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Analysis of calretinin immunoreactivity in the rat piriform cortex after open field stress during postnatal maturation

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In our study we used c-Fos protein to identify whether cells containing calretinin (CR) in the rat piriform cortex are engaged in the response to stress stimulation and to find out how this expression changes during maturation (PC). The material consisted of Wistar strain rats of between 0 and 120 days of age divided into 9 groups. Each group consisted of 5 experimental and 3 control rats. Animals from the experimental groups were exposed to the open field test throughout 10 minutes. The control animals were kept in a home cage. In all age-related control rats weak c-Fos immunoreactivity was observed. Our results showed that cells containing c-Fos following an acute open field test were observed predominantly in layers II and III of the PC just after birth. Their number then increased and stabilised on P30. We had already observed immature CR-ir cells at birth. In the 4th week of life these neurons achieved maturity. Their number increased to P90 and decreased in older animals. CR-ir neurons were localised mainly in layer II and to a lesser degree in layers III and I of the PC. Double immunostaining c-Fos/CR revealed that the level of co-localisation was low. Only small differences were observed between the anterior and posterior parts of the PC. In the anterior part a higher number of CR-ir neurons was found. The difference in the level of co-localisation between the anterior and posterior parts was age-related and differentiated. Our results may suggest that during maturation CR-ir neurons of the piriform cortex are not the main population engaged in response to the open field test.

Key words: c-Fos, calcium binding proteins, development, open field test

INTRODUCTION

The piriform cortex (PC), the main area of the primary olfactory cortex, is composed of 3 neuronal layers. Layer I is characterised by the presence of cells which are round in shape and which contain only a few GABAergic neurons occupying the most superficial portion of the layer. These reach a network of ascending dendrites and axonal fibres from the deeper layers [8, 21, 25]. Layer II consists of a great number of densely packed cell bodies. These neurons are thought to be glutamatergic. Layer III contains neuronal cell bodies at lower density and with a considerable quantity of fibres. The cells of this layer are large GABAergic multipolar cells [8, 21, 25]. The piriform cortex receives direct input from the olfactory bulb [14]. This projects into areas that play a role in mediating functions related to the integration of sensory cues with behaviour (the prefrontal

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cortex), assessment of the emotional or motivational significance of sensory cues (the amygdala), and multisensory association and memory (the entorhinal and perirhinal cortex) [22]. The PC interacts with these areas and other parts of the limbic system (the hippocampus, hypothalamus and thalamus) and the prefrontal cortex during the mediation of olfactory-guided behaviour [10, 14].

The piriform cortex is usually divided by the anterior commissure into the anterior and posterior parts on the basis of ontogeny, efferent projections, and the intrinsic organisation of the connections [10, 21, 25]. Neurons of the posterior part are older than those of the anterior part [3].

The neuronal population of the PC consists of GABAergic interneurons and non-GABAergic projecting cells. The population of GABAergic interneurons in this structure appears to correspond to the neurons containing calcium-binding proteins (CaBPs): parvalbumin, calbindin and calretinin [8, 17, 18], although the presence of CaBPs was also described in the projecting neurons [6, 11].

Calcium-binding proteins are interesting from the neuro-anatomical point of view, as they are observed in neurons belonging to multi-functional systems in mammals [13]. Calretinin is an EF-hand calcium-binding protein, the exact function of which is still unclear. CR regulates the intracellular calcium balance and its level may reflect differential metabolic needs [6].

In the development and maturation of the brain calretinin regulates fast synaptic transmission [27]. It has been suggested that calretinin protects neurons against an increase in the calcium level during periods of high frequency discharge or neurotransmitter-induced depolarisation and in pathological conditions such as ischaemia or epileptic seizures [4, 19].

It has been found that neurons of the piriform cortex are involved in the response to a variety of stress stimulations [9, 16, 23]. Proto-oncoprotein Fos, which is encoded by the immediate early gene c-fos, has attracted much attention because Fos has been shown to be induced by strongly activated neurons [9, 21]. As c-Fos expression is a consequence of neural activity, it can indicate neuronal ability to undergo plastic change [24].

Although there are some data concerning the morphology of the PC, the functions of this area is still not clear. Evaluation of the differences between the two parts of the PC in their activity after stress stimulation and in the distribution of calretinin appears, therefore, to be a matter of interest. In the present study we have used c-Fos expression to investigate whether acute open field stress has an effect on calretinin-containing neurons in the piriform cortex and to assess the possible influence of age.

