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MORPHOLOGY, SIZE AND DISTRIBUTION OF CORTICOTROPIN RELEASING FACTOR (CRF) IMMUNOREACTIVE NEURONS IN THE CENTRAL NUCLEUS OF THE RAT AMYGDALOID COMPLEX

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The amygdaloid complex (AC) is a heterogenous group of cortical and nuclear structures. As an important component of the limbic system contains numerous neurotransmitters including neuropeptides. In this study we have investigated the types, distribution, and morphometric characteristics of corticotropin releasing factor (CRF) immunoreactive neurons in the central nucleus (Ce) of the AC. We have also identified CRF- immunoreactive fibers in this nucleus. This research was performed on 5 adult rats kept in standard conditions. Animals were perfused 48 hours after application of colhicin. The removed brains were postfixed and cut into free floating sections. The sections were treated with antibodies against the rabbit CRF and ABC immunohistochemstry was applied. The neurons were measured and drawn with a camera lucida. We found uniform distribution of CRF immunoreactive neurons in the Ce of AC of rat. According to morphological type dominant were triangular (50%), while bipolar (25%) and multipolar (25%) types were also present. The most of the CRF immunoreactive neurons had 2-5 primary dendrites. These findings will contribute to understanding the role of CRF neurons in rat Ce during stress and in other behavioural functions.

Key words: amygdala, CRF, rat, central nucleus, neurons

INTRODUCTION

The amygdaloid complex (AC) plays an important role in the control of the endocrine and autonomic components of species-specific behaviour and in complex mechanisms of behaviour, such as defence, nutrition, agression, affect, reproduction, memory and learning (Zhang *et al*., 1986; Parent, 1995; Aggleton, 2000). These nuclei are interconnected, they function in conjuction with each other and have numerous connections with other structures in the central nervous system, especially with the hippocampus and hypothalamus (Krettek and Price, 1978; Price *et al*., 1987; Aggleton, 2000; Pitkanen, 2000).

The central nucleus (Ce) belongs to the so called deep nuclei of the AC (Krettek and Price, 1978). In our previous studies we found moderate numbers of CART peptide and of CB1 receptors in the rat Ce (Puškaš *et al.*, 2004; Puškaš *et al*., 2005). At the same time the Ce is also the main region of the motor divergence of the AC (Maren, 1999).

The corticotropin releasing factor (CRF), neuropeptide which consists of 41 aminoacids was first isolated from the ovine hypothalamus (Vale *et al*., 1981), and was later discovered in other species (Hashimoto *et al*., 1984), as well as in human AC, telencephalon, cerebrospinal fluid, and especially in locus coeruleus (Gold and Rubinow, 1987). The largest concentration of CRF immunoreactive bodies and fibers in AC is present in Ce (Cassell 1986; Cassell 1989; Sakanaka *et al.,* 1986). This peptide is a member of the "stress hormone" group along with ACTH and cortisol (Burlet, 1988). It is a mediator in various effects caused by stress: activation of the hypothalamo-hypophysial axis, stimulation of the sympathetic system, motor activities, general behavioural activation, anorexia, suppression of sexual behavior, anxiety and sleep regulation (Ehlerss *et al.*, 1986; Burlet, 1988; Parent, 1985; Palkovits *et al*., 1996; Craiset *et al*., 2000).

The aim of our study was to investigate the morphology and the distribution, as well as to classify the CRF imunoreactive neurons of Ce in rat AC, according to the shape of their pericarya.

MATERIAL AND METHODS

Experimental animals

We performed this study on 5 male adult Wistar rats with body weight from 250-300 gr. The animals were kept in individual cages, and fed standard food with free access to water. Optimal room temperature (21-23^{o}C) and air humidity were maintained.

Operational techniques for colchicine application

Colchicine possesses the ability to destroy microtubules, and thus blocks axonal transport. As a result of this blockage, the substances that are being examined remain in the neurons for a longer period, thus enabling a more efficent immunohistochemical visualisation.

