

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response to stress stimulation through the hypothalamic projection and implication in the activation of the hypothalamic-pituitary-adrenal (HPA) axis [12, 23, 29, 32]. They have an important role within the neural circuitry, controlling responses to psychological stressors such as open-field exposure [10, 12, 41, 44].

Exposure to a novel environment is one example of an animal model of limbic-mediated stress. In such cases lesions of the central or/and medial nuclei of the amygdala impair hormonal responses to this kind of stressor in the rodent [12]. Such stressors appear to be relayed primarily through limbic forebrain inputs to the hypothalamus [39]. Although the neuronal activity of amygdaloid nuclei is related to stress duration [45], many issues concerning the mechanism of these responses still remain unclear.

Certain authors have noted a change in c-Fos--immunoreactivity in the central and/or medial nuclei of amygdala following exposure to various kinds of stressors, both acute and chronic (alarm pheromone, noise, restraint, forced swim, footshock and open-field) [6, 11, 13, 25, 48].

Endogenously released (also during stress) nerve growth factor (NGF) may contribute to structural changes in the mature brain by promoting cell repair and the remodelling of damaged tissues [1, 2, 5]. Apart from this neurotrophic function, NGF is implicated in the activity of HPA axis and may regulate the response of neurons to stress stimulation [1, 40, 47].

Most of the effects of NGF are elicited through TrkA receptor [17, 36, 38]. Several lines of evidence from intact brain structures have shown that NGF and its receptor are expressed in the nuclei of the amygdala [28, 42, 43, 47]. It may be involved in neuroendocrine functions, thereby regulating behavioural outcomes [1, 27].

In the present study we investigated whether c-Fos activity in the central and medial amygdaloid nuclei of the amygdala does remain under the influence of acute and chronic stress (exposure to the open-field test) in adult rats and whether there is any influence of the duration of stressful stimuli on the level of NGF- and TrkA-immunoreactivity in these nuclei.

## MATERIAL AND METHODS

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

Following the two-week handling period, 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 performed once for 15 min) and an experimental group exposed to chronic stress (the open-field test performed for 15 min daily over 21 days). Each group consisted of seven animals.

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

At an interval of 90 min after the final exposure all the rats were deeply anaesthetised with lethal doses of Nembutal (80 mg/kg of body weight) and then transcardially perfused with 0.9% solution of NaCl with heparin followed by 4% paraformaldehyde solution in 0.1 M phosphate buffer (pH 7.4). The brains were postfixed in 4% paraformaldehyde fixative 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 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) or 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 in 4°C. After multiple rinses in PBS the sections were incubated (for 2–3 hours 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 40x objective lens, whereas the 568 nm line of this laser was applied to excite Cy3 dye. The optimal iris was used for each magnification.

The number of cells in the amygdaloid nuclei investigated was estimated semiquantitatively and classified into the following: –/+ few, + moderate, ++ large, +++ very large.

**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: –/+ few; + moderate; ++ large; +++ very large

| Nucleus | Non-stressed (control group) | Acute stress | Chronic stress |
|---|---|---|---|
| c-Fos-immunoreactivity | | | |
| Medial (MeA) | +/– | +++ | + |
| Central (CeA) | +/– | + | + |
| TrkA-immunoreactivity | | | |
| Medial (MeA) | ++ | +++ | ++ |
| Central (CeA) | ++ | +++ | ++ |
| NGF-immunoreactivity | | | |
| Medial (MeA) | + | ++ | ++ |
| Central (CeA) | + | ++ | ++ |

## RESULTS

Only single c-Fos-ir cells were observed in the control animals in both the amygdaloid nuclei examined. The acute exposure to the open field resulted in an increase in the number of c-Fos-ir neurons predominantly in MeA, in comparison to that of the control group.

Exposure to chronic stress resulted in an inconsiderable and similar increase in the number of c-Fos-ir cells in both nuclei (Table 1, Fig. 1).

In the control animals the number of TrkA-ir cells in both the amygdaloid nuclei investigated was large. Exposure to acute stress caused an increase in the number of TrkA-ir neurons in both CeA and MeA. However, we did not observe any significant change in the number of TrkA-ir cells following chronic stress stimulation in comparison with those in the control rats (Table 1, Fig. 2).

A moderate number of NGF-ir neurons was observed in the control animals. Acute exposure to the open-field stimulation resulted in an increase in the number of NGF-ir cells in both the nuclei of the amygdalae under examination. However, the number of NGF-ir neurons in MeA and CeA following acute stress simulation was similar to that of TrkA. Chronic stress stimulation caused a similar increase in the number of NGF-ir cells. These also corresponded in number to TrkA-ir neurons in both the nuclei of the amygdalae investigated (Table 1, Fig. 3).

## DISCUSSION

In our study acute exposure to the open field caused a differentiated increase of c-Fos expression in the nuclei of the amygdale investigated, strong in MeA, but only moderate in CeA. Our results are in concordance with those of Day et al. [10], Dayas et al. [12], Fiquiredo et al. [15], Emmert and Herman [14] and Kiyokawa et al. [25]. However, Martinez et al. [33] found that exposure to an intruder caused intense c-Fos expression involving both medial and central amygdaloid nuclei.

The number of c-Fos-ir neurons in MeA and CeA depends on the nature of stress stimulation [6, 11]. c-Fos induction in MeA can be a result of restraint, novelty, forced swim and noise, whereas in CeA it may result from immobilisation, hypovolaemia or ether inhalation [7, 23].

Many reports have confirmed that the open-field test activates a specific circuitry in the amygdaloid nuclei [3, 14]. It is known that CeA and MeA play a key role in a regulation of stress response by HPA axis activity [23–25]. Moreover, Dayas et al. [12] observed that the medial, rather than the central, amygdala is critical to hypothalamic activation during an emotional stress response.

We have noted that chronic open-field exposure caused a decrease in the number of c-Fos-ir cells in comparison to that following acute stress, but only in MeA. However, the number of c-Fos-ir neurons following chronic stress was still higher than that in the control groups in both MeA and CeA.

Different kinds of chronic stress strain can cause an increase or decrease in c-fos mRNA or c-Fos-ir in the amygdaloid nuclei, although most authors agreed that the latter effect is more common [16, 26, 48]. Persistent or increased c-Fos expression as an effect of a chronic emotional stressor in the amygdaloid nuclei was observed in mice by Matsuda et al. [34] and in rats by Dayas et al. [12].

The decreased number of c-Fos-ir cells in MeA observed by us 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 the limbic-HPA axis [37]. Most probably neuroendocrine changes in HPA-axis activity underlie the habituation process [26, 35]. In contrast, the maintained level of c-Fos-ir in CeA following repeated stress may indicate little involvement in the response to the open-field stimulation.

In the studied amygdaloid nuclei of the control group we observed numerous TrkA-ir neurons. The presence of such a population of cells in the amygdala has not hitherto been reported, although some investigations concerning forebrain structures have been carried out [31, 43].

