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Orexinergic Innervation of the Rat Extended Amygdala

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The orexinergic system participates in the regulation of emotions, stress, hunger, wakefulness and behavioral arousal. Despite their limited number and restricted origin from the lateral hypothalamus, orexin-containing neurons give out vast projections that innervate the entire neuraxis. To identify the orexinergic innervation of the amygdala, which is substantially involved in the hypothalamic output, the distribution of orexin A- and orexin B-containing fibers and terminals was mapped in the rat amygdaloid nuclear complex by using immunohistochemistry. We observed the most prominent axonal immunolabelling in the nuclei of the corticomedial group. The orexin-immunoreactive axons were dense in the basolateral amygdaloid nucleus while the basomedial nucleus contained moderately labelled axons in its all subdivisions. Besides, our results showed that subdivisions and subnuclei of the extended amygdala were specific targets of the orexinergic system as well. The present data suggest that the orexinergic amygdala projections may exert excitatory modulatory effects on the expression of emotional behavior.

Key words: amygdaloid nuclear complex, hypothalamus, immunohistochemistry, orexin, rat.

Introduction

Orexin A and B, also named hypocretin 1 and 2, are two recently described excitatory hypothalamic neuropeptides shown to influence a wide range of physiological and behavioral processes. Orexins are solely synthesized in a restricted population of neurons located in the caudal aspects of the lateral hypothalamus (LH) and the adjacent areas. These neurons project extensively to multiple cerebral regions throughout the entire neuraxis by four major pathways [13]. It has been proposed that orexin (OX) serves as a master switch within multiple efferent pathways that mediate the defense response [15]. Thus, we focused on the amygdala (Am) orexinergic target that is substantially involved in the LH output and contributes most to the functional outcome of the defense responses closely related to emotional behavior.

The amygdaloid nuclear complex in rats consists of several structurally and functionally distinct nuclear groups located deeply in the temporal lobe [6]. It is traditionally divided, on the basis of cytoarchitectonic, hodological, histochemical, and immunohistochemical studies, into a corticomedial division and a basolateral division. The former is evolutionarily newer and encompasses the centromedial and cortical nuclei, while the latter is phylogenetically older and comprises the lateral, basolateral and basomedial amygdaloid nuclei [reviewed in 3]. The rat Am has a wide variety of afferent and efferent connections throughout the central nervous system (CNS) and is involved in a vast range of normal behavioral functions (for a recent comprehensive review see [14]. Furthermore, the extended Am, which includes the bed nucleus of the stria terminalis (BST) and its sublenticular extension into the centromedial Am, is implicated in complex motivational responses [1].

Not long ago, it has been shown that OX neurons in the LH mediate cardiorespiratory responses induced by disinhibition of the Am and BST [15]. More recently, it has been suggested that the OX system is one of the essential modulators required for orchestrating the neural circuits controlling autonomic functions and emotional behaviors [8].

From these backgrounds, we have paid in this study special attention to the orexinergic innervation of the rat extended Am, with a particular focus on the excitatory modulatory role of orexins in autonomic and emotional functions.

Materials and Methods

The experiments were carried out on adult male rats of both sexes, weighing 250-300 g. All housing facilities were supervised and approved by the Ethic Commissions at the Medical University-Sofia, Bulgaria, and the University of Rostock, Germany. The animals were transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, the brains were removed, cut on a Reichert Jung freezing microtome at 30 μm thick sections and they were subsequently processed for ABC (avidin-biotin-horseradish peroxidase complex) immunohistochemistry. Briefly, the tissue sections were treated with hydrogen peroxide (1.2% in absolute methanol; 30 min) to inactivate endogenous peroxidase, and the background staining was blocked with 2% normal goat serum (NGS) in PBS for 30 min. Thereafter, the sections were incubated for 24 h at room temperature with the respective primary antibodies, rabbit anti-OX-A (diluted 1:2000) and rabbit anti-OX-B (1:500; both from Oncogene, Cambridge, MA, USA). After rinsing in 0.1 M PBS, the sections were incubated with the secondary antibody, biotinylated goat anti-rabbit IgG (Dianova, Hamburg, Germany) at a dilution of 1:500 for 2 h at room temperature and finally the ABC complex (Vector Laboratories, Burlingame, CA, USA) was applied for 2 h at room temperature. The peroxidase activity was visualized using 2.4% SG substrate kit (Vector) for 5 min. The sections were then mounted on chrome-gelatin slides, air dried, dehydrated in a graded series of ethanols, cleared in xylene, and coverslipped with Entellan (Merck, Darmstadt, Germany). Each third from the immunostained sections was counterstained with 1% Neutral Red (Sigma, St. Louis, MO, USA) to reveal the precise cytoarchitectonic orientation of the stained neuronal population. The delineation of the investigated structures was made according to the stereotaxic atlas of the rat brain [Paxinos and Watson, 2007]. The specimens were examined and photographed with a Zeiss Axioplan 2 research microscope, and the digital images were saved in a TIF format. Negative controls included an omission of the primary antibody and/or its replacement with a non-immune normal serum as well as antigen-antibody preabsorption experiments with the respective native antigens.

Results

In general, the distribution of OX-A and OX-B immunoreactive cell bodies and fibers was almost identical. The OX-containing perikarya were distributed exclusively in the tuberal part of the hypothalamus. A prominent group of orexinergic neurons was symmetrically located on both sides in the central portion of the LH (Fig. 1A). Most of the immunostained cells were medium in size (25.4 ± 2.3 μm maximal diameter and 16.2 ± 1.6 μm minimal diameter, mean ± S.E.M., n=120) and fusiform or multipolar in shape. A considerably lower number of OX-immunopositive neurons were located immediately ventral to the fornix. In rostral and caudal directions the number of OX neurons gradually diminished.

