Acta Veterinaria (Beograd), Vol. 55, No. 5-6, 423-434, 2005.

UDK 619:612.822.5

LOCALISATION AND MORPHOLOGY OF COCAINE- AND AMPHETAMINE-REGULATED TRANSCRIPT (CART) PEPTIDE IMMUNOREACTIVE NEURONS IN RAT AMYGDALA

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(Received 25. May 2005)

Cocaine- and amphetamine-regulated transcript (CART) is a recently discovered mRNA in rat brain, upregulated in the nucleus accumbens after administration of cocaine and amphetamine. All data until now suggest a widespread distribution of this mRNA in the brain and endocrine tissues. The aim of this study was to precisely map and desrcibe this mRNA product - CART peptide immunoreactive (IR) neurons in rat amygdala (AMY) by using immunohistochemical methods.

Distribution and density of CART -IR neurons in AMY was very different. The greatest density was in the lateral nucleus (La) and basolateral nucleus (BL) of AMY, and in Anterior Amygdaloid Area (AAA), moderate was in the central nucleus (Ce) and a small density was in the cortical posterior nucleus (CoP).

By analysing the morphology of CART- IR neurons in AMY, we found that the greatest number (43.75%) of these neurons has a bipolar shape. About 15% of investigated CART IR neurons are "bitufted" neurons, while an equal percentage is of multipolar (12.5%) and of ovoid neurons (12.5%). CART-IR neurons of pyramidal shape are present in about 9%, while the smallest number is of triangular neurons (6.25%).

The largest CART-IR neurons are in La and, neurons with the smallest long diameter are present in BL AMY. Even if there is not any morphologically distinct CART–IR neuron type in rat AMY, these neurons mainly are small and oval in most of AMY nuclei where they are usually present. This suggests the functional role of a majority of CART-IR neurons in rat AMY, especially in BL, as small interneurons. The small number of large neurons could be projection neurons.

Key words: CART, neurons, immunohistochemistry, amygdaloid nuclei, rat

INTRODUCTION

Cocaine and amphetamine regulated transcript (CART) is a recently discovered mRNA in the rat's brain, upregulated in the nucleus accumbens after administration of cocaine and amphetamine. This mRNA encodes a novel polypeptide of 116 or 129 amino acids (Douglass *et al.* 1995). Both of these variants were found in the brain of rats (Couceyro *et al*., 1997). Distribution of CART mRNA found in human brain is similar to that observed for rats (Douglas and Daoud, 1996).

A high level of conserved peptide seqences between the species, typical for the majority of neuropeptides, is evident also in the case of CART. There is 95% homology between the rat and human CART amino-acid sequence level (Douglas and Daoud, 1996). Although details about this peptide in the brain of rats were reported in 1995. (Douglas *et al*. 1995), much earlier, Spiess *et al*, in 1981 identified two peptide fragments in the extract of bovine hypothalamus. Sequences of these peptides were identical to later discovered C-terminal sequences of CART in rats (Koylu *et al*., 1998). This high level of conservation within the mammals and similarity in distribution suggests that CART peptide plays a conserved functional role across mammalian species (Douglas and Daoud, 1996).

All data until now suggest a widespread distribution of this neuropeptide in the brain and endocrine tissues. According to functional investigations of CART peptides and of several CART peptide fragments (CART-55-102 and CART 62-102) identified in the last few years (Thim et al., 1998; Kuhar and Yoho, 1999), it may be concluded that CART peptide is deeply involved in the regulation of food intake, body weight and energy balance (Thim *et al*., 1998; Vrang *et al*., 1999, 2000; Wang *et al*., 2000). There is evidence that CART administration induces c-Fos expression in the central nucleus of amygdala and in the paraventricular nucleus of the hypothalamus (Vrang *et al*., 1999, 2000). It is shown that administered CART (55-102) increases the release of CRH and TRH from hypothalamic explants, and that i.c.v. aplication of CART (55-102) significantly increases ACTH and corticosterone levels (Stanley *et al*., 2001). Thanks to these findings it is posible to conclude that CART may play a role in the activation of the hypothalamus-pituitary-adrenal axis, and that it can be involved in stress response. Different CART fragments (CART 55-102, CART 62-102, CART 82-103, CART 42-89, CART 49-89) are not involved only in food intake, but they have even some physiological roles like: locomotor activity (Kimmel *et al*., 2000), behavioral effects (Bannon *et al*., 2001), arterial pressure changes (Matsumura *et al*., 2001) and anxiogenic-like activity in mouse and rat (Kask *et al*., 2000). Several papers describe its distribution in the brain in rats, but detailed mapping and desciption of CART immunoreactive (IR) neurons was not reported for rat amygdaloid complex (AMY). The aim of this study was to describe the localisation and morphology of CART peptide immunoreactive neurons in the rat amygdala.

