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Projections from the central nucleus of the amygdala to the nucleus pontis oralis in the rat: An anterograde labeling study

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HIGHLIGHTS

- PHA-L was iontophoresed into the central nucleus of the amygdala of rats.
- PHA-L-labeled axons/terminals were found in the NPO ipsilateral to the injection site.
- PHA-L-labeled axons/terminals in the NPO were glutamatergic.

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ABSTRACT

The present study was designed to elucidate the neuronal projections from the amygdala to the nucleus pontis oralis (NPO). We propose that glutamatergic cells in the central nucleus of the amygdala (CNA) activate neurons in the NPO, which is the critical brainstem site that is responsible for the generation and maintenance of active (REM) sleep. Phaseolus vulgaris-leucoagglutinin (PHA-L), an anterograde transported neuronal tracer, was iontophoresed into the CNA of adult male Sprague-Dawley rats. After a survival time of 7–8 days, the animals were perfused with a fixative and brain tissue was prepared for histological analysis. Sections of the NPO and CNA, which were immunostained with an antibody against PHA-L, were examined with light microscopy. In addition, in order to identify the phenotype of PHA-L-labeled fibers and terminals in the NPO, a double immunohistochemical technique was employed with antibodies against PHA-L and the vesicular glutamate transporter type 2 (VGluT2). Numerous PHA-L-labeled axons and terminals were found in the NPO ipsilateral to the injection site in the CNA. Within the NPO, the majority of labeled fibers were located in the dorsolateral portion of the caudal part of the nucleus. Double-labeling immunostaining studies revealed that PHA-L-labeled axons and terminals in the NPO were glutamatergic. The present demonstration of direct, excitatory (glutamatergic) projections from the CNA to the NPO provide an anatomical basis for the amygdalar control of active sleep.

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1. Introduction

The amygdala is a complex structure, comprised of more than a dozen nuclei, located deep within the medial temporal lobe of the brain [13,14]. It is well known for its involvement in cognition and decision-making and in the emotional states [14,18]. In addition to these functions that are expressed during wakefulness, there is a growing body of data that suggests that the central nucleus of the amygdala (CNA) also participate in the generation of active (REM) sleep [17,23]. Using an in vivo rat and guinea pig preparations, we previously determine that neurons in the CNA are capable of activating neurons in the NPO which is the key brainstem site that is responsible for initiating and maintaining active sleep [11,19,26,27]. We also reported that glutamatergic neurons in the CNA project directly to the NPO [5]. However, the precise location of the terminals of these projections within

Abbreviations: 3V, third ventricle; ACo, anterior cortical amygdaloid nucleus; *AV*, anterior nuclei of thalamus, ventral part; BLA, basolateral amