In the present study we wanted to check whether the expression of the c-Fos protein (the marker of cellular activity) appears in cells containing calcium-binding proteins (CaBPs) in animals exposed to the open field test. Eight adult Wistar rats were examined. In the first step the open field test was applied throughout 10 minutes. After perfusional fixation brains were frozen and cut on the cryostat in the coronal plane and stained with the standard immunohistochemical method. Sections were double stained for c-Fos and CaBPs: parvalbumin (PV), calbindin (CB), calretinin (CR). c-Fos positive cells were localized predominantly in layers II and III of the piriform cortex (PC). The double labeling study showed that neurons containing CaBPs are rarely c-Fos-immunoreactive. Often PV-positive and CB-positive fibers surround c-Fos-positive neurons in layers II and III in a form of a basket. It seems that cells containing CaBPs are not directly involved in the response to aversive stimuli but cells containing those calcium-binding proteins might influence directly c-Fos positive neurons of PC.

Key words: piriform cortex, c-Fos, calcium-binding proteins, open field

INTRODUCTION

The piriform cortex (PC) is the largest cortical olfactory region. Within its cortical layers systems of fibers and neuronal cell types are highly segregated. Besides the obvious involvement in olfactory perception and discrimination, PC has been implicated in memory processing, the spread of excitatory waves and some brain disorders such as epilepsy (1,2). Moreover PC has close reciprocal connections with many limbic areas including the amygdala and hippocampus. Neurons of PC are divided into two populations: projection neurons and GABA-
ergic interneurons. The latter express immunoreactivity for different calcium-binding proteins considers of its marker, and probably fulfill somewhat different functions (3,4).

PC is usually divided into anterior and posterior parts. The border between them is located at the level of the anterior commissure. The differences between these two divisions of PC concern not only morphology but also connections and function (5-7). Several lines of evidence suggest that neurons of that area are involved in the response to various aversive stimulations (8). One way to identify brain areas exhibiting stress-related changes in functional neuronal activity is to determine the expression of the immediate early genes such as c-fos (9-11).

Indeed c-fos and its protein product c-Fos is expressed in different centers of CNS including PC, after the induction of various types of both normal and pathological stimuli (7,8,12-15).

Therefore it has been proposed that c-Fos accumulation is not a mere indicator of functional neuronal activity, but reflects a predisposal to a plastic change (16-18).

The expression pattern of the c-Fos protein in each neuronal pathway depends on the type of stimulus and environmental conditions. The expression of c-Fos is especially intensive after the exposition of a rat in an "open field". According to Ramos and Mormede (19) it is one of the most common behavioural tests used with the aim of inducting a stress response. A new open field, especially the big, white and brightly lighted one, may be regarded as a stress factor responsible for causing proper conditions for the appearance of a stress reaction in neurogenic character. Furthermore, this test is regarded as one of the so called "anxiety tests" and it is used in research concerning animal models of depression and anxiety. In the present study we used c-Fos immunohistochemistry to identify whether the piriform cortex internal system of GABA-ergic interneurons containing different calcium-binding proteins (CaBPs) exposed to "the open field test" were engaged in the stress response.

MATERIALS AND METHODS

The material consisted of eight adult rats from the Wistar strain. Group consisted of five experimental animals and three control ones. Care and treatment of animals 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 experimental group was exposed to the "open field test" throughout 10 minutes. The open field was constructed of wooden white floor and walls (100x100x40 cm) and was illuminated with 500 watt halogen light. Control group was treated with procedures as described by Dielenberg et al. (8). That means their home-cage control group of animals was left undisturbed in their home cages until perfusion. After 90 min. all animals were deeply anesthetized with lethal doses of Nembutal (80 mg/kg of body weight), then transcardially perfused with 0.9% solution of NaCl with heparin, followed by 4% paraformaldehyde solution in 0.1M phosphate buffer (PBS; pH 7.4). The brains were postfixed in 4% paraformaldehyde fixative for 3-4 hours. They were then placed in 15% sucrose in 0.1M PBS
overnight at 4°C) followed by 30% sucrose in 0.1M PBS until sunk. Coronal 40-µm-thick, serial
sections of the brain were cut on JUNG 1800 cryostat (Leica, Germany). The sections were then
stained with double immunohistochemical method. The secondary antibodies used in this study
were fluorophore conjugated. The free floating sections were blocked with 3% normal goat serum
(NGS) in 0.1M PBS or 3% normal donkey serum (NDS) in 0.1M PBS containing 0.3% Triton X-
100 for 1 hour and then incubated with the mixture of polyclonal rabbit anti-c-Fos antibody (Santa
Cruz; dilution 1:1000) together with either polyclonal goat anti-calretinin (Chemicon; dilution
1:500) or monoclonal mouse anti-parvalbumin (Sigma; dilution 1:500), or monoclonal mouse anti-
calbindin-D28 k (Sigma; dilution 1:1500) diluted in 3% NGS in 0.1M PBS or 3% NDS in 0.1M
PBS for 48 hours in 4°C. After multiple rinses in PBS, sections were incubated (2-3 hours, room
temperature) with the mixture of appropriate secondary antibodies: Alexa Fluor 488 goat anti-
mouse or Alexa Fluor 488 donkey anti-goat (Symbios; dilution 1:150) or Cy3-conjugated donkey
anti-rabbit or Cy3-conjugated goat anti-rabbit (Jackson ImmunoResearch; dilution 1: 600) diluted
in 3% NGS in 0.1M PBS or 3% NDS in 0.1M PBS.