MATERIAL AND METHODS

The material consisted of Wistar rats of postnatal ages between 0 and 120 days (P0 was defined as the day of birth). The rats were divided into 9 groups: P0, P4, P7, P10, P14, P21, P30, P90, P120 days old. Each group consisted of 5 experimental animals and 3 control ones. The care and treatment of the rats were in accordance with the guidelines for laboratory animals established by the National Institutes of Health as well as those of the Local Ethical Committee of the Medical University of Gdańsk. The experimental groups were exposed to an „open field test" throughout 10 minutes. 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 control animals remained in their home cages. After 90 minutes all the rats were deeply anesthetised with lethal doses of Nembutal (80 mg/kg of body weight), transcardially perfused with a 0.9% solution of NaCl with heparin, followed by a 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 brain were cut on a JUNG 1800 cryostat (Leica, Germany).

The sections were then stained with the double immunohistochemical method. The free floating sections were blocked with 3% NGS (Normal Goat Serum) containing 0.3% Triton X-100 for 1 hour and then incubated with a mixture of polyclonal rabbit anti-c-Fos antibody (Santa Cruz; dilution 1:500) together with polyclonal goat anti-calretinin (Chemicon; dilution 1:500) in 3% NGS for 48 hours in 4°C. After multiple rinses in PBS, sections were incubated for 2 to 3 hours at room temperature with a mixture of appropriate secondary antibodies: Alexa Fluor 488-conjugated donkey anti-goat (Molecular Probes; dilution 1:150) and Cy3-conjugated donkey anti-rabbit (Jackson ImmunoResearch; dilution 1:600).

The immunohistochemically stained slides were examined by a fluorescent microscope BX-51 (Olympus, Japan) equipped with a Color View II (Olympus, DK) camera or an Eclipse TE300 (Nikon, Japan) microscope and the Radiance 2100 (Bio-Rad, UK) confocal system equipped with a Krypton/Argon laser

and mounted on a light microscope Eclipse 600 (Nikon, Japan). The confocal microscopy (CLSM) images were obtained using 40 × and 60 × oil immersion objective lenses of N.A. = 1.3 and 1.4 respectively. An Argon laser produced dichromatic light at 488 and 568 nm. The 488-nm line of the laser was applied for excitation of Alexa dye. The 568-nm line of this laser was applied to excite Cy3 dye. The optimal iris was used for each magnification. A reconstruction of the image analysis program LaserSharp 2000 v. 4.0 (Bio-Rad; UK) was used. In each case only sections completely stained with fluorescence were taken into account.

RESULTS

Control groups

No c-Fos-positive neurons or single scattered ones were observed in any of the age-related groups of control rats in the piriform cortex.

Experimental groups

P0–P7. In the group of newborn rats strong c-Fos expression was observed in response to the acute open field test and this increased during the first week of life. Cells containing c-Fos-ir nuclei were localised predominantly in layer II and to a lesser degree in layer III in both the anterior and posterior parts of the PC.

A few calretinin-ir cells as well as immunoreactive neuropil were observed in both parts of the PC on the day of birth (Fig. 1A, B). These cells were small and resembled immature neurons. They possessed a large nucleus surrounded by a narrow rim of cytoplasm. Until the 7th postnatal day CR-ir neurons in the PC were predominantly oval and fusiform, and of a unipolar or bipolar type. Triangular cells occurred rarely. The CR-ir cells possessed short, thick single unbranched fibres (Fig. 2A, B). During this week of life CR-ir cells prevailed in the anterior part of the PC. They were heterogeneously distributed, being largest in number in layer III, whereas in layers I and II the numbers were markedly smaller (Fig. 1A, B).

The double immunolabelling study showed that the cells containing calretinin and c-Fos constituted separate populations of neurons, both in the anterior and posterior regions of the PC (Fig. 2A, B). However, very few cells were observed with co-localisation of these substances.

P10–P14. In comparison with the previous groups there was an increase in the c-Fos expression observed in response to the acute open field test. The c-Fos-positive cells were localised mainly in layer II and, to a lesser degree, in layer III of the PC.

CR-positive neurons were found predominantly in layers I and II in both parts of the PC (Fig. 1C, D; 2C, D). There were only single CR-positive cells in layer III of the PC. Although the shapes of the CR-ir cell bodies remained unchanged in comparison with the former age groups, more cells of a triangular and fusiform shape were observed in layers II and III. The CR-ir neurons, moreover, had longer fibres. CR-ir neurons were heterogeneously distributed among parts of the piriform cortex. More cells occupied the anterior part of the PC.