After applying anaesthesia with a mixture of SBH-ketamine (0.3 mL) and 0.2% xylazinhydrochloride (0.2 mL) the head of the rat was fixed into the stereotaxic frame. In order to find and clean the bregma a scalpel incision was carried out at the head at the level of the central suture. The coordinates for the lateral ventricles, into which we applied colchicine were determined using Paxinos' atlas (Paxinos and Watson, 1998). While marking the coordinates on the skull, we used the bregma as a starting point. The coordinates were:

AP:-0,8 (anterior-posterior); Lat:1,3 (lateral) and DV:-4,2 (dorsal-ventral).

After marking the first two coordinates on the skull the dental drill was used to open the bone and insert the needle to extract the dura. This provided an opening needed for the needle to enter the ventricle, enabling us to place a

Hamilton needle into the appropriate coordinate. We injected 7 μL of colchicine solution (1 μL solution = 10 μg colchicine). Each injection lasted 5 minutes. After removing the needle, we closed the scull with the extracted piece of bone, fibrinogen gel, and sutured the skin with clips. These animals were kept in standard conditions and perfused after 48 hours.

Perfusion of the rats

We performed the perfusions in sterile conditions. Anaesthesia was achieved using a mixture of ketamine (0.2 mL) and xylazinhydrochloride (ROMPUN 2%, BAYER) (0.2 mL). The Zamboni fixative (250-300 mL/rat) was used.

After anaesthetizing the animals, the abdominal cavity below the xyphoid process was opened-up with surgical scissors. The diaphragm and part of the thoracic wall were dissected in order to access to the heart. We placed a tube into the aorta through the left chamber, fixated it with a clamp, and simultaneously opened the right auricle. Perfusion with 50 mL of saline solution (0.9% NaCl) was initiated, and then we injected 250-300 mL of the Zamboni fixative into the perfusion system. The perfusion took 60 minutes. After perfusion we removed the brains and left them in the same fixative until further treatment.

Immunohistochemical procedures

After perfusing and extracting the brains, fixation was continued in the same fixative, diluted with a phosphate buffer (1:2), followed by 24 h refrigeration, at +4°C. For the sake of cryoprotection, the samples were stored in a 20% saharose solution for the next 24h. The brains were cut on cryocut (frigomobil) at -18°C into 50 μm thick sections. This thickness was convenient for immunohistochemical reactions on the free floating slices. After treatement with triton, the slices were washed with a phosphate buffer and the procedure was continued by adding 3% H_2O_2 in order to block the endogenous peroxidase. After washing the cuts with a phosphate buffer, we added a 10% normal goat serum which in one hour blocks all unspecific antigene sites. A primary policlonal antibody (anti-rabbit serum), specific for CRF was added into the bottles with the slices after having washed them. The slices were left for 48 hours at +4°C, on a mixer. After that period, and after the slices were washed in the phosphate buffer, biotinylated anti-rabbit secondary serum was placed in the bottles with the slices. The secondary serum was also washed with the phosphate buffer, and then VECTASTAIN®ABC complex was added. The slices were kept in the ABC complex for an hour, and then they were washed with 0.1 M phosphate buffer and TRIS. Visualisation of the immunoreactive sites was performed in a solution of DAB (3, 3'-diamino-benzidine).

The histological sections were analysed using a light microscope and the CRF immunoreactive cells were drawn under the *camera lucida*.

RESULTS

Our immunohistochemical study of Ce of rat AC showed a significant population of CRF-immunoreactive neuronal bodies and also a dense network of these fibers (Fig. 1). Immunoreactive structures (cells and fibers) showed uniform distribution in all parts of Ce. The density of these elements was so high that Ce could be observed on a histological slide even by naked eye. By analysing the shape of the body, as well as the number and distribution of the primary dendrites, three types of CRF immunoreactive neurons in rat Ce have been described: triangular, multipolar and bipolar neurons. The number of their primary dendrites varied between 2 and 5 (Fig. 2).