MATERIALS AND METHODS

Five adult male Wistar rats, 250-300 gr of body weight were used in this study. Animals were housed in single cages with palleted food and drinking water *ad libitum*. Rats were kept at room temperature (22 ± 1°C) and in an air humidity controlled enviroment. Light- dark cycle was 12 hours.

Application of colchicine was done 48 hours before sacrification (perfusion) of animals. Rats were first anesthetisied by a cocktail of ketamine (100 mg/kg b.w.) and Rompun (15 mg/kg b.w.). After that, the head was fixed on a stereotaxic table. In order to approach the bregma, incision was done on the level of the middle suture. Administration of colhicine was unilateral into the lateral ventricle (AP:-0.8; L:1.3; DV:-4.2) according to the stereotaxic atlas (Paxinos and Watson, 1998). A Hamilton needle was placed into the coordinate point and about 7 μl of colchicine solution (1 μl of solution = 10 μg of colchicine) was injected during 5 minutes. After removal of the needle, the skull was closed using the removed part of the bone and gelatine with fibrinogen, hence the skin was sutured. The so prepared animals were kept under standard conditions. The rats were sacrificed 48 hours after colchine application.

Perfusion of rats was performed under sterile conditions and under anesthesia with 250-300 ml of Zamboni fixative per rat. The approach to the heart was enabled by thoractomy. Perfusion started with 50 ml of physiological solution (0,9% NaCl), and was continued with 250-300 ml of Zamboni fixative. After 60 minutes of perfusion, the brains were removed and post-fixed overnight in the same fixative. Next day the brains were transferred into the Zamboni fixative diluted with phosphate buffer (PB), for 24 h at +4°C and then for the next 24 h the brains were left in a 20% solution of saccharose for cryoprotection. Sections, 50 ìm thick, were prepared on cryocat (Frigomobile) on -18°C, which is convenient for immunohistohemical reactions on free floating sections. All sections were collected in 0,1M PB with Na-azid until immunoreaction.

Immunohistochemical studies included the identification and distribution of CART-IR neurons in the nuclei of the amygdaloid complex by ABC immunohistohemical method.

After washing in 0,1M PB and after treatment by 0,5% Triton X-100 for 1 h, sections were washed again by 0,1 M PB and the procedure was continued by adding 3% H_2O_2 in order to block endogenous peroxidase. After washing, the sections were incubated one hour in 10% normal goat serum. After washing once again in 0,1M PB the sections were incubated in the primary antiserum specific for CART peptide (1:5 000). In the primary serum the sections were left on a mixer for 48 hours at +4°C. After this period and after washing in PB, sections were incubated with biothinizied anti-rabbit IgG and therafter treated with Vectastatin Elit ABC-peroxidase Kit (Vector Labs). In ABC complex the sections were left for 1h, and after that were washed in 0,1 M PBS and then in TRIS buffer. Visualisation of immunoreactive places was performed in the DAB (3,3-diaminobensidine) solution. Sections were mounted on gelatin-coated slides.

Exact anatomical sites were determined using the atlas of rat brain (Paxinos and Watson, 1998). Images were captured with an Olympus microscope and photographed with a CoolSnap camera.

We studied 50 neurons of each type. All neurons were drown with *camera lucida* on magnification 40x. The number of primary and secondary dendrites was determined, as well as the length of the longer and shorter diameters of perikaryon. The longer diameter (D1) was defined as a longitudinal one and the shorter diameter (D2) was defined as the transverse one, mostly perpendicular to D1. All results were statistically evaluated by SPSS 10.0.

RESULTS

CART immunoreactive (IR) cells in AMY were observed in the lateral and basolateral nucleus, anterior amygdaloid area, central nucleus and bed nucleus striae terminalis (BNST), while a smaller number of these cells was present in the posterior cortical nucleus.