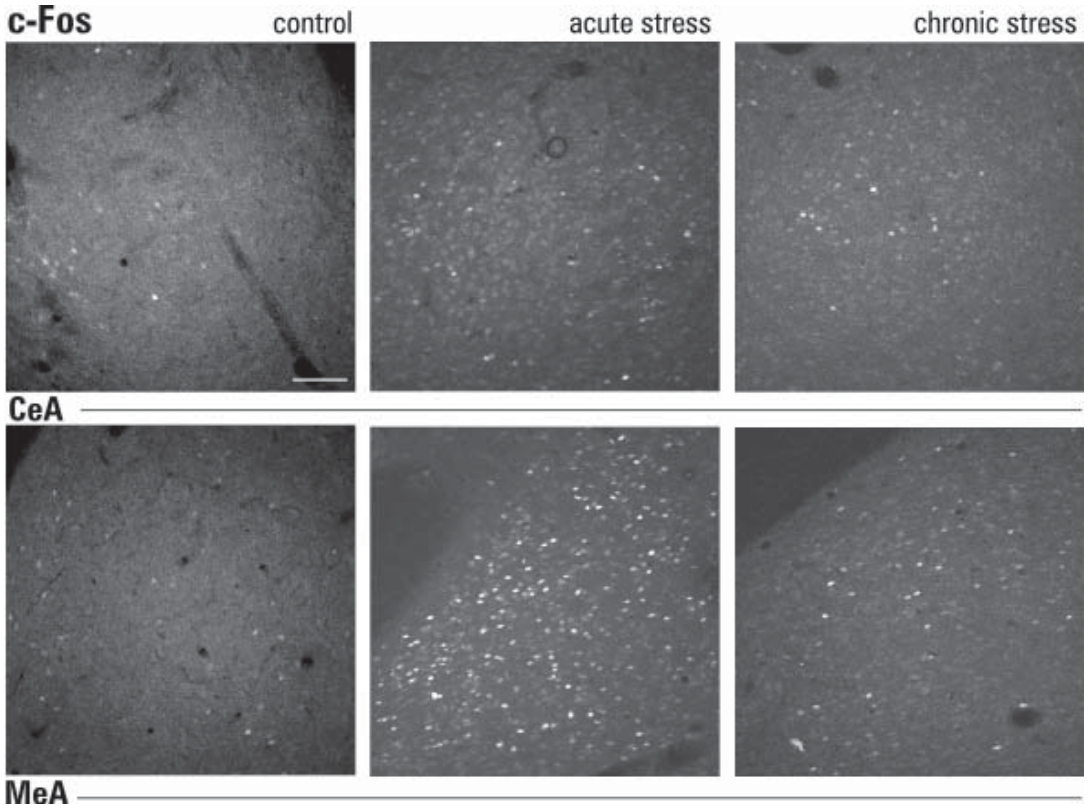


**Figure 1.** c-Fos-immunoreactivity in the studied nuclei of the amygdala in rats exposed to acute or chronic open field test and in the control group; CeA — central nucleus, MeA — medial nucleus. Scale bar: 100 $\mu$m.