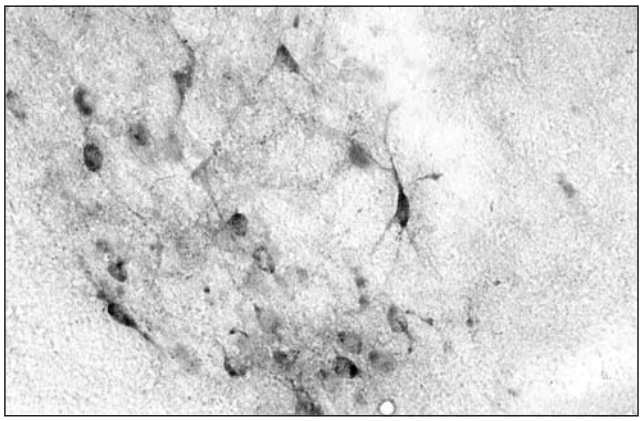

Figure 1. CRF immunoreactive neurons and fibers in Ce, ABC method, 40x

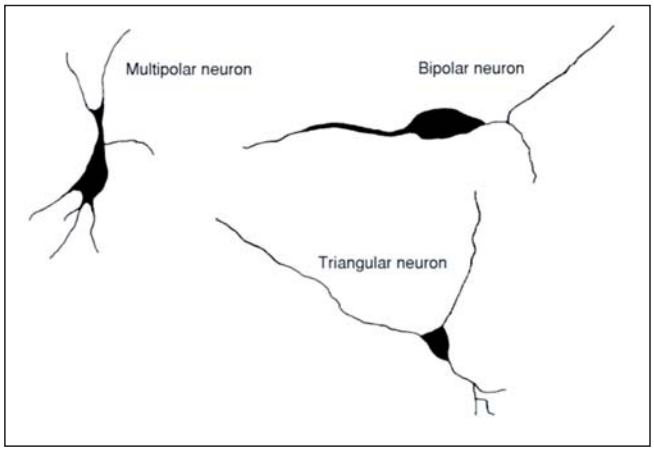

Figure 2. Reconstructions of 3 types of CRF immunoreactive neurons

In Ce of the rat AC the triangular neurons were the greatest population (50%) of CRF immunoreactive neurons. This type of neuron has a triangular cell body, and primary dendrites arisied from neuronal poles.

Multipolar neurons contributed to 25% to the total population of CRF immunoreactive neurons in the Ce of rat AC. They were characterised by an irregular, i.e. multipolar shape of the cell body, with several primary dendrites which arised from different parts of the cell body and with different directions. Secondary dendrites followed the same direction as the primary ones, but few of them had a recurrent course, namely, they were directed back to the cell body.

The third type of CRF immunoreactive neurons in Ce were bipolar ones, and like multipolar neurons they represented 25% of the population of CRF immunoreactive neurons in this nucleus. Each of their primary dendrites airsed from opposite ends of their spindle-shaped body. We were able to follow their secondary dendrite branches only in few cases. Basic morphometric parameters of the CRF immunoreactive neurons in Ce of rat AC are presented on Table 1.

Table 1. Basic morphomteric parameters of CRF-immunoreactive neurons in central nucleus of rat amygdaloid complex

CRF neurons	maximal	minimal	X	SD
maximal diameter of neuronal body	41.28 μm	19.61 μm	30.06	6.64
minimal diameter of neuronal body	24.77 μm	10.32 μm	16.25	3.86
number of primary dendrites	5	2	3.25	1.03
number of secondary dendrites	2	2	2	0

DISCUSSION

After long and numerous investigations of the AC we can say that many facts are known about its influence on different functions such as the behaviour of the individual, the memory and drives, etc., but in this relationship the specific nuclei of AC were less investigated. Recent research of the AC is headed towards morphometric and functional analysis of these nuclei. Central nucleus is the most interesting nucleus in AC because it contains a wide spectrum of peptides and other neurotransmitters (Roberts, 1981; Price *et al.,* 1987; Asan *et al*., 2005).

