Expression of CART Peptide in the Paleoamygdala Neurons and Its Relationship with Sex Hormone Levels

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The location of CART peptide in the paleoamygdala neurons was studied by immunocytochemical reaction. Significant differences in the number of immunoreactive cells and optical density of CART-positive neurons detected over the course of the estrous cycle indicate modulating effects of sex steroids on the expression of CART peptide.

Key Words: *paleoamygdala; cerebral amygdaloid complex; sex steroids; CART peptide; narcotic addiction*

CART (cocaine-amphetamine-regulated transcript) peptide is synthesized in certain zones of the brain under the effect of cocaine and amphetamine, which suggests it as a marker of structures involved in the pathogenesis of drug abuse [6]. This peptide is involved in the development of anxiogenic reactions during narcotic use [5] and formation of narcotic addition [11,13]. It modifies neuroendocrine relationships determining the regulation of alimentary and sexual behavior and the mechanisms of energy homeostasis [8-10,12,15].

Paleoamygdala is a neuroendocrine center of the brain playing an important role in the organization of alimentary, sexual, aggressive, and defense behavior [3,4]. It should be noted that shifts in these behavioral forms are characteristic of clinical picture of drug abuse. Possible involvement of the paleoamygdala in the mechanisms of narcotic addiction is essential for the development of therapeutic methods based on intranasal drug administration.

We characterized the location and cytological features of CART-positive neurons, detected for the first time in the paleoamygdala, and evaluated the possible relationship between neuronal immunoreactivity and fluctuations in sex steroid levels.

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MATERIALS AND METHODS

The study was carried out on adult Wistar rats (7 estrous and 7 metestrous females) grown under standard vivarium conditions. The estrous status was determined by the cytology of vaginal smears. Immunocytochemical detection of CART peptide was carried out on cryostat frontal sections of the brain (30 μ) after brain perfusion with 1 M phosphate buffer (pH 7.4) and 4% paraformaldehyde solution in 0.1 M phosphate buffer. The reaction was carried out on free floating sections simultaneously for all groups of animals. After elimination of endogenous peroxidase and exposure of the sections in blocking solution, they were incubated with primary anti-CART polyclonal rabbit antibodies (dilution 1:8000, 55-102; H-003-62, Phoenix Pharm Incorp) for 48 h. After washout the sections were incubated with second goat anti-rabbit antibodies conjugated with avidin complex (ABC-kit 689321, ICN Biomedicals Inc.) and transferred for 2 h into peroxidase-antiperoxidase complex conjugated with streptavidin (ABC-kit 689321, ICN Bio-

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medicals Inc.). Then the sections were washed and all sections from all groups were simultaneously put into 0.05% diaminobenzidene solution (Sigma) with 0.015% H_2O_2 in phosphate buffer. The sections were then stretched on polylysine-coated slides, dried, and embedded into Canadian balm under coverslips.

Structures of the amygdaloid complex were identified on sections stained after Nissl using the criteria developed for highly informative sections of this brain formation [1]. The same sections were used for quantification of neurons in the paleoamygdala structures (neurons in these structures exhibited positive immunoreactivity to CART peptide). The preparations were studied under an MC-300 triocular light microscope with objectives 10 and 40. Microphotographs were made with a Nicon CoolPix 4500 digital photocam. The images were analyzed using Image J software. In order to evaluate the level of neuronal immunoreactivity to CART peptide in the studied paleoamygdala structures, the neurons were counted on the cytoarchitectonic and immunocytochemical preparations in the microscope visual field (35,000 μ^2) under an objective 40. Optical density was evaluated using PhotoM software in 15 neurons of each paleoamygdaloid structure of each animal using images obtained with objective 40.

The data were statistically processed using Student's *t* test.