Distribution and density of CART -IR neurons in AMY was very different. The greatest density was in La and BL AMY nuclei, AAA; moderate was in Ce and small density in CoP. Other parts of AMY did not show presence of CART immunoreactivity (Fig. 1).

According to the shape of perikarion and number and distibution of primary dendrites it is possible to describe the following types of CART-immunoreactive neurons: pyramidal, triangular, multipolar, bipolar, "bitufted", and ovoid (Figs. 2, 3 and 4).

Pyramidal neurons have pyramidal (conical) shape of perikarion, one apical dendrite and two basal dendrites wich arise from the base of the cell body.

Triangular neurons are cells with triangular or pyramidal shape, but there are visible only one or two primary dendrites.

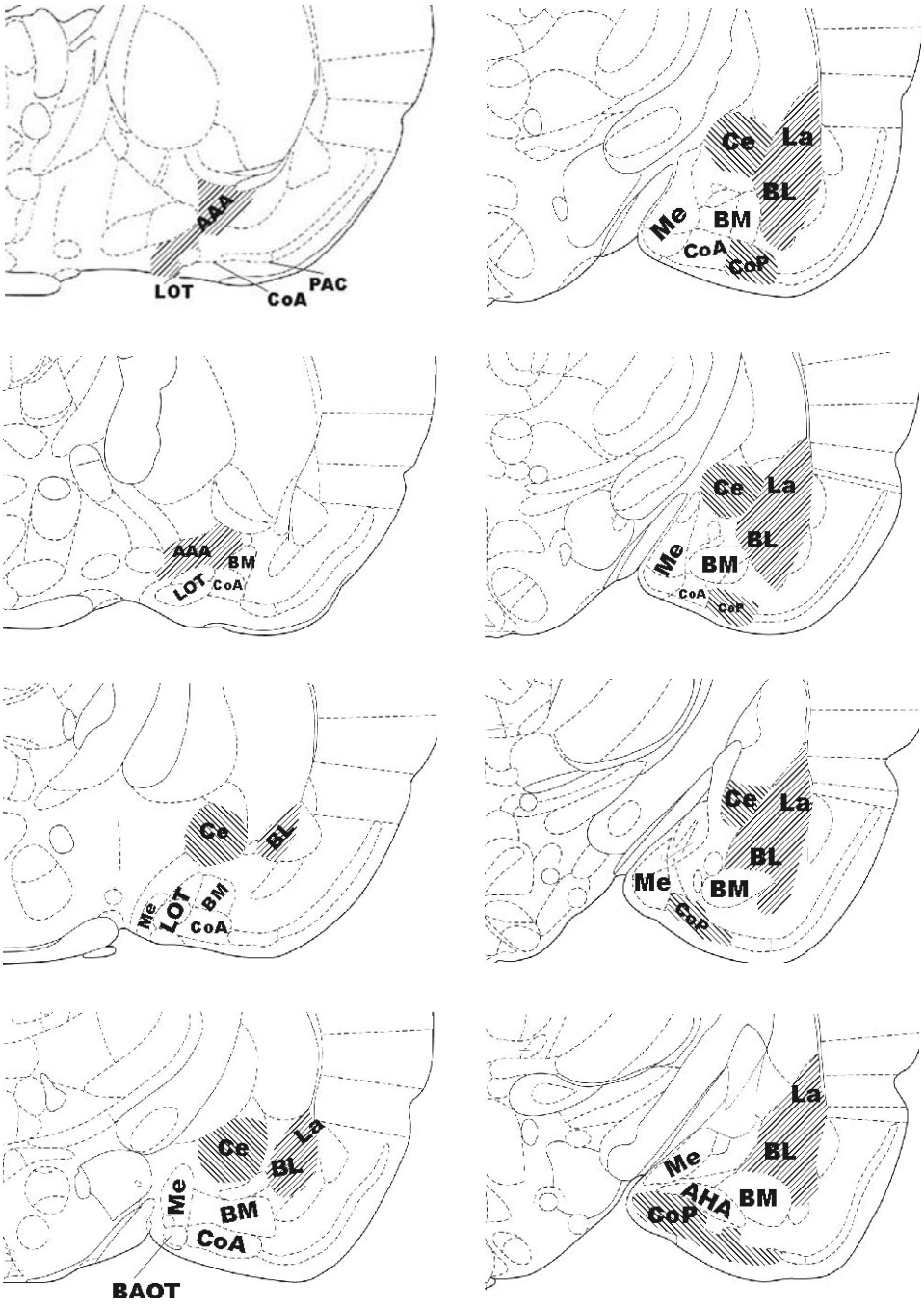
Multipolar neurons have ovoid or round perikarion and three or more primary dendrites wich arise from different sides of the soma.

