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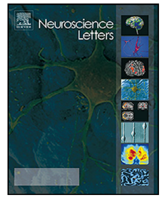

Research paper

Orexin neurons in the lateral hypothalamus project to the medial prefrontal cortex with a rostro-caudal gradient

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HIGHLIGHTS

- Lateral hypothalamic (LH) fibers project to the medial prefrontal cortex (mPFC) with a rostro-caudal hierarchy.
- LH orexinergic fibers distributes in the mPFC with a rostro-caudal increasing pattern.
- Distribution of type 1 orexin receptors increased from rostral to caudal mPFC.

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ABSTRACT

Orexin neurons in the lateral hypothalamus (LH) play an important role in arousal, guaranteeing the execution of medial prefrontal cortex (mPFC)-related higher cognitive functions. The mPFC is anatomically and functionally a rostro-caudal hierarchy. Little is known about the innervation pattern, especially in the rostro-caudal model, from the arousal-promoting orexin system in the LH to the mPFC subregions, including the anterior cingulate cortex (AC), prelimbic cortex (PL) and infralimbic cortex (IL). Here, we used an anterograde tracing method and immunohistochemistry and found that the density of the LH, as well as orexinergic, fibers increased from the rostral part to the caudal part of the mPFC, regardless of AC, PL or IL. Similarly, the distribution of type 1 orexin receptors in the mPFC follows a rostro-caudal increasing gradient hierarchy. These data suggest a rostro-caudal hierarchy of LH orexinergic innervation to the mPFC. We hope to provide anatomical and morphological evidence for the regulation pattern of the arousal-promoting orexin system on the cognition-related mPFC system.

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1. Introduction

The lateral hypothalamus (LH) is involved in diverse functions, such as arousal [1], stress [2], motivation [3], and energy homoeostasis [4]. Orexin neurons in the LH secret neuropeptides orexin-A and orexin-B and are the key regulators of wakefulness [5,6]. Earlier studies have revealed a widespread distribution of orexin receptors throughout the central nervous system, including medial prefrontal cortex (mPFC) [7,8]. Orexins selectively excite non-specific thalamocortical projection neurons [9], enhancing their glutamatergic input into the mPFC pyramidal neurons [10]. Our previous studies demonstrated a much higher density of orexin receptors type 1 (OX_1Rs) than OX_2Rs in layers II/III and V of the mPFC, suggesting a more important role of OX_1Rs in orexin-mediated function in the mPFC [11]. OX_1Rs showed a preferential affinity for orexin-A. The application of orexin-A increased the excitability of pyramidal neurons and maintained the basic excitatory levels of these neurons [11,12].

The mPFC plays an important role in decision making, attention, learning and memory. It can be dorso-ventrally divided into the anterior cingulate cortex (AC, BA 24b), prelimbic cortex (PL, BA 32) and infralimbic cortex (IL, BA 25) [13]. Several studies have revealed subregional differences in projections from the LH orexin neurons to the mPFC neurons. LH neurons send a moderate density of fibers to the AC but less dense projections to the PL and IL cortices [14]. Regarding orexin fibers, most of the orexin axons are ipsilat-

Abbreviations: AC, anterior cingulate cortex; BA, brodman area; BDA, biotinylated dextran amine; DAB, diaminobenzidine; HRP, horseradish peroxidase; IL, infralimbic cortex; LH, lateral hypothalamus; mPFC, medial prefrontal cortex; OX_1R, type 1 orexin receptor; PBS, phosphate buffered solution; PFA-DMH, perifornical area and dorsomedial hypothalamus; PL, prelimbic cortex; SD, Sprague-Dawley.

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erally projected to the mPFC, with few collaterals [15]. A moderate amount of labeling was detected in the PL and IL, but not the AC [16]. Interestingly, the mPFC displays a rostro-caudal difference in cognitive functions. The rostral, but not caudal, part of the mPFC is essential for incentive contrast [17]. Consumption-related theta phase-locking during reward activity is prominent in the rostral mPFC and decreases progressively towards the caudal mPFC [18]. It remains largely unknown whether the LH orexin fibers or the distribution of orexin receptors in the mPFC follows a rostro-caudal gradient model.

In this study, LH fibers were anterogradely labeled, and their respective distributions in the rostral, middle, and caudal parts of the mPFC were quantitatively calculated. Immunohistochemical studies identified and quantified the co-localization of labeled fibers and orexin-A. Additionally, we explored the distribution of OX_1Rs in the three subdivisions of the mPFC. We hope to provide evidence for the anatomical and morphological pattern of the arousal-promoting orexin system's regulation of the cognition-related mPFC system.

2. Material and methods

2.1. Animals

Male Sprague-Dawley (SD) rats (250–300 g) were group-housed in accordance with the Third Military Medical University Guide for the Care and Use of Laboratory Animals, which certifies compliance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. Rats were maintained on a 12 h light/dark cycle with ad libitum access to food and water.