The double immunolabelling study revealed that cells containing calretinin were not co-localised with c-Fos-immunolabelled-neurons either in the anterior or in the posterior parts of the PC (Fig. 2C, D). However, very few cells were observed with co-localisation of these substances.

P21–P30. In comparison with the previous groups there was an increase in c-Fos expression. It was especially high by the end of the 4th week of life.

The number of CR-ir cells was also relatively high and, moreover, increased from P21 to P30. On P21 the calretinin-ir neurons reached mature morphology (Fig. 2E, F). They were characterised by intense immunolabelling and often possessed neuronal fibres with extensive arborisation (Fig. 2E–H).

Fusiform bipolar and triangular CR-ir cells comprised neurons with long thin dendrites arranged perpendicularly to the cortical surface. Some bipolar cells were also horizontally oriented. The neuronal fibres often possessed varicosities (Fig. 2E–H). Both parts of the PC were also characterised by intense immunoreactive neuropil.

The distribution of cell types among the layers of the PC was differentiated. Fusiform bipolar neurons were characteristic of layer II, whereas triangular cells were characteristic of layer III. In comparison with the former groups, only some CR-positive neurons of an oval shape were noticed in layer I. Layer I was also characterised by a band of CR-ir fibres and puncta.

The double immunolabelling study showed some CR-positive neurons co-localising with cells containing c-Fos-ir nuclei (Fig. 2E–H). These neurons were observed in layers III and II, predominantly in the anterior part of the PC.

P90. There was no significant change comparable to that in the previous groups in c-Fos expression in response to the acute open field test. Their distribution did not change either.