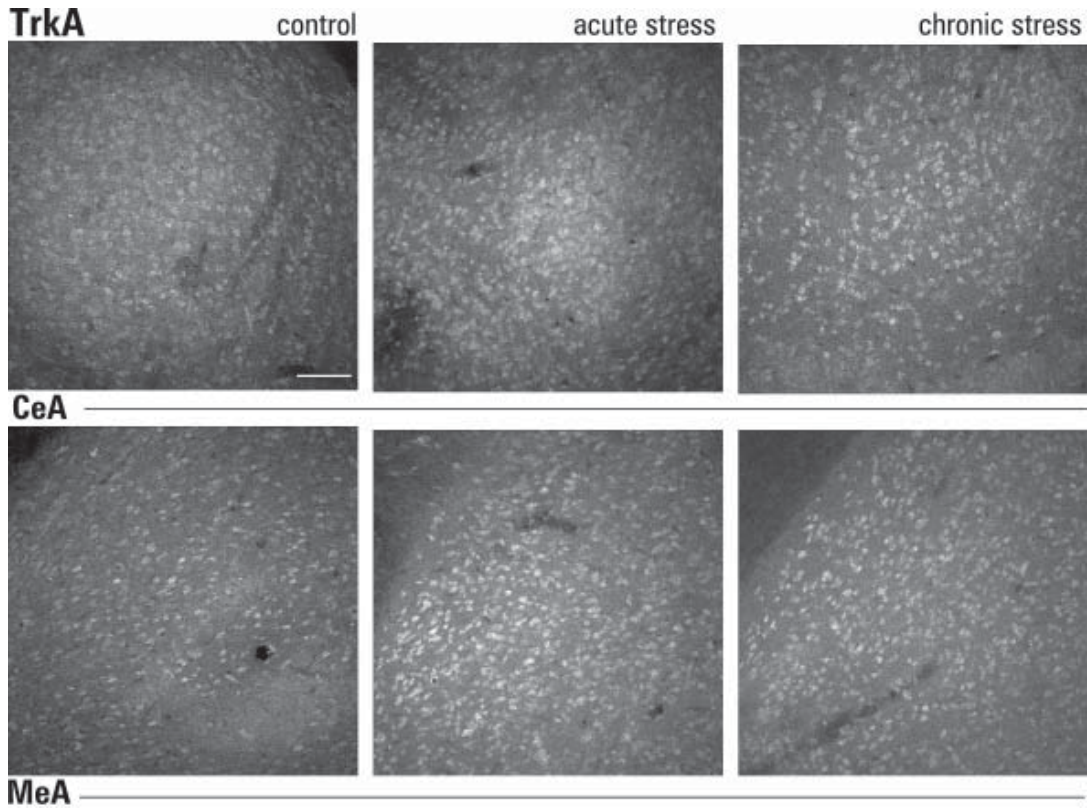


**Figure 2.** TrkA-immunoreactivity in the studied nuclei of the amygdala in rats exposed to the acute or chronic open-field test and in the control group; CeA — central nucleus, MeA — medial nucleus. Scale bar: 100 $\mu$m.