Bipolar neurons are cells with oval perikarion and they have two primary dendrites extending from opposite ends of the cell body.

Bitufted neurons have similar shape of perikarion like bipolar neurons, but from opposite sites of the soma arise one or two short primary dendrites, which ramify and look like "tufts".

Ovoid neurons have an ovoid perikarion and from it arises one short primary dendrite.

By analysing the morphology of CART- IR neurons, we found that the greatest number (43.75%) of these neurons has a bipolar shape. About 15% of investigated CART IR neurons are "bitufted" neurons, equal is the presence of multipolar (12.5%) and ovoid neurons (12.5%). CART- IR neurons of pyramidal shape are present in about 9%, while triangular neurons are present in the smallest number (6.25%) (Fig. 5).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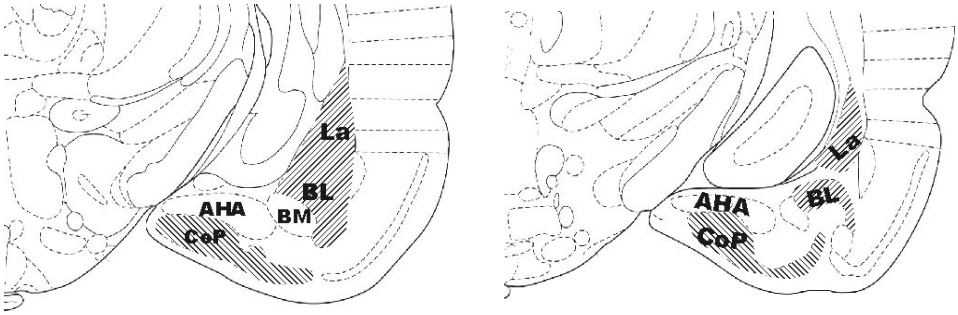

Fig 1. Distribution of CART-IR neurons in AMY

Legend
AAA - Anterior Amygdaloid Area, La - lateral nucleus, BL - basolateral nucleus, BM – basomedial nucleus, Ce - central nucleus, Me – medial nucleus, LOT – nc. of lateral olfactory tract, BAOT - bed nc. of the accesory olfactory tract, CoA – cortical anterior nucleus, CoP- cortical posterior nucleus, PAC - periamygdaloid cortex, AHA - amygdalo-hippocampal area