Our findings about the presence of CRF-immunoreactivity in Ce of AC are in partial accord with the findings of other researchers on this nucleus of the rat (Cassell *et al.,* 1986; Cassell, 1989). Namely, in these studies CRF immunnoreactive elements were found in largest numbers in the lateral region of Ce, a bit less in the medial and ventral part, and there was no trace of their presence in the lateral capsular part of this nucleus. Contrary to this, our study

showed a uniform distribution of immunoreactive neurons in all parts of the nucleus. However, the morphological types of neurons and their morphometric characteristics showed no significant deviations from those described in literature: the dominant type is the triangular neuron with a maximal diameter of the body between 16 i 22 μm, with 3 primary dendrites, and without significant branches. Cassel and Gray (1989) described the piriform neurons as the dominant CRF immunoreactive cell population in Ce, with rare dendritic spines, and this finding could be related to our results. In the available literature detailed morphometric data about the CRF immunoreactive neurons in the nuclei of AC are rare.

Most of the available data indicate the role of CRF in individual behaviour, as well as in acute and chronic stress, and endocrine and vegetative reactions in an organism under stress which are in direct correlation with the level of released CRF (Aggleton, 2000). Studies of the changes of CRF in Ce during different kinds of stress showed mostly an increase in concentration of this peptide during stress. It has also been proven that stress leads to maladaptive responses which may result in psychiatric sindromes, such as anxiety and depression disorders (Brady and Sinha, 2005). The CRF may have a role in some psychiatric disorders such as schizophrenia and depression (Banki *et al.,* 1987), as well as in certain types of learning and memory processing (Liang and Lee, 1988). It has been proven that CRF shows a positive effect on memory retention by increasing gene expression for the brain-derived neurotrophic factor (BDNF) gene (Ma *et al.,* 1999).

It is known that 60% of CRF immunoreactive neurons from Ce project to the parabrachial nucleus, 30% to the central gray matter, and about 15% into the dorsal vagal complex (Gray *et al.,* 1989). These multiple projections of the CRF immunopositive neurons of CE into the brain stem may explain coordinated effects of the different target regions as a response to stressful situations.

Morphometric studies of CRF immunoreactive cells and fibers in Ce of the AC are significant because together with the findings about the function of this peptide, they represent a basis for further research. It is very probable that changes of volume or of distribution of CRF immunoreactive neurons can be associated with various behavioral disorders or various disorders of the autonomic nervous system. We expect that potential changes of density, and/or of types and of distribution of CRF immunoreactive neurons in Ce in different animal models of disorders may contribute to beter understanding of different disorders.

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MORFOLOGIJA, VELIČINA I DISTRIBUCIJA KORTIKOTROPIN RILIZING FAKTOR (CRF) IMUNOREAKTIVNIH NEURONA U CENTRALNOM JEDRU AMIGDALOIDNOG KOMPLEKSA PACOVA

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SADRŽAJ

Amigdaloidni kompleks (AK) kao heterogena grupa kortikalnih i nuklearnih struktura i važna komponenta limbičkog sistema, sadrži brojne neurotransmitere uključujući i neuropeptide. U ovom radu su prikazani rezultati istraživanja tipova, distribucije i morfometrijskih karakteristika CRF (corticotropin releasing factor) imunoreaktivnih neurona u centralnom jedru AK. Takođe, u ovom jedru su uočena i CRF- imunoreaktivna vlakna.

Istraživanje je obavljeno na 5 odraslih pacova koji su perfundovani nakon 48 sati od aplikacije kolhicina, a potom su izvađeni mozgovi postfiksirani i formirani slobodno plutajući rezovi. Rezovi su tretirani antitelima CRF kunića i ABC imunohistohemijskom metodom. Neuroni imunoreaktivni na CRF su crtani pomoću *camerae lucidae.*

Rezultati su ukazali da je distribucija CRF imunoreaktivnih neurona u svim delovima Ce ravnomerna. U odnosu na morfološki tip, dominiraju triangularni neuroni (50%), a u manjem procentu su ravnopravno zastupljeni bipolarni (25%) i multipolarni (25%) tip neurona. Najveći broj CRF imunoreaktivnih neurona poseduje 2-5 primarnih dendrita. Podaci o obliku, distribuciji i veličini CRF imunoreaktivnih neurona u centralnom jedru AK su od značaja za dalja istraživanja o njihovoj ulozi u odgovoru na stres i u drugim funkcijama.