Immunohistochemically stained slides were examined with a fluorescent microscope BX-51
(Olympus, Japan) and a 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 microscopy (CLSM) images were obtained using
40X and 60X oil immersion objective lenses of N.A.=1.3 and 1.4, respectively. Argon laser
produced dichromatic light at 488 and 514 nm. The 488-nm line of this laser was applied to excite
the Alexa, using a dichronic beam splitter FT 505 and an emission long-pass filter LP 515. The 514-
nm line of this laser was applied to excite Cy3. The optimal iris was used for each magnification.
For reconstruction of the image analysis program LaserSharp 2000 v. 2.0 (Bio-Rad; UK) was used.
In each case only sections completely stained with fluorescence were taken into account.

RESULTS

Single scattered c-Fos-immunoreactive (c-Fos-ir) cells were observed in the
group of control rats. The significant increase of c-Fos expression took place in
the response to the open field exposure. c-Fos-ir cells were localized predominantly
in layers II and III of PC. There were no major differences in the distribution of
c-Fos-ir cells between anterior and posterior parts of PC (Fig. 1D-E). Calretinin
immunoreactivity (CR-ir) in PC was weak and cortical layers were weakly
distinguishable, except the subpial portion of layer I (Fig. 1A). The number of
immunoreactive cells was small, they were localized predominantly in the layer
II and less in layers III and I. Calbindin immunoreactive (CB-ir) cells were found
mainly in the layer III, whereas in the layer II they were less numerous (Fig. 1B).
There were many labeled fibers in the layer II of PC identified; in remaining
layers their amount was smaller. In layers II and III fibers formed basket-like
structures. The distribution of parvalbumin immunoreactivity (PV-ir) was similar
to CB-ir one, but its intensity was stronger. PV-ir cells occupied mostly the layer
III of PC, whereas the layer II characterized intense immunoreactivity of neuropil
(Fig. 1C). Some of PV-ir fibers, which were more numerous than CB-ir fibers in
the layer II had varicosities. Especially in the layer II a big number of basket-like
structures was present. Cartridges - short, perpendicularly to the surface of brain-oriented rows of PV-ir puncta were observed in the layer III of PC.

The double immunolabeling study revealed that cells containing calcium binding proteins and these showed c-Fos expression constituted distinct populations. Very few cells showed colocalization (Fig. 2A-F). But characteristically PV-ir and CB-ir fibers in layers II and III of PC often

Fig. 1. Distribution of calcium-binding proteins immunoreactivity cells in layers I, II, III of the rat piriform cortex: A-calretinin, B-calbindin D28k, C-parvalbumin. Distribution of c-Fos immunoreactivity in anterior (D) and posterior parts (E) of the rat piriform cortex after the open field test. Scale bar - 100µm.
Fig. 2. Fluorescence immunohistochemistry for c-Fos (red) and calcium-binding proteins (green). Double immunolabeled sections of the rat piriform cortex: A, B - calretinin/c-Fos; C, D - calbindin D28k/c-Fos; E, F - parvalbumin/c-Fos. Arrows indicate cells with colocalization. G - PV immunoreactive fibers surrounding c-Fos positive cells of the layer II of PC form basket-like structures (empty arrow heads), H - Cartridge - PV-ir immunoreactive puncta on proximal parts of dendrites of neurons with c-Fos positive nuclei (white arrow heads). Scale bar -25 µm.
surrounded c-Fos-ir neurons forming basket-like structures (Fig. 2G). Morphology and localization of cartridges observed in double PV/c-Fos staining suggested that there were immunoreactive points on proximal parts of dendrites of neurons with c-Fos-ir nuclei (Fig. 2H).