High density of CART-IR neurons Low density of CART-IR neurons

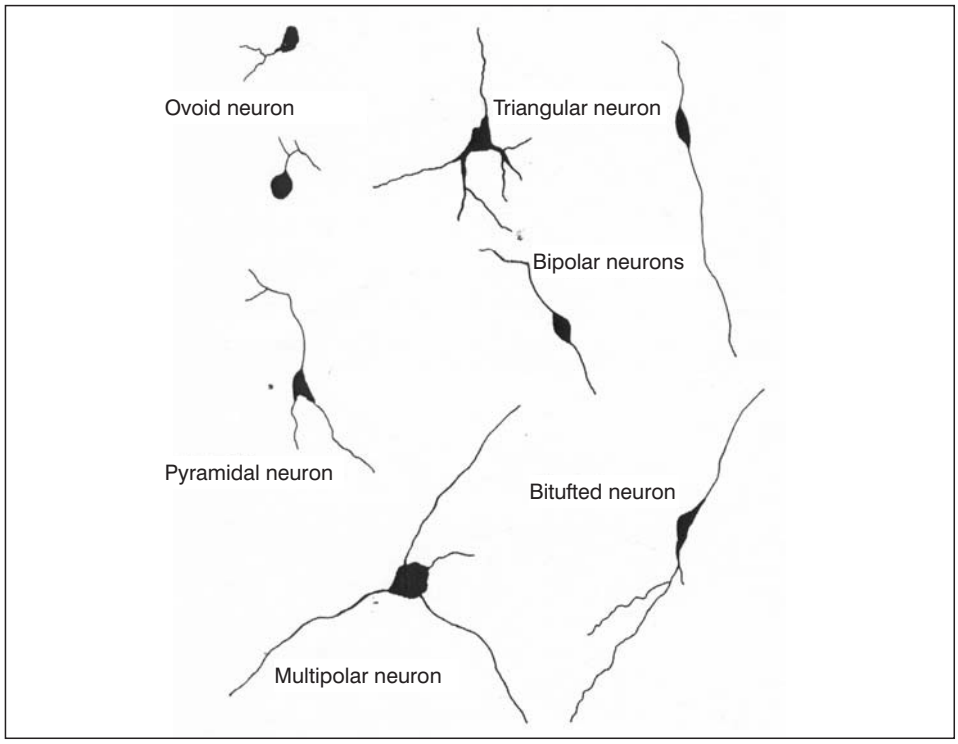

Figure 2. Reconstructions of CART-IR neurons

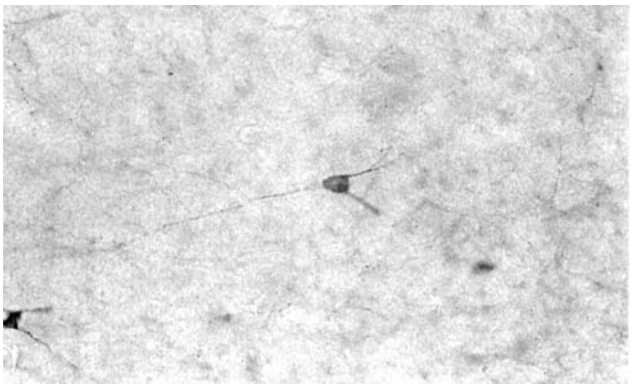

Figure 3. CART-IR immunoreactive neurons, ABC method, 40x

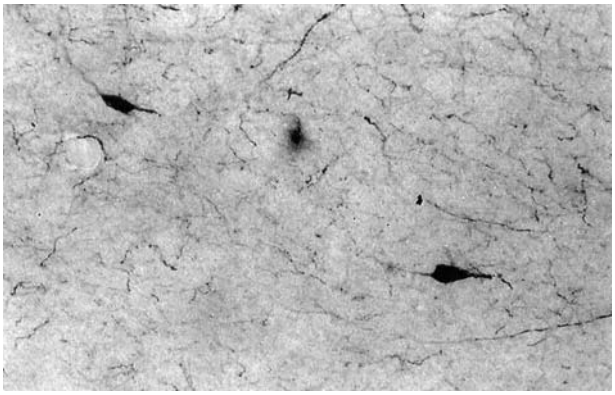

Figure 4. CART-IR immunoreactive neurons, ABC method, 40x

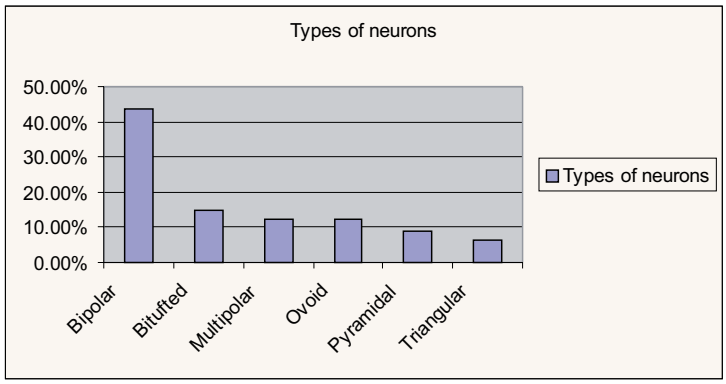

Figure 5. Percentage of different type of CART-IR neurons

Table 1. Basic morphometric characteristics of CART IR neurons in AMY

CART-IR neurons	X (μm)	Min (μm)	Max (μm)	SD
D1	21.82	13.73	30.96	± 4.00
D2	10.20	6.19	14.45	± 2.14
PD	2.55	1	4	± 0.89
S	2.67	2	4	± 0.98

(D1-longer diameter of perikaryon, D2-shorter diameter of perikaryon, PD- number of primary dendrites, S-number of secondary dendrites, SD-standard deviation).

