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The influence of open field exposure on neurons containing nitric oxide synthase in the basolateral complex and paracapsular intercalated nerve cells of the rat amygdala

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Our intention in the present study was to ascertain whether NO-producing cells in the basolateral complex (BLC) and paracapsular intercalated nerve cell groups (Ip) of the amygdala are activated in the open field (OF) test. The material consisted of 8 adult rat brains. The OF test was applied throughout 10 min and 90 min before the death of the animals. The brain sections were double stained using the antibodies against c-Fos (marker of neuronal activation) and against nitric oxide synthase (NOS — marker of NO-producing cells).

The neurons containing NOS and those revealing c-Fos activity constituted distinct populations within both the BLC and Ip but NOS-immunoreactive fibres often surrounded the c-Fos-immunoreactive neurons. Our results suggest that (1) neurons of the basolateral complex of the amygdala and paracapsular intercalated islands are involved but probably not crucial for the open field stress response and (2) NOS-immunoreactive cells in the BLC and Ip are not activated after OF exposure.

Key words: stress, c-Fos protein, NO-producing neurons

INTRODUCTION

The stress system co-ordinates the adaptive responses of the organism to stressors of any kind [31]. Stressors can be divided into two categories: (1) systemic, which directly threaten survival and activate the paraventricular hypothalamic nucleus (PVH) through the ascending pathway from the brainstem and (2) processive (emotional, psychological), which activate the cortical and limbic areas before the PVH is activated [28]. The amygdala, throughout its connections, activates the hypothalamic-pituitary-adrenal axis by processive stressors such as an unfriendly and unfamiliar environment [28].

The basolateral complex (BLC), composed of lateral (L) and basolateral nuclei (BL) [5], is a critical component of the amygdaloid body. The lateral nucleus receives most of the cortical and subcortical input from the visual, auditory and somatosensory modalities and may integrate this sensory information [9, 18, 19, 25]. Direct and indirect projections from the lateral nucleus connect it with the central nucleus (Ce) of the amygdala [25] and by this pathway can generate the fear response [19]. The intercalated masses of the amygdala (Ip) [2] surround the BLC. The cell group lying between the basolateral complex and the central nucleus receives inputs from the BLC and sends them to the central nucleus [11], representing an inhibitory interface between the input and output nuclei of the amygdala.

Nitric oxide (NO) is well-known as a gas that acts in the nervous system as a neurotransmitter or a neuromodulator [5]. Neuronal NOS (nNOS) is the

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brain-specific isozyme that makes NO in the brain [13, 22]. The role of NO is not confined to a cellular level but is also involved in regulation of the activity of specific physiological systems [17].

It has been demonstrated that NO can mediate the stress responses in the amygdala [16, 29]. In view of this, our intention was to ascertain whether NOS-containing cells in the basolateral complex of the amygdala and paracapsular intercalated nerve cells are activated by the open field (OF) test, a model of an unfamiliar and unfriendly environment. The engagement of these cells in the stress response can be evaluated by the detection of c-Fos protein, commonly recognised as a marker of neuronal activation [15, 26, 30].

MATERIAL AND METHODS

The material consisted of 8 adult rats of the Wistar strain. The experimental group consisted of 5 animals, while the control consisted of 3. Care and treatment of the animals were in accordance with the guidelines for laboratory animals established by the National Institutes of Health as well as by the Local Ethical Committee. The rats of the experimental group were exposed to the open field test throughout 10 minutes. The open field was constructed of a white wooden floor and walls (100 × 100 × 40 cm) and was illuminated with a 500--watt halogen light. The control animals were kept in a home cage. After 90 min all the animals were deeply anesthetised with lethal doses of Thiopental (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-μm-thick serial sections of the brain were cut on a JUNG 1800 cryostat (Leica, Germany). The sections were then double stained with the immunohistochemical method. The free floating sections were blocked with 3% normal goat serum (NGS) containing 0.3% Triton X-100 for 1 hour and then incubated with a mixture of rabbit anti-c-Fos antibody (Santa Cruz; diluted 1:1000) together with mouse anti-NOS (Sigma, diluted 1:1500) in 3% NGS for 48 hours in 4°C. After multiple rinses in PBS, sections were incubated (2–3 hours, at room temperature) with a mixture of secondary fluorophore conjugated antibodies: Alexa Fluor 488 goat anti-mouse (Symbios; dilution 1:150) and Cy3-conjugated goat anti-rabbit (Jackson ImmunoResearch; dilution 1: 600).

The immunohistochemically stained slides were examined with fluorescent microscope BX-51 (Olympus, Japan) and the confocal system MicroRadiance (Bio-Rad, UK), equipped with an Argon ion laser and mounted on a light microscope Eclipse 600 (Nikon, Japan), using the software LaserSharp 2000 (Bio-Rad, UK). The confocal laser scanning microscopy (CLSM) images were obtained using 40 × and 60 × oil immersion objective lenses of NA = 1.3 and 1.4, respectively. The optimal iris was used for each magnification. For the reconstruction of the images the program LaserSharp 2000 v. 2.0 (Bio-Rad; UK) was used.