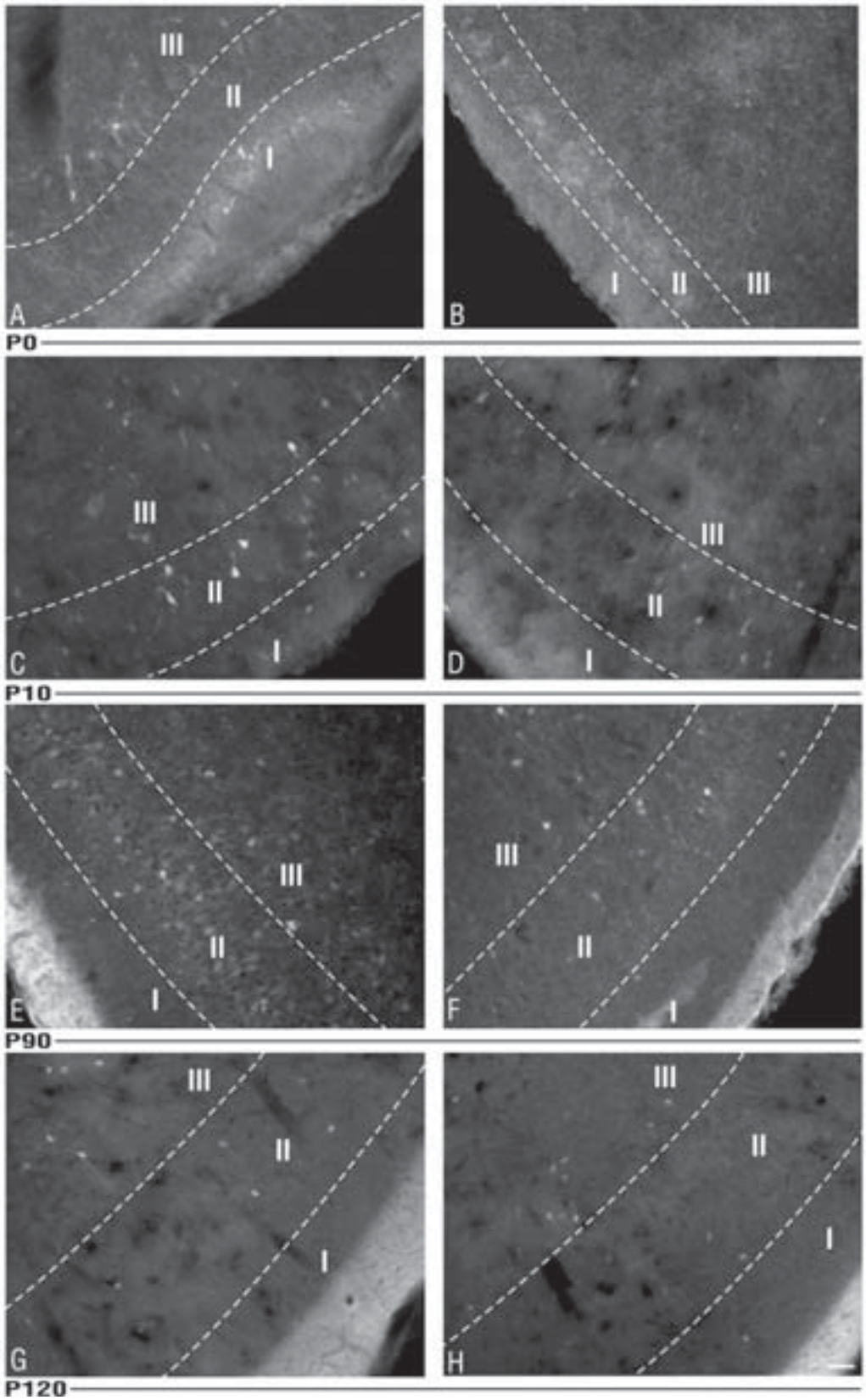

Figure 1. Age-related calretinin immunoreactivity in the anterior (A, C, E, G) and posterior (B, D, F, H) part of the piriform cortex following the acute open field test in the rat. Age: **A, B.** At birth; **C, D.** 10th postnatal day; **E, F.** 90th postnatal day; **G, H.** 120th postnatal day. I, II, III — layer of PC. Scale bar = 0.05 mm.