The results in Table 1 suggest that measured values of longer and shorter diameters of CART –IR perikarya in rat AMY greatly deviate (SD_{D1} = ±4,00 and SD_{D2} = ±2,14) from their mean values. Expressed for the complete AMY, the longer diameter of the cell body varies between 13.73 – 30.96 μm, and the shorter between 6.19 – 14.45 μm.

The mean values of these parameters in different nuclei of AMY were following: the greatest value of longer diameter (x=25.32 μm) had the CART-IR neurons in the La, and the smallest value (x=14.73 μm) those in BL nucleus. Average values of longer diameters in AAA (21.09 ìm) and Ce (23.73 ìm) do not deviate significantly from the mean value obtained for the CART- IR neurons in the whole AMY complex. Mean values of shorter (D2) diameters of perikaryons in AMY nuclei did not show a significant deviation from the mean value even in cases of great differences between the minimal and maximal values.

DISCUSSION

A number of recent papers try to elucidate the details of synthesis, distribution and function of the newly discovered CART neuropeptide, (Douglas *et al*., 1995; Couceyero *et al*., 1997; Koylu *et al*., 1998; Hurd *et al*., 1999; Vrang *et al*., 1999; Hurd and Fagergren, 2000; Elias *et al*., 2001). By *in situ* hybridizaton was found that the distribution of CART mRNA is completely overlapping with the distribution of the CART peptide (Koylu *et al*., 1998).

The functional significance of this peptide, as well as its relationship to other transmitter systems and substances, are not completely known. Behaviour related to consumption of cocaine is sensitive to specific concentrations of dopamine in nucleus accumbens, as well as in AMY. The difference in the expression of CART mRNA in the mesolimbic system between male and female rats was found as a significant elevation of the level of CART mRNA after administration of cocaine in males (Hurd *et al*., 1999).

According to our findings, cortex-like AMY nuclei (Mc Donald, 1992) containing CART-IR neurons are La, BL, AAA, CoP, while non- cortex like nuclei (Mc Donald, 1992) with CART-IR neurons are Ce, and BNST. Even if present in a

great number of AMY nuclei, the density of CART- IR perikarya was different. We found their greatest density in La, BL, AAA, Ce, in BNST and in CoP, respectively. In previous studies the highest density of CART-IR fibers was observed in Ce, Me and CoP, moderate density in La, BL, BM and corticalis anterior, and low density was described in AAA and intercalated nuclei (Koylu *et al*., 1998). Hurd and Fagergren (2000) found in rats the greatest number of CART mRNA in neurons of Me and CoP nucleus, compared to anterior cortical and amygdaloid hippocampal nucleus. This being different to our findings. These differences can be caused by the use of serum against different fragments of CART peptide, with or without colchicine and by different parcellation of AMY.

With the exception of some studies of distribution of CART mRNA and CART peptide, in the available literature we could not find data about the shape and/or morphometric characteristics of CART-IR neurons in AMY of rat.

According to our results the range of longer (13.73 μm - 30.96 μm) and shorter (6.19 μm- 14.45 μm) diameters of CART- IR neurons significantly deviates from the mean value.

Because of this great variability in diameters, we studied the mean values of these parameters for particular AMY nuclei and we found CART- IR neurons with the greatest maximal diameter (x=25.32 μm) in La and the smallest in BL (x=14.73 μm). The size of pyramidal or piriform somata of neurons in rat La is 16 μm - 12 μm and in basal nucleus 20 μm - 12 μm (McDonald, 1992).

Size variation among different classes of Golgi impregnated neurons is the greatest in the BL, and smallest in La (McDonalds, 1985). Most of the cells in rat BL that contains cholecistokinin (CCK), somatostatin, and vasoactive intestinal peptide (VIP) immunoreactive neurons correspond to class II neurons described in Golgi studies. CCK IR cells are large piriform or multipolar neurons (18.9 ± 3.5 x 13.5 ± 1.5 μm). VIP IR neurons have small perikarya 10.6 ± 1.1 x 7.9 μm ± 0.9 μm, and somatostatin IR neurons have medium sized fusiform perikarya of 15.1 ± 2.7 μm x 11.1 ± 1.4 μm (Cassel and Gray, 1989).

