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Parvalbumin containing neurons of the piriform cortex in open field stress — a developmental study in the rat

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In our study we used c-Fos protein (as a marker of cellular activity) to identify whether cells containing parvalbumin (PV) in the piriform cortex (PC) are engaged in the response to stress stimulation and to discover how this expression changes during maturation. The material consisted of Wistar rats of postnatal (P) ages between 0 and 120 days divided into 9 groups: P0, P4, P7, P10, P14, P21, P30, P90, P120. Each group consisted of 5 experimental and 3 control animals. Rats of the experimental groups were exposed to the "open field test" throughout 10 minutes. The control animals were kept in a home cage. Our results showed that c-Fos activity in the open field test was observed in layers II and III of PC after birth. It then increased and stabilised on P30. In the second week of life PV-positive cells were also observed in those layers. These achieved maturity in the 4th week of life. After this time basket-like structures appeared but the level of PV/c-Fos co-localisation was low. Only small differences were observed between the anterior and posterior parts of PC. In the anterior part a higher number of PV-positive neurons, neuropil threads, and basket-like structures and a larger degree of PV/c-Fos co-localisation were observed. Our results suggested that during maturation PV cells are not directly activated in response to stress stimuli but PV neurons via their numerous endings influence the activation of c-Fos-positive cells predominantly in the anterior part of PC.

Key words: c-Fos, maturation, open field test

INTRODUCTION

The piriform cortex (PC), the largest area of the mammalian olfactory cortex, is a paleocortical structure consisting of 3 layers. Layer I is characterised by the presence of ascending dendrites from deeper layers, layer II is formed by a compact zone of neuronal bodies, while layer III contains neuronal cell bodies and a large number of dendrites and axons [6]. The piriform cortex receives direct projection from the olfactory bulb, has a close reciprocal connection with many limbic areas including the amygdala, hippocampus and hypothalamus and additionally has access to the motor system in the diencephalon. It has been considered that neurons of the piriform cortex are involved in the response to a variety of stressful stimulations [14, 20].

The neurons of PC are divided into 2 subpopulations, namely GABAergic interneurons and non-GABAergic cells. The former expresses immunoreactivity for different calcium-binding proteins [15, 19]. Parvalbumin (PV), like other calcium-binding proteins (calretinin or calbindin), plays an important role in

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buffering the level of intracellular calcium ions in neurons and can be involved in neurotransmitter release, neuronal excitability, regulation of neurogenesis and neuronal growth, survival and plasticity [2, 16].

The level of c-fos or Fos protein has commonly been used for the identification of neuronal activation in various structures (including PC) following various stimuli [3, 9, 14, 25]. However, c-fos accumulation is not only an indicator of neural activity, but also reflects the capability of neurons for plastic changes [23]. Fos expression is intensive after the exposition of a rodent to a new "open field" [21]. According to Ramos and Mormede [22], this test is widely used to study the anxiety and emotionality of animals and investigates behavioural changes during the stress exposure in a novel environment.

So far little is known about the neurochemical specificity of piriform cortex neurons excited in response to stress stimulation. In this study we used Fos immunoreactivity to identify whether the cells containing parvalbumin in the piriform cortex are engaged in the stress stimulation and to find out how this dependence changes during postnatal development.

MATERIAL AND METHODS

The material consisted of Wistar rats of postnatal (P) ages between 0 and 120 days. The rats were divided into 9 groups: P0, P4, P7, P10, P14, P21, P30, P90 and P120. Each group consisted of 5 experimental and 3 control animals. 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 by the Local Ethical Committee of the Medical University of Gdańsk. The experimental groups were exposed to the "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 cage. After 90 min all the rats were deeply anaesthetised with lethal doses of Nembutal (80 mg/kg of body weight) and then transcardially perfused with a 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 brain were cut on JUNG 1800 cryostat (Leica, Germany). The sections were then stained with the double immunohistochemical method. The free floating sections were blocked with 3% NGS containing 0.3% Triton X-100 for 1 hour and then incubated with a mixture of policlonal rabbit anti-c-Fos antibody (Santa Cruz; dilution 1:500) together with monoclonal mouse anti-parvalbumin (Sigma; dilution 1:500) in 3% NGS for 48 hours in 4°C. After multiple rinses in PBS, sections were incubated with a mixture of appropriate secondary antibodies: Alexa Fluor 488-conjugated goat anti-mouse (Symbios; dilution 1:150) and Cy3-conjugated goat anti-rabbit (Jackson ImmunoResearch; dilution 1:600) for 2–3 hours, at room temperature.