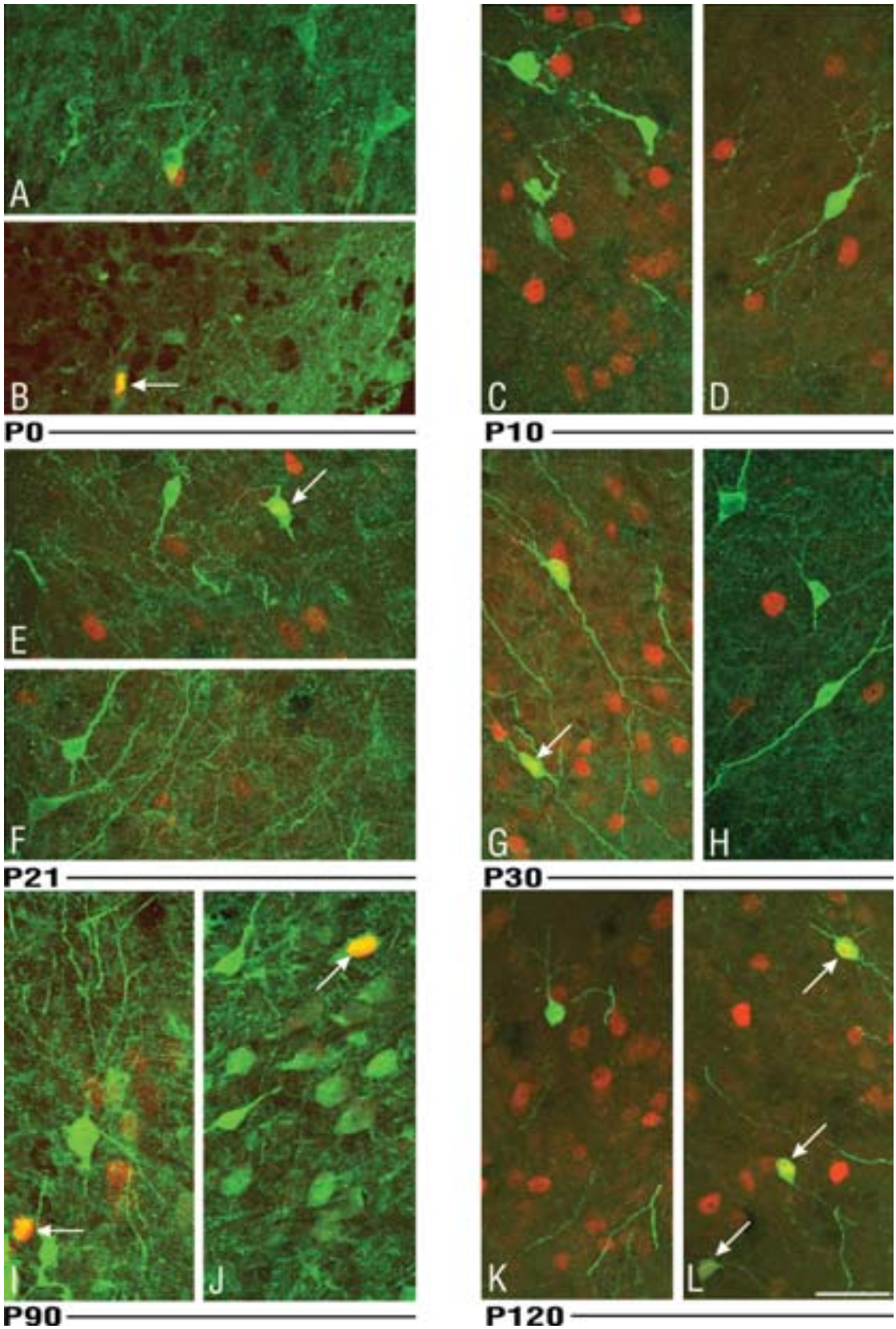

Figure 2. Age-related changes in c-Fos protein (red) and calretinin (green) immunoreactivity in the anterior (A, C, E, G, I, K) and posterior (B, D, F, H, J, L) parts of the piriform cortex following the acute open field test in the rat. Age: **A, B.** Birthday; **C, D.** 10th postnatal day; **E, F.** 21st postnatal day; **G, H.** 30th postnatal day; **I, J.** 90th postnatal day; **K, L.** 120th postnatal day. Arrows indicate cells with CR/c-Fos co-localisation. Scale bar = 0.05 mm.

Calretinin immunoreactivity in the PC was quite strong and the cortical layers were well distinguished. Although the number of CR-positive neurons was considerably larger in comparison to previous groups, their distribution among the layers, as well as between the anterior and posterior part of the PC, did not change significantly (Fig. 1E, F). The cells often possessed very long dendrites. The piriform cortex (especially layer I) was also characterised by intense neuropil staining. A large number of fibres had varicosities (Fig. 2I, J).

The double immunolabelling study revealed that the vast majority of cells containing c-Fos did not show expression of calretinin. However, c-Fos/CR colocalisation was observed occasionally, both in the anterior and posterior parts of the PC (Fig. 2I, J).

P120. Neither the number, nor the distribution of cells containing c-Fos-ir nuclei changed in response to the acute open field test in comparison with those in the previous groups.

The piriform cortex of this age group was characterised by an almost total lack of CR-ir cells. Moreover, these possessed very poor dendritic arborisation. The immunoreactivity of the neuropil was moderate or low in layers II and III, whereas in layer I it was high.

There was no difference in the distribution of CR-ir cells between the anterior and posterior parts of the PC (Fig. 1G, H).

Double immunolabelling data showed that there were only a few neurons containing both c-Fos and CR and these were observed predominantly in the posterior part of the PC (Fig. 2K, L).

DISCUSSION

In the present study all the age-related control rats exhibited weak c-Fos immunoreactivity. In contrast, c-Fos activity was noticed in the PC of rats exposed to the acute open field test. Both the distribution among the layers and the appearance of the c-Fos-ir nuclei (even at birth) corresponded to the data obtained by Datiche et al. [5].

c-Fos immunoreactivity increases gradually and stabilises at P30. In older groups of animals no significant changes in c-Fos expression are observed in relation to that on P30. These findings correspond to the reports of Kellogg et al. [16], who noted strong Fos immunoreactivity in adult rats and, in contrast, only a few Fos-positive cells in juveniles. However the lower level of c-Fos stimulation in late juvenile rats appears not to be related to deficiencies in primary olfaction because olfactory-guided behaviours are evident in rats from the first week of postnatal life. Thus the influence of age on the differences in c-Fos immunoreactivity is more likely to be linked to the immaturity of these regions than to deficiencies in the information coming through the primary olfactory system [16]. Nagahara and Handa, who examined the expression of c-fos mRNA following the exploration of a new environment, also found age-related changes in response to exposure to a novel open field [23].

Our findings indicated that distribution of the c-Fos-labelled nuclei was similar in the anterior and posterior parts of the PC. Tronel and Sara did not find any differences in the intensity of c-Fos immunoreactivity between the two parts of the PC either [26]. They observed abundant fos-labelled cells in adult rats, both in the anterior and posterior PC, after odour-reward associative learning.