It is interesting that the principal neurons in BL, pyramidal ones (including triangular or piriform) are about 17 μm long and about 15 μm wide, with 1-2 primary dendrites (McDonald, 1982), are represented only 9% of all CART- IR neurons. It is possible in BL to recognize as nonpyramidal varietes multipolar, bitufted and bipolar neurons. Small neurons in BL are nonpyramidal and are present as only 5% of all impregnated neurons in BL AMY (Mc Donald, 1982), however small neurons comprise a greater part of CART- IR neurons in AMY. Most of nonpyramidal neurons in BL have small ovoid perikarya (10 μm -14 μm) and 2-6 primary dendrites (McDonald, 1992).

Average values of maximal diameters we found in AAA and Ce do not deviate significantly from the mean value and are 21.09 μm in AAA and 23.73 μm in Ce. Principal neurons of Ce are ovoid or fusiform (18x12 μm) with 3-4 primary dendrites (Mc Donald, 1992). In Ce medial part Cassel and Gray (1989), found most frequent pyramiform (16/22 μm long axis) neurons with 3-4 primary dendrites and some fusiform neurons, while in the lateral part of rat Ce most often they found round or oval perikarya (major axis length 12-18 μm) with 2- 5 primary dendrites. Peptidergic types of rat Ce neurons are present within two or more

morphologically distinct types of neurons and major morphological classes of neurons identifiable with Golgi stains are each associated with more than one neuropetide, indicating heterogenous morphologies of most peptide containing neurons in rat Ce AMY (Cassel and Gray, 1989).

Our conclusion is that the greatest density of CART- IR neurons in rat AMY is in La, BL, and AAA, and that they exhibit great variability in the longer diameter. It is remarakble the greater variability in size of CART-IR neurons in those AMY nuclei with greater density of CART-IR neurons.

The largest CART-IR neurons are in La and, neurons with the smallest long diameter are present in BL AMY. Even if there is not any morphologically distinct CART – IR neuron type in rat AMY, these neurons mainly are small and oval in most of AMY nuclei where they are present. This suggest the functional role of a majority of CART-IR neurons in rat AMY, especially in BL as small interneurons. The small number of CART-IR neurons could be due to large, size of neurons.

Acknowledgement:
This study was supported by grant NR 135011 of Ministry of Science and Ecology Republic of Serbia.

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LOKALIZACIJA I MORFOLOGIJA KOKAIN I AMFETAMIN-REGULISANIH TRANSKRIPT (CART) PEPTIDA IMUNOREAKTIVNIH NEURONA U AMIGDALOIDNOM KOMPLEKSU PACOVA

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SADRŽAJ

Nedavno je otkrivena mRNA u mozgu pacova, koja pokazuje ushodnu regulaciju u *nc. accumbensu* nakon davanja kokaina i amfetamina. Dosadašnji podaci ukazuju na široku distribuciju ove mRNA u mozgu i endokrinim organima. Cilj ove studije bio je mapiranje i detaljan opis mRNA produkta – CART peptid imunoreaktivnih (IR) neurona u amigdalama pacova korišćenjem imunohistohemijske procedure.

Distribucija i gustina CART-IR neurona u amigdaloidnom kompleksu pacova je vrlo različita. Najveća gustina je zabeležena u lateralnom (La), bazolateral-

nom (BL) jedru i prednjoj amigdaloidnoj arei (AAA), umerena u centralnom jedru (Ce) dok je mala gustina prisutna u zadnjem kortikalnom jedru (CoP).

Analizom morfologije CART IR neurona zaključeno je da je najveći broj tih neurona bipolarnog oblika (43.75%). Oko 15% ispitivanih neurona su "bitufted" neuroni, dok je isti procenat multipolarnih (12.5%) i ovoidnih neurona (12.5%). CART-IR neuroni piramidalnog oblika su zastupljeni sa oko 9%, dok je najmanji broj triangularnih neurona (6.25%).

Najkrupniji CART-IR neuroni su u La, dok su neuroni sa najmanjim dužim dijametrom u BL. Generalno posmatrajući možemo zaključiti da su u većini jedara AK prisutni uglavnom mali i ovalni neuroni, posebno u BL, što ukazuje na njihovu funkciju - malih interneurona. Manji broj CART IR neurona bi mogli biti veliki projekcioni neuroni.