RESULTS

NOS-immunoreactivity

The distribution of NOS-immunoreactive (-ir) neurons in the basolateral complex was similar in all the brains studied. The lateral nucleus showed the highest number of labelled cells. There were considerably less NOS-ir neurons in the basolateral nucleus and many of these cells were located along the ventral and medial borders of the nucleus. There were also numerous stained NOS-ir cells surrounding the basolateral complex — located in the paracapsular intercalated nerve cell groups (Fig. 1).

Intensely stained NOS-ir neurons both in BLC and Ip typically had ovoid or fusiform cell bodies and 3 to 4 primary dendrites, although some were bipolar (Fig. 1A, B).

NOS-ir structures in neuropil comprised fibres and axon terminals. NOS-ir fibres in the lateral nucleus were of two kinds, (1) thin and (2) thick with varicosities (Fig. 1A). The latter were less numerous. In the lateral part of the basolateral nucleus the thin fibres predominated (Fig. 1C).

NOS-ir neurons interposed between the basolateral complex and the central nucleus and located in the adjacent external capsule were intensely stained, with elongated ovoid or fusiform cell bodies and with dendrites oriented parallel to the basolateral complex borders. NOS-ir fibres were intensely stained, thick and with varicosities (Fig. 1B).

c-Fos-immunoreactivity

Single scattered c-Fos positive cells were observed in the basolateral complex of the control rats. Both the number and the distribution of c-Fos-ir cells in the response to the open field exposure were differentiated among individual animals. c-Fos-ir cells were found in the lateral and basolateral nuclei and in the

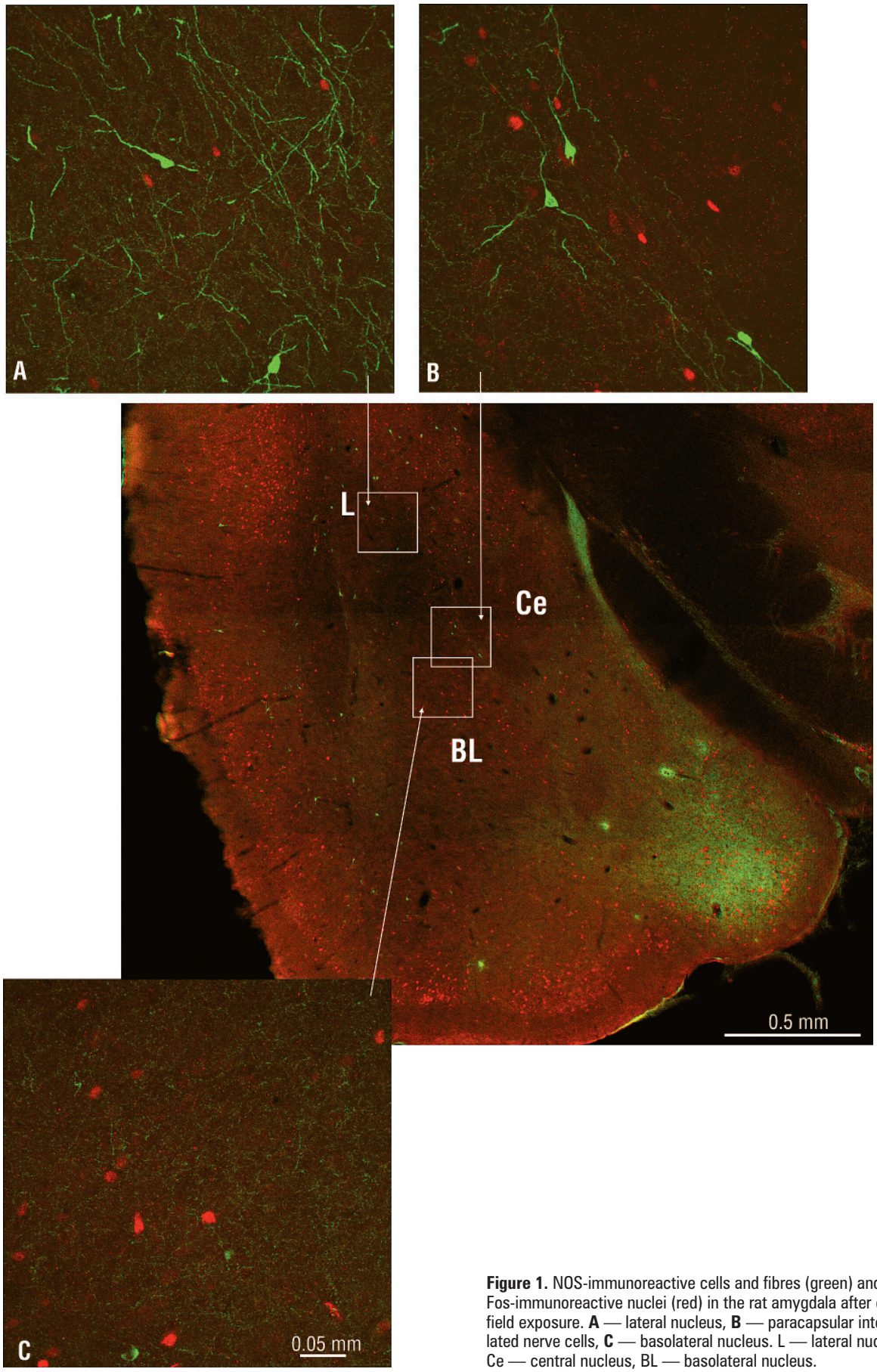

Figure 1. NOS-immunoreactive cells and fibres (green) and c-Fos-immunoreactive nuclei (red) in the rat amygdala after open field exposure. **A** — lateral nucleus, **B** — paracapsular intercalated nerve cells, **C** — basolateral nucleus. L — lateral nucleus, Ce — central nucleus, BL — basolateral nucleus.

paracapsular intercalated cells (Fig. 1). Their intensity characterised the diverse level of immunolabelling, from high to low. No clearly visible pattern of distribution of c-Fos-ir cells was observed within BLC in the response to the open field exposure, but it seems that they were more often localised in the medial part of the basolateral nucleus (Fig. 1).

Double NOS/c-Fos immunolabelling

The double immunolabelling study revealed that cells containing NOS and those containing c-Fos constituted distinct populations within the BLC (Fig. 1A, C). Very few cells showed co-localisation — they were observed mostly in the medial part of the basolateral nucleus. Among cells of paracapsular intercalated groups no co-localisation with c-Fos was observed (Fig. 1B).

Relatively often we observed thin NOS-ir fibres surrounding cells containing c-Fos-ir nuclei (sometimes they formed basket-like structures; Fig. 1).

DISCUSSION

The results of the present study demonstrate that the nitric oxide synthase-containing neurons in the basolateral complex of the amygdala do not reveal c-Fos activity in response to open field exposure.

The exposure to the open field did not significantly influence either the morphology or the distribution patterns of NOS-ir cells in BLC. They were similar to those reported previously [20, 33].

The morphology and localisation of NOS-ir neurons belonging to the paracapsular intercalated cell groups surrounding BLC suggest that they can be GABAergic neurons [11, 23] receiving glutamergic input from the basolateral complex and sending GABAergic fibres to the central nucleus [11].

The relatively low number of c-Fos-ir cells both in the BLC and paracapsular intercalated islands were confirmed earlier, revealing that after exposure to a processive stressor c-fos expression was more prominent in the medial (Me) than in other amygdaloid nuclei [7, 8, 10, 24, 28]. In contrast to the consistency of Me activation by processive stressors, activation of other amygdaloid nuclei is, according to various authors, differentiated, even for the same stressor. For example, restraint generally elicited very little c-Fos expression in BLC [8], although, in contrast, Bhatnagar and Dallman reported substantial c-fos expression throughout the whole amygdala in the same conditions [3]. In the present study we observed relatively low c-Fos expression in BLC and Ip. However, in the case of an unconditioned emotional stressor, namely open field exposure, it appears that these structures are in fact not critical. It seems that BLC, Ce and paracapsular intercalated nerve cell groups are more important for the generation of responses of conditioned emotional stressors [8, 32].

The c-Fos-ir cells observed by us in the BLC support the hypothesis that the basolateral complex of the amygdala could be a locus of neuroplasticity underlying fear-based memory formed and stored within this structure [21]. These c-For-ir neurons in the BLC probably "mark" their readiness for memory processing. According to Kaczmarek and Chaudhuri [14] and Savonenko et al. [27], c-Fos accumulation is not a mere indicator of functional neural activity but reflects a predisposition to plastic change. This hypothesis is additionally supported by the presence of long-term potentiation (LTP) of excitatory synaptic transmission, which is a form of activity-dependent plasticity which may underlie learning and memory [1, 4]. It has been demonstrated that synapses in the amygdala (especially in the lateral and basolateral nuclei) display LTP [1, 6, 12, 34], which confirms the hypothesis concerning memory-related plasticity in this structure [35]. It has also been revealed that NO plays a role in facilitating the induction of LTP in the medial amygdala [1]. It is known, that neurons cannot sequester NO; thus the key to regulating NO activity is to control NO synthesis [13]. In this case, the question arises as to why NOS-ir neurons in BLC are hardly activated after open field stress. Some reasons have been put forward by Abe et al. [1]. According to them, long-term potentiation in the lateral amygdala is NO-independent. Additionally, the presence of NOS-ir endings located around the cells containing c-Fos-ir nuclei suggests that the nitric oxide may influence (modulate) the action of stress-activated neurons of BLC.

CONCLUSION

Our results suggest that (1) neurons of the basolateral amygdaloid complex and paracapsular intercalated islands are involved but probably not crucial for open field stress response and (2) NOS-ir cells in these structures are not activated after open field exposure.

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