DISCUSSION

In our study the control rats kept in their home cages exhibited weak c-Fos expression. In the comparison with these control animals, the open field exposed rats showed a large c-Fos production. There were no major differences in the distribution of c-Fos positive cells between anterior and posterior parts of PC. The two subdivisions of PC - anterior and posterior areas, differ in cell morphology, connections and physiology (5,20,21) and could differentially contribute to the olfactory guided behavior but not to the visual behavior. Löscher and Ebert (2) showed that c-Fos induction following amygdala-kindled focal seizures was much more marked in the posterior than anterior PC. Thus, a high c-Fos expression could be expected in the posterior PC because it is strongly reactivated by intrinsic fibers. Furthermore the posterior PC is assumed to be mainly involved in synaptic plasticity and memory processes. On the other hand Datiche et al. (7) demonstrated a statistically higher c-Fos immunoreactivity in the anterior PC in comparison with the posterior one after acquisition of olfactory learning. The anterior PC is known to receive more afferent activity than the posterior part because the density of bulbar afferent fibers decreases from rostral to caudal (1). The sniffing behavior elicits a strong activity in the olfactory bulb. Subsequently, dense bulbar messages reach the anterior PC and could account for the high c-Fos immunoreactivity observed within this area. The afferent bulbar activity is redistributed to the whole PC and particularly to the posterior area via rostro-caudal association fibers system (20). However according to our study the exposition of rats in the open field test did not cause differences in the expression of c-Fos between these two regions of PC. The lack of these differences might be connected with two main reasons: firstly - a rat placed in a new open field treats it as a new environment which causes a strong exploratory reaction and olfactory stimulation, this behavior is correlated with the activation of the anterior part of PC. Secondly, the unknown environment and bright light used in our test induce a strong stress neurogenic reaction, which is expressed in the activation of the posterior area of PC. Our dates are supported by report of Tronel and Sara (22) which described the lack of differences between anterior and posterior region of PC after odor-reward associative learning. Those experiments used olfactory bulb stimulation as a conditioned stimulus and extensive training overall several sessions.

c-Fos positive cells were localized predominantly in the layer II and III of PC what is consistent with the study of Datiche et al. (7). On the basis of literature
the layer II of PC consists of a great number of cells. These cells have semilunar or pyramidal pericaryon and send dendrites into the layer I (1,23,24). Both semilunar and pyramidal cells are probably glutaminergic (2). The layer III consists of large size multipolar cell bodies and fibers, which are presumably GABA-ergic (24). Besides GABA-ergic cells with globular somata are localized in all layers of PC (24).

Taking into account the localization of c-Fos-ir neurons and the fact that they did not colocalize with PV and CB, let us predict that the population of activated cells do not belong to the inner neuronal net of PC but they are supposed to be projection neurons. However this thesis needs to be proved by further investigation. Similar findings have been presented by Filipkowski (25) who showed that majority of cells expressing c-Fos after mechanical stimulation of vibrissae turned out to be parvalbumin-negative. On the basis of Frassoni et al. (26) studies and very rare colocalization of CR and c-Fos protein in the layer II of PC, we can draw conclusions that projecting neurons of that layer must be engaged in the reaction to the exposition in the open field.

The distribution pattern of PV and CB immunoreactivity and also morphology type of cells in the piriform cortex were similar to that described by Kubota and Jones (3), as well as CR immunoreactivity in PC was consistent with results of Frassoni et al. (26). Double immunolabeling study revealed that very few cells containing calcium-binding proteins show colocalization with c-Fos. Our results as far as colocalization of CaBPs and c-Fos are concerned; differ significantly from the Hiscock et al. (27) results achieved in rats after the systemic infusion of picrotoxin and after restricted or generalized seizures. The previously mentioned authors achieved colocalization of c-Fos in about 40% of PV-ir, 55% of CB-ir and 33% of CR-ir neurons. Probably the usage of different models of stimulation may explain differences of results. Because the c-Fos expression occurred in a greater proportion of total spiny cells in PC the above authors conclude that seizures induce c-Fos expression predominantly in excitatory cortical neurons. In our material many PV- and CB-ir fibres in the layer II and III outlined c-Fos positive neurons forming basket-like structures. This observation suggests that at least part of the population of PV-ir cells as well as CB-ir ones are GABA-ergic cells and can be treated as interneurons (28,29) which may play an inhibitory role in the control of cell activity in PC. This theory also supports the presence of PV-ir cartridges. Their morphology and relation to neurons possessed c-Fos-ir nuclei correspond to previously described axon terminal portions of GABA-ergic chandelier cells forming symmetrical synapses on axon initial segments of pyramidal cells (29).

In conclusion, it seems that cells containing calcium-binding proteins are not directly involved in the response to the "open field" stimuli. In the stress reactions other cell populations, presumably projection neurons of the piriform cortex are activated. Taking into account that many PV, CB-ir endings on c-Fos positive
cells are found, the cells containing those calcium binding proteins might influence directly c-Fos positive neurons of PC.

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REFERENCES


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