The immunohistochemically stained slides were examined by a fluorescent microscope BX-51 (Olympus, Japan) equipped with camera Color View II (Olympus, DK) or microscope Eclipse TE300 (Nikon, Japan) and the confocal system Radiance 2100 (Bio-Rad, UK), equipped with Krypton/Argon laser and mounted on a light microscope Eclipse 600 (Nikon, Japan). The confocal laser scanning microscopy images (CLSM) 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 image analysis program LaserSharp 2000 v. 4.0 (Bio-Rad; UK) was used.

RESULTS

The piriform cortex of the control rats in all age-related groups was characterised by an almost total lack of c-Fos-positive neurons.

P0–P4

In these groups there was only slight c-Fos expression, mainly involving layer II of PC. Neither Parvalbumin-immunoreactive cells nor neuropil were observed.

P7–P10

Between P7 and P10 a significant increase in c-Fos expression was observed in response to the open field stimulation. These positive cells were localised predominantly in layer II with fewer in layer III of PC. At P7 sporadically PV-immunoreactive neurons were found, but only in layer III. They were small, mainly round in shape and with large nuclei surrounded by very bright cytoplasm. Apart from PV-positive neurons of immature morphology in layer III, only single PV-positive cells in layer II in the anterior part of PC appeared at P10. At that age there was no neuropil immunoreactivity in both parts of PC. A double im-

munolabelling study revealed that cells containing parvalbumin and c-Fos constituted distinct populations of neurons both in the anterior and posterior parts of PC.

P14–P21

In these groups Fos protein expression was significantly higher after stimulation than in the previous groups. Cells containing c-Fos were localised in both layer II and layer III (Fig. 1A, B). On P14 more PV-positive neurons, still immature, were noticed in layer III and considerably fewer in layer II (Fig. 1A–C). At the end of the 3rd week of postnatal life the morphology of PV-immunoreactive neurons changed. There appeared more intense cells of different forms (oval, triangular or multipolar) with processes (Fig. 1F, G). The fibres often possessed varicosities (Fig. 1G). In addition to cell bodies, PV-positive fibres and endings were also present (Fig. 1F, G). A large number of PV-immunoreactive neurons were found in layer III, whereas in layer II they were less numerous, both in the anterior and posterior parts (Fig. 1D, E). At P21 small differences appeared in the number of PV-immunoreactive cells between the anterior and posterior parts of PC (Fig. 1D, E). In this group neuropil expressed weak immunoactivity (Fig. 1F). From P21 the basket-like structures were predominantly present in the anterior part of PC (Fig. 1F). In this part the double immunolabelling study displayed a few PV-positive neurons co-localising with cells containing c-Fos (Fig. 1G).

P30–P120

In these groups an increase in c-Fos expression was evident in response to the open field exposure. Abundant c-Fos cells were localised in both layers of the anterior and posterior part of the piriform cortex (Fig. 1H, I). The parvalbumin immunoreactivity in PC was strong and the layers were easily distinguishable. From the 4th week of life PV-positive cells were identified as mature. The cells were mostly small and round with radiating dendrites, stellate, fusiform, chandelier and large multipolar (Fig. 1J). A large number of PV-immunoreactive cells occupied predominantly layer III and, to a lesser degree, layer II of PC (Fig. 1H, I). These possessed numerous, often very long, PV-positive dendrites. Some of them, mainly in layer III, had varicosities (Fig. 1J). In layer II, in particular, a large number of basket-like structures were present, mostly in the anterior part of PC (Fig. 1J, M). Some parvalbumin-immunoreactive fibres and endings were also found in layer I. Parvalbumin cells were more numerous and intense in the anterior part of PC than in the posterior (Fig. 1K, L). The double immunolabelling study revealed that the vast majority of cells containing c-Fos did not show expression of parvalbumin after stimulation in the open field. However, cells containing both c-Fos and PV were occasionally observed in PC. We found single PV-positive neurons co-localised with c-Fos cells predominantly in the anterior part of PC (Fig. 1N). However, characteristically PV-positive fibres in layers II and III of PC often surrounded c-Fos-positive neurons, forming basket-like structures (Fig. 1M, N).

DISCUSSION

In all age-related home-cage control rats no c-Fos immunoreactivity was observed. In contrast, in rats exposed to the open field we noted an age-related increase in c-Fos expression, which reached a plateau in the 4th week of life. The increase in immunoreactivity suggests that the new environment exerted a strong influence on c-Fos expression in the piriform cortex. Similarly, Filipkowski et al. [10] in their experiment confirmed that even a very brief exposure of animals to a new environment results in an activation of Fos protein in the rat barrel cortex. Other authors have shown a strong Fos immunoreactivity in young adult (P60) rats in the piriform cortex after 15 min of restrain stress in comparison with juvenile (P28) and control animals [14]. According to a study by Kellogg et al. [14], these differences could be related to the different behavioural responses observed between juvenile and young adult rats. Age-related changes in c-fos mRNA levels in PC in response to exposure to a new open field have also been reported by Nagahara et al. [21].