Datiche and colleagues demonstrated a significantly higher Fos immunoreactivity in the anterior PC in comparison with that in the posterior PC after olfactory learning in rats [5]. These authors described a rostro-caudal heterogenity of the Fos expression pattern in the PC and also differential involvement of this structure in the processing of sensory information.

The absence of observable differences in the expression of c-Fos between the anterior and posterior regions of the PC after open field stress might be explained by fact that, the anterior PC receives more afferent olfactory activation than the posterior [12]. The sniffing behaviour elicits strong activity in the olfactory bulb. Dense bulbar information subsequently reaches the anterior PC and could be responsible for the high level of Fos immunoreactivity observed within this area.

In turn, the unknown environment and bright light used in our open field test induces a strong stress neurogenic reaction, which is expressed by the activation of the posterior part of the PC, as this area has extensive connections with the limbic system [14].

Immediately after birth we observed CR-positive neurons of immature morphology in the piriform cortex. These neurons achieved maturity between the 3rd and 4th weeks of life. During maturation, the number of CR-positive cells increased until P90 and then decreased. The presence of calretinin in neurons even at the early stages of postnatal life indicates that this protein takes part predominantly in the maturation of neurons and in the formation of new neuronal connections [13, 15]. Moreover calretinin takes part in the remodelling of synaptic connections [27].

The lower level of CR in older animals indicates that neuron maturation in the given structure of the central nervous system has been completed. Bu and colleagues, for example, showed a decrease in CR--positive neurons in the mature human cerebral cortex brain as compared to the young one [4]. Similarly, changes in CR expression during maturation have also been described for various animals [1, 15].

We found that calretinin-containing neurons in the PC belong to the population of small cells, unlike the remaining subpopulations of neurons containing calcium-binding proteins described in our previous study [2] and by Andressen et al. [1].

The calretinin immunoreactivity observed in our study comprised fibres and puncta predominantly in layer I and cells in the remaining layers. The largest number of CR-ir neurons was observed in layer II and only some cells in layer III.

The distribution of calretinin immunoreactivity in the rat piriform cortex as well as the morphology of CR-ir neurons was in accordance with the results of other authors [1, 11, 13, 15, 17, 28]. We have observed small differences between the anterior and posterior parts of the piriform cortex. The CR-positive neurons of the anterior part of the PC were more numerous and also more dense (predominantly in layer II) than those of the posterior part. This phenomenon can be partially explained by the regulation of calretinin expression by afferent input from the olfactory bulb [20]. The heterogeneous distribution of CR-ir cells influences the variability of their neuroprotective effect between parts of the PC.

The double immunolabelling study revealed that only a few cells containing calretinin also showed c-Fos-positive nuclei. This c-Fos/CR co-localisation was observed in the cells of layers II and III of the PC. Until P21 co-localisation was found predominantly in the anterior part of the piriform cortex, whereas on P30 and P90 it was found in both subdivisions of the PC. In older rats co-localised cells were found mainly in the posterior part. This age-related change in the spatial distribution of co-localised cells may reflect the engagement of calretinin in the different regulatory processes of these two regions of the PC.

Our findings strongly support the assumption that the PC may be treated as a functionally heterogeneous structure [14, 21]. The two subdivisions of the PC can provide distinct information processing and therefore may participate differentially in olfactory guided behaviour.

It is known that calretinin is expressed in morphologically heterogeneous cell populations which include both GABAergic and non-GABAergic neurons and also that this protein has been observed in both excitatory and inhibitory cells in the PC [11, 28]. Our observations have indicated that the largest number of CR-ir neurons can be observed in layer II. The data from different studies demonstrates that the number of GABAergic cells in this layer is smaller than that of the CR-positive cells, which could suggest that at least a subpopulation of the CR-ir cells did not contain the inhibitory transmitter GABA [7, 11, 21]. In turn, the CR-positive cells in layer II, which often possess long dendrites with a perpendicular orientation, are known to utilise glutamate and aspartate as neurotransmitters [11]. According to our data most CR-positive neurons in the PC were of fusiform and oval shape. This subpopulation of neuronal cells is very rarely GABAergic [7, 11].

We assume that GABAergic neurons are not engaged in the acute open field test, indicating that it is projecting neurons that are mainly involved. CR-ir cells may also take part in the stress reaction.

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