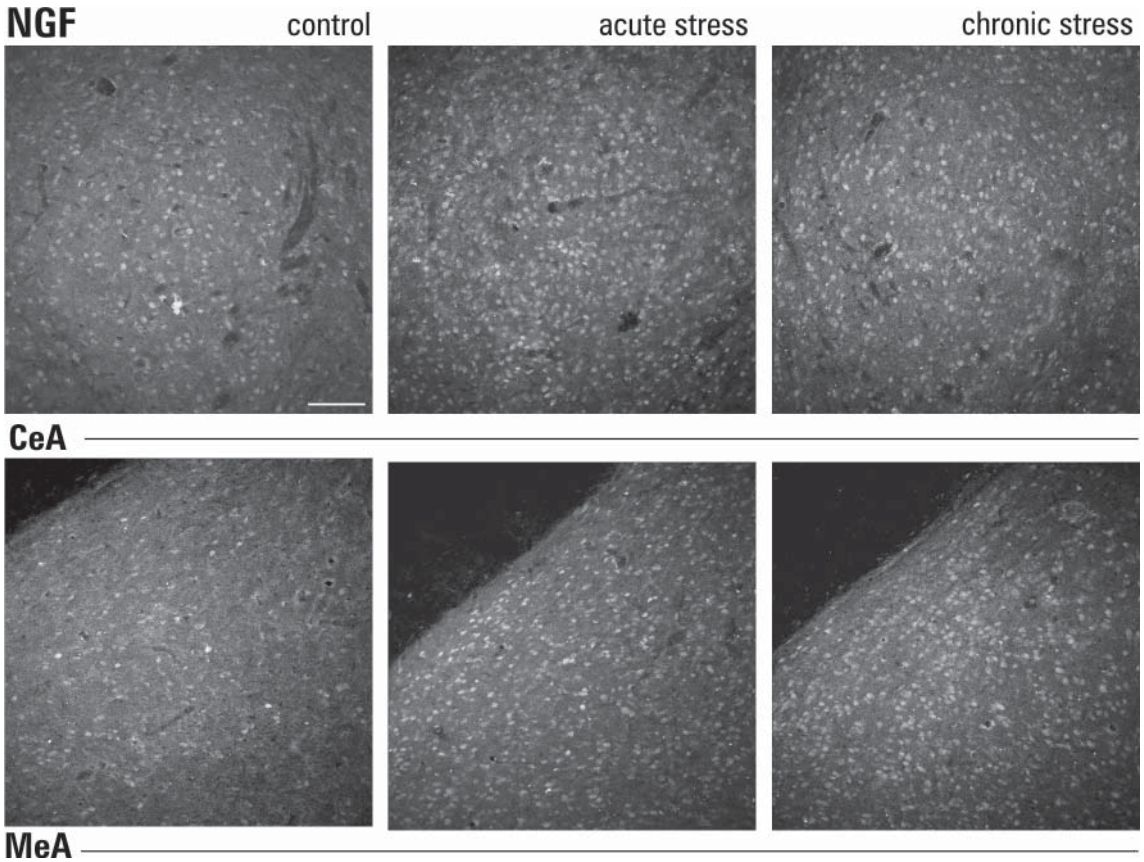


**Figure 3.** NGF-immunoreactivity in the studied nuclei of the amygdala in rats exposed to the acute or chronic open-field test and in the control group; CeA — central nucleus, MeA — medial nucleus. Scale bar: 100 $\mu$m.

We have noted a very large number of TrkA-ir neurons in MeA and CeA in response to acute stress. Several studies now suggest that TrkA receptors, activated by stress, can serve as retrograde NGF signal carriers after endocytosis at the axon terminals [2, 8, 9, 20, 46].

We showed that after chronic stress the number of TrkA-ir cells was lower in comparison to those in the animals exposed to acute stress but similar to those of the control rats. Our results are in concordance with those of Ueyama et al. [46], who noted a reduced level of NGF-high affinity receptors in the brain after exposure to long-lasting immobilisation stress. Such a decrease in TrkA protein level in some amygdaloid nuclei may be explained by utilisation of receptors as a result of habituation after open-field exposure [8].

In our study we found that in the control rats the number of NGF-ir neurons in the nuclei studied was lower in comparison to the number of TrkA-ir neurons. NGF and its receptor TrkA are present in the neurons of control animals, indicating engagement of trophic factors in normally functioning of cells [30]. Lines of evidence from both injured and intact brain structures have shown that NGF is expressed in the amygdala [4, 18, 21, 47, 49].

We have noted that in the animals exposed to acute stress the number of NGF-ir neurons (both in MeA and CeA) was larger as compared with those in the control rats. Our results are in accordance with the data of other authors who have shown that NGF levels in the brain areas such as the basal forebrain are enhanced by emotional stress in adult rodent specimens [5]. However, the level of NGF-ir depends on the nature of the stressor. Von Richthofen et al. [47] found that after forced running the level of NGF immunoreactivity did not change. Moreover, the same authors have shown that acute physical stress, namely the experience of physical threat and pain, resulted in NGF reduction in the amygdala.

In the amygdaloid nuclei examined we found no significant changes in NGF-ir after chronic stress as compared with those after acute stress; however, the level of immunoreactivity was higher than that in the control animals. It has been demonstrated that

exposure to long-lasting psychological stressors can also induce changes in NGF concentrations in other brain regions [19, 46]. Zhu et al. [49] have noted that long-term social experience may influence neurotrophin levels in the amygdalae of adult mice.

Neurotrophins elicit numerous brain neuroprotective effects during stress [5, 21, 28]. A persistent number of NGF-ir cells may indicate its role in suppression of the hypothalamo-neurohypophyseal system in response to long-term stress stimuli.

Our data concerning the pattern of changes in NGF-ir in the central and medial amygdaloid nuclei after acute and chronic open-field exposure in the adult rat are a novelty. It is known that the amygdala is implicated in the processing of fear responses as well as in the activation of HPA axis [22]. However, the change in neuronal activation during stress does not always correspond to changes in NGF concentration [40]. While the level of c-Fos-ir and TrkA-ir indicated adaptation after chronic open field, the observed increase in NGF-ir following both acute and chronic stress was similar.

## CONCLUSIONS

Our results suggest that neurons of MeA and CeA show differentiated levels of activation in response to open-field exposure, which is probably related to their functional heterogenity and participation in different neurosecretory pathways.

The decrease in both c-Fos-ir and TrkA-ir observed in the studied nuclei of the chronically stressed animals may indicate the phenomenon of habituation. This phenomenon does not involve activation of NGF.

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