The rat Am was richly supplied with intensely stained OX fibers. Numerous labelled axons entered the Am through the central amygdaloid nucleus. All its three subdivisions, central medial, central lateral and central capsular, contained a substantial to moderate number of thin varicose OX-immunostained axons (Fig. 1B). Prominent labelling was also observed in the medial amygdaloid nucleus, with a decreasing density from its posterodorsal to posteroventral parts. In the cortical amygdaloid nuclei, anterior, posteromedial and posterolateral, the density of OX axons was moderate (Fig. 1C). In the basolateral group the number of OX-positive axons was lower than in the corticomedial group. Somewhat larger was the number of OX axons in the basolateral nucleus while the lateral

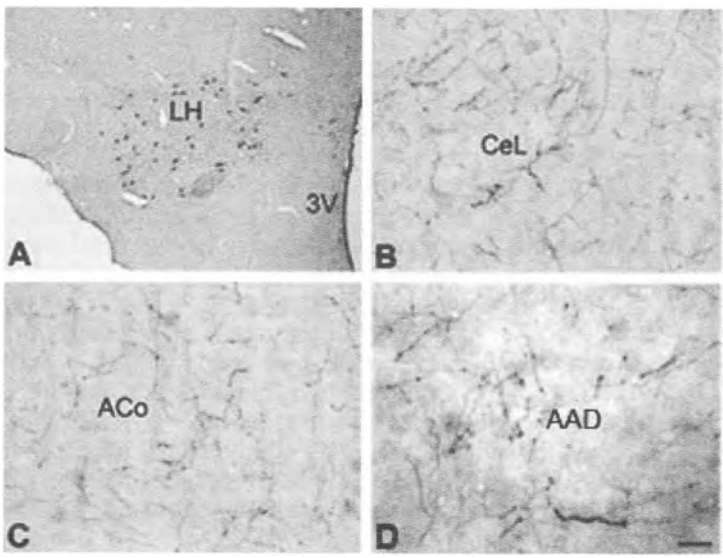

Fig. 1. (**A**) Distribution of orexin-containing cells bodies in the tuberal part of the rat lateral hypothalamus (LH). Note that the immunoreactive perikarya are mostly medium in size with multipolar or fusiform morphology. 3V, 3rd ventricle. (**B**) Dense orexinergic innervation of the central amygdaloid nucleus, lateral division (CeL). Thin varicose axons are also seen in the other subdivisions of this nucleus. (**C**) OX immunoreactive fibers in the anterior cortical amygdaloid nucleus (ACo). Note that their density is comparable to those of the central amygdaloid nucleus. (**D**) Distribution of OX-immunoreactive fibers in the extended amygdala. Numerous densely arranged axonal varicosities and their terminals are observed in the anterior amygdaloid area, dorsal part (AAD). Scale bars = 100 μm (A); 50 μm (**B-D**)

nucleus contained the smallest number of OX axons in the amygdaloid nuclear complex. Conversely, the anterior amygdaloid area was the most heavily innervated by OX axons component of the Am (Fig. 1D).

In the extended Am many new orexinergic targets were found in the anterior cortical nucleus (moderate), amygdalostriatal transition region (moderate) and BST (significant).

Discussion

Despite the low number of OX-containing neurons in the hypothalamus, orexinergic fibers project widely in the CNS. Our previous research has shown that the whole subcortical motor network from motor regions over the basal ganglia and back over the thalamus to the motor cortex receives orexinergic afferents (unpublished data). In this study we confirm and further extend the data of Nambu et al. [11] on the distribution of OX neurons in the adult rat brain. More specifically, the present results provide evidence that the rat amygdaloid nuclear complex is a specific target of orexinergic projections.

The orexinergic input appears to be involved in the modulation of different functional responses that have been attributed to Am, including behavioral and autonomic responses to stressors such as fear and anxiety, assigned to the extended Am. It has recently been reported by Bisetti et al. [2] that the OX system exerts a direct action on neurons in the central medial nucleus, the major output structure of the Am. The authors also demonstrated that Am neurons are depolarized by OX through the activation of postsynaptic OX-2 receptors. which are also excited by vasopressin. Thus, it seems likely that the orexinergic innervation may modulate emotionality and memory consolidation through the excitatory action it exerts on several neurotransmitter and neuromodulatory systems affecting the Am neurons. Besides, we found OX-fibers in the amygdalostriatal transition area that may act as a relay with functions of positive enhancement or negative filtering for the inhibition of specific signal patterns.

On the other hand, it is proposed that the Am is implicated in various aspects of emotional behavior through an extensive network of projections to other brain regions [reviewed in 9]. Specifically, the direct projections from the central nucleus of the Am to the LH [7] appear to be involved in the activation of the autonomic concomitants of conditioned fear and anxiety [cf. 10]. Moreover, there are dense connections from the amygdala to the BST [5] and vice versa [4]. Therefore, input from the Am and BST might be important in modulating the activity of orexin neurons upon emotional stimuli and thus could participate in evoking emotional arousal or fear-related responses [15].

In conclusion, combined with our previous data, it can be inferred that the hypothalamic orexinergic projections may act as an excitatory modulator of motor signals in the Am. In turn, the Am contributes most to the functional outcome of the OX system.

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