Our results additionally showed that there was no difference in the intensity of Fos expression between the anterior and posterior parts of PC in response to stress stimuli, although electrophysiological studies have provided strong evidence of the functional dissociation of these two regions [13, 18, 24]. Datiche et al. [4] demonstrated markedly higher c-fos reactivity in the anterior PC in comparison with the posterior in the rats after acquisition of olfactory learning. This is a result of the fact that the anterior area of PC receives more afferent activity than the posterior (a decrease in the density of the bulbar afferent fibres in rostro-caudal order [12]). Subsequently, dense bulbar information reaches the anterior part of PC and makes a contribution to the high Fos immunoreactivity also observed by us within this area. The afferent bulbar activity is then redis-

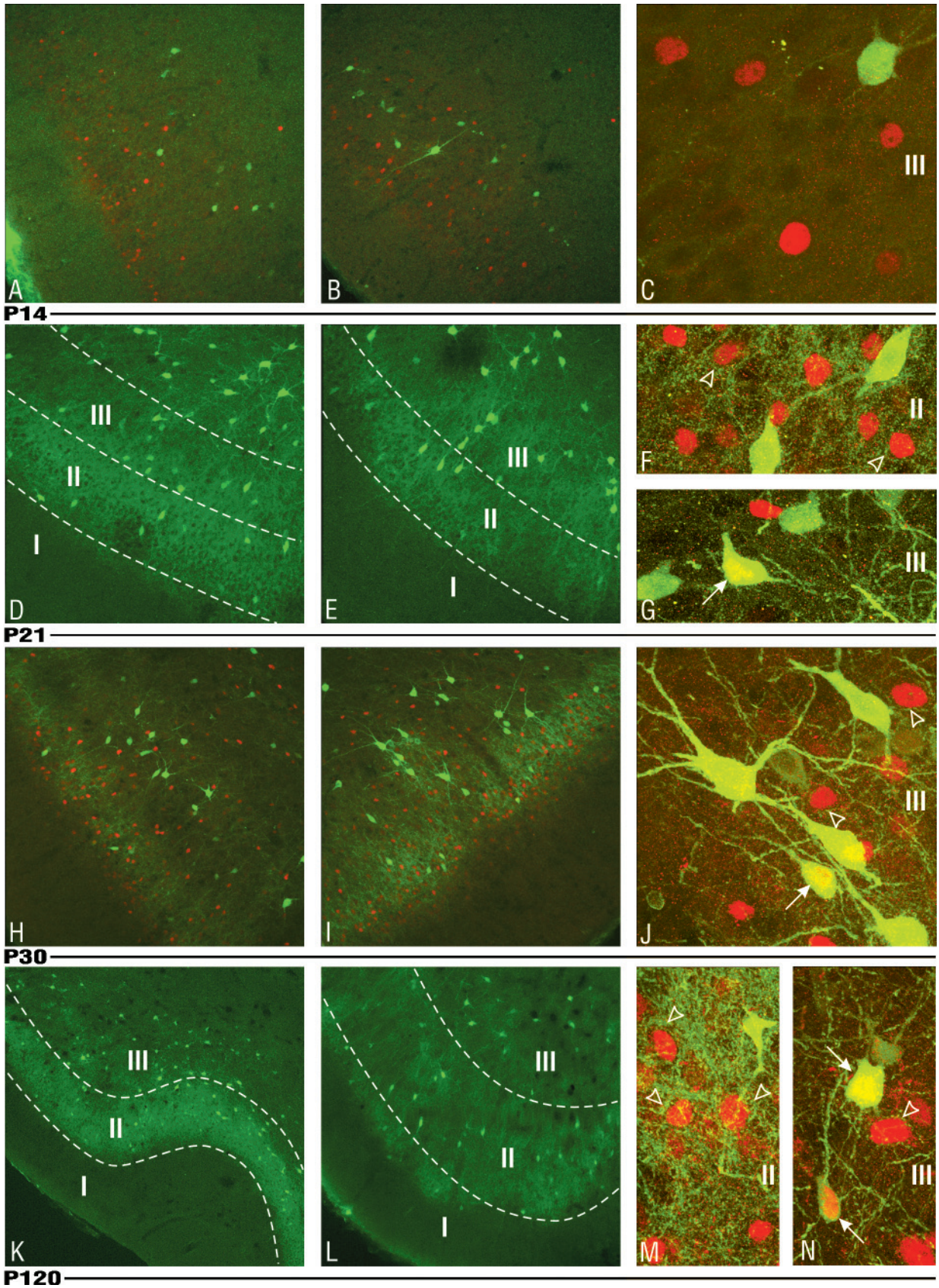

Figure 1. Developmental changes in fluorescence immunohistochemistry for c-Fos protein (red) and parvalbumin (PV; green) in the rat piriform cortex (PC) after the open field test. P14 — double immunolabelling for PV/c-Fos of the anterior (**A, C**) and posterior (**B**) parts of the PC; P21 — distribution of PV-immunoreactive cells in the anterior (**D**) and posterior (**E**) parts of the PC; double immunolabelling for PV/c-Fos of the anterior PC (**F, G**); P30 — double immunolabelling for PV/c-Fos in the anterior (**H, J**) and posterior (**I**) parts of the PC; P120 — distribution of PV-immunoreactive cells in the anterior (**K**) and posterior (**L**) parts of the PC; double immunolabelling for PV/c-Fos in the anterior PC (**M, N**). Arrows indicate cells with PV/c-Fos co-localisation. Arrow heads indicate basket-like structures; P-day of post-natal age, I, II, III — layers of the rat PC.

tributed to the whole piriform cortex through a rostro-caudal association fibre system [13]. Thus the Fos activation shown in the posterior part of the mature PC was also high. Our findings are also supported by Tronel and Sara [25], who noticed no difference in the distribution of c-fos-positive neurons between the anterior and posterior areas of PC after odour-associative learning and after reactivation of the well-consolidated olfactory memory in a retrieval test.

Only a small number of immature PV-positive cells in the piriform cortex appeared for the first time in the 2nd week of life. From the 4th week of life PV-positive neurons had a mature morphology. We observed an age-dependent increase in the number of PV-positive neurons in the PC. Andressen et al. [1] also claimed that the expression of PV correlated well with functional maturation in the central nervous system. These significant changes in PV protein may potentially play an important role in the survival of neurons [16]. The distribution pattern of parvalbumin immunoreactivity and the morphology of cells in the piriform cortex were similar to those described by Kubota and Jones [15], Frassoni et al. [11] and Ekstrand et al. [8].