2.2. Anterograde tracing experiments

SD rats were anaesthetized with sodium pentobarbital (50 mg/kg, i.p.). Under stereotaxic guidance, a glass microelectrode (20–50 μm tip) attached to a microsyringe was lowered into the LH orexin field (n = 7; AP–3.2, ML ± 1.6, DV −8.8) [19] and held in place for 5 min. The injection area of each animal was determined by referring to the atlas by George Paxinos and Charles Watson (1998) to identify the approximate area. For anterograde experiments, the rats received unilateral (right) pressure injections of biotinylated dextran amine (BDA; MW 10,000; 10%, 0.5 μl, kit, Invitrogen) for 5 min. After tracer delivery, the microelectrode remained in place for a further 5-min period before being slowly withdrawn. After a survival period of 7 days, animals were deeply anaesthetized with an overdose of sodium pentobarbital (100 mg/kg, i.p.). They were perfused with saline (0.9%), followed by perfusion with 4% paraformaldehyde. The rat brains were then carefully removed from the skulls and tissue prepared for subsequent immunocytochemical and histochemical study. Serial coronal sections (40 μm) were cut with a freezing stage microtome and were collected sequentially in 5 sets and stored in cryoprotectant solution at 4 °C prior to immunohistochemical processing. One set of brain sections was processed immunohistochemically using the biotin–avidin–peroxidase technique (kit, Invitrogen), according to the method of Gerfen and Sawchenko [20].

To reveal anterogradely BDA-labeled neurons and their terminal axonal arbors, biotin-avidin-horseradish peroxidase (HRP) histochemistry was performed. First, sections were incubated free-floating with 3% H_2O_2 to quench endogenous peroxidase activity. After three washes in phosphate buffered saline solution (PBS; 0.01 M, pH 7.4), the sections were permeabilized for 1 h in PBS containing 0.5% Triton-X (Sigma). Then, the sections were placed in an avidin–HRP mixture (1:800, kit, Invitrogen) at 4 °C overnight. After washing in 0.01 M PBS, the sections were reacted in 0.05% diaminobenzidine (DAB)/0.015% H_2O_2. The sections were then washed in PBS, mounted onto poly-lysine coated slides and dehydrated at 25 °C overnight. Finally, the slices were counterstained with hematoxylin to distinguish the target regions conveniently.

2.3. OX_1R and orexin-A immunohistochemistry

Slices from 5 SD rats were used to perform OX_1R immunohistochemical staining. The protocol for OX_1R immunohistochemistry was similar to the protocol for BDA except the slices were 20 μm, and the goat antibody against OX_1R (1:100; Santa Cruz Biotechnology) was used as the primary antibody, while anti-goat-biotin was used as the secondary antibody. For orexin-A immunohistochemical staining, first, BDA was stained with FITC-Avidin (1:200; Boster Biotechnology). Then, goat antibody against orexin-A (1:1000; Santa Cruz Biotechnology) was applied, and anti-goat-568 (1:200; Invitrogen) was subsequently used as secondary antibody. The specificity of the immunohistochemical reaction was corroborated with controls in which either the primary antibody or secondary antibody was omitted. In all these negative controls, the immunostaining was eliminated.

2.4. Data acquisition

Photomicrographs of injection sites were taken through the apparent center of the injections. Digital images were prepared using a Leica microscope in a blind manner. Adobe Photoshop (Adobe Systems, San Jose, CA, USA) was used to adjust the brightness, contrast and color balance. Somas were counted at 4× magnification using imaging analysis software (Image-Pro Plus 6.0), and positive fiber lengths were calculated at 40× magnification in rostral mPFC sections (Bregma 5.2–3.7 mm), middle mPFC sections (Bregma 3.7–2.7 mm) and caudal mPFC sections (Bregma 2.7–1.7 mm) by imaging analysis software (cellSens Standard 1.7). The size of the analyzed area was 192 μm × 145 μm and was consistent across all sections.

2.5. Statistical analysis

All the data were presented as the mean ± SEM. Fibers in mPFC subdivisions were compared using two-way ANOVA and Turkey's test. Significant differences were accepted at $p < 0.05$.

3. Results

3.1. LH fiber density increases from rostral to caudal part of the mPFC

To investigate whether neurons located in the LH projected differently to subregions in the mPFC, we first injected the anterograde tracer biotinylated dextran amine (BDA) into the LH. The injection site is shown in Fig. 1A. Both the densely labeled center and the less densely labeled peripheral part of the injection site were located within the LH, except for slight diffusion in the perifornical area and dorsomedial hypothalamus (PFA-DMH). The injection sites of all 7 samples were located in the LH (Fig. 1A). Although BDA-labeled fibers were bilaterally observed in the mPFC, the ipsilateral projections (Fig. 1B, C and D) were much higher than the contralateral components (Fig. 1G). Fig. 1B, C, and D show representative examples of BDA-positive fibers in the AC, PL, and IL, respectively, in the coronal plane 3.2 mm from the Bregma. Numerous varicosities and collaterals were observed for the scatter-labeled fibers in the three subregions. The schematics of the fiber distribution indicated the differences in LH-labeled fibers in the AC, PL and IL (Fig. 1E). We calculated the length of BDA-positive fibers in the rostral, middle, and caudal parts of these three subregions, and the results showed

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