RESULTS

The paleoamygdala includes the dorsomedial (DMN), posteromedial (PMN), and posterior cortical nuclei (PCN). In the DMN, small and medium-sized round neurons expressing CART peptide are evenly distributed and form a network with moderate immunoreactivity. Groups of large neurons with angular bodies containing an appreciable amount of immunoreactive precipitate (accumulation of fine granules in the perikaryon) were easily discernible against the background of a fine network of immunoreactive DMN neurons. The precipitate was detected not only in neuron bodies, but also in thick first dendrites originating from them. Large neurons with positive immunoreactivity were located on the ventral pole of the nucleus and in its lateral zones. In some neurons, CART peptide was detected also in the axons, which had varicose dilatations. In some medium-sized neurons CART peptide was seen in the two initial segments of dendrites originating from the opposite poles of the cell. These neurons were spindle-shaped. Some large neurons had 3 processes, these cells being polygonal. Previous studies of the cytoarchitectonics and neuronal organization of this nucleus [2-4] indicate that CART peptide is mainly expressed in the reticular and short-dendrite neurons of DMN.

The morphology of CART peptide-containing neurons in the PMN was different. The peptide was detected in medium-sized and large neurons evenly distributed in the nucleus. Neurons varied in shapes from oval to cone-shaped and polygonal, these latter ones often with an immunoreactive precipitate in the initial segments of dendrites. The intensity of the precipitate was different; some neurons contained much of it, and it shielded the cell nucleus, others contained less, and the clear nucleus was discernible against the background of stained cytoplasm.

The PCN has the medial and lateral parts. The greater parts of neurons positively reacting to the studied peptide are located in the lateral part of the nucleus. High content of CART peptide was observed in the surface zone of the PCN medial zone containing, as we know, axons originating from the accessory olfactory bulb. The sizes of immunopositive neurons varied from small to large. These neurons had different shape (oval, spindle-shaped, and polygonal) and were characterized by different intensity of immunoreactive precipitate. Neurons

TABLE 1. Percentage of Immunoreactive Neurons of the Total Number of Neurons in Rat Paleoamygdaloid Structures at Different Stages of Estrous Cycle (*M*±*m*)

Structure		Stage of estrous cycle	
		estrous	metestrous
DMN		62.53±4.01	39.19±3.52*
PMN		41.19±2.32	31.91±1.88
PCN	medial part	42.16±0.69	32.10±0.95***
	lateral part	45.58±1.04	26.54±1.24***

Note. Here and in Table 2: *p<0.05, **p<0.01, ***p<0.001 compared to estrous.

TABLE 2. Optical Density of Immunoreactive Precipitate in Paleoamygdaloid Neurons Expressing CART Peptide during Estrous and Metestrous (arb. units; *M*±*m*)

Structure		Stage of estrous cycle	
		estrous	metestrous
DMN		385.88±32.33	121.88±24.26**
PMN		385.00±52.83	166.92±10.56**
PCN	medial part	263.95±36.47	110.14±16.34*
	lateral part	299.87±57.36	104.03±15.59

containing CART peptide were located unevenly, but formed small groups of 2-3 to 10 cells. In the lateral part of PCN, CART peptide was detected not only in the surface zone, but also in groups of neurons in the surface cellular and deep zones, whose perikaryons were shaped as pyramids.

The immunoreactivity of CART peptide-containing neurons during the estrous and metestrous stages was evaluated by the percentage of immunopositive neurons of the total number of neurons in the respective paleoamygdaloid zones and by optical density of the immunocytochemical reaction product (Tables 1, 2). Increase in the levels of sex steroids during estrous led to a significant increase in the number of immunoreactive neurons in the DMN and in both parts of PMN.

Evaluation of optical densities of neurons expressing CART peptide (Table 2) showed significant differences in the neurons of the DMN, PMN, and medial part of PCN. This implies that despite the increase in the number of immunoreactive neurons in the lateral PCN part during the estrous, many neurons exhibit low immunoreactivity.

Hence, we showed for the first time that paleoamygdaloid neurons expressed CART peptide and this process was regulated by sex steroids, this suggesting their therapeutic use.

It is known that DMN and PCN are directly related to the receptor and conductor centers of the olfactory analyzer [1], which can be used for intranasal (noninvasive and rapid) drug administration. Application of WGA conjugated with horseradish peroxidase to the olfactory epithelium leads to axonal transport of this complex to the nuclear and shielding structures of the amygdaloid complex. The intranasal route of administration can be used for introduction of some genes into the brain by means of virus vector carriers [7,14]. This approach makes it possible to realize effective gene therapy, leading to synthesis of proteins, whose shortage caused disease, in certain areas of the brain. Intranasal route for introduction of new genes into CNS is the most promising route.

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