In our study we observed that the number and staining intensity of PV-positive neurons and neuropil were greater in the anterior part of PC than those in the posterior part. This may suggest a greater neuroprotective effect of the parvalbumin in cells of the anterior region. Lephart and Watson [16] and Bu et al. [2] attempted to explain the enhanced expression of calcium-binding proteins as compensation for an increase in the intracellular level of Ca^{2+} in different brain sites. Lim and Brunjes [17], on the other hand, demonstrated data that the expression of parvalbumin in the rat olfactory cortex is regulated partially by the afferent input from the olfactory bulb. Furthermore, the increase in parvalbumin immunoreactivity may provide neuroprotective support by possible alterations in intracellular calcium ion concentration.

According to Kubota and Jones [15] and Frassoni et al. [11], parvalbumin appears in the piriform cortex only in the interneurons. The localisation of c-Fos-positive cells and the low level of co-localisation with parvalbumin in the mature brain led us to predict that activated cells were probably either projection cells or PV-negative interneurons.

The double immunolabelling study revealed that only a very few cells containing parvalbumin showed co-localisation with c-Fos-positive neurons in layers III and II of the PC only visible after the 4th week of life. This co-localisation was found predominantly in the anterior part of the piriform cortex. Perhaps this reflects the requirements for a large number of regulating processes for optimal operation in this region of PC. Filipkowski et al. [10] ascertained that the vast majority (i.e. > 90%) of cells containing c-Fos in the rat barrel cortex did not show expression of PV after stimulation.

In our material when PV-immunoreactive cells achieved maturity many of the fibres in layers II and III of PC outlined c-Fos-positive neurons forming basket-like structures, mainly in the anterior part of PC. Basket cells are commonly defined as neurons that give rise to axosomatic synapses on the appearance of their terminal arbours [7]. According to De Felipe [5] and Ekstrand et al. [7, 8] at least part of the population of PV-positive cells are GABAergic interneurons, which can play an important inhibitory role in regulating excitatory processes in the piriform cortex and may control cell activity in this structure.

It would appear that during postnatal development cells containing parvalbumin are not directly involved in the response to the "open field" reaction. In the stress stimulation other cell populations (probably projecting neurons) are mainly activated in the piriform cortex. In view of the fact that many PV-endings were found on c-Fos-positive nuclei, we assume that neurons containing parvalbumin protein might exert a direct influence on c-Fos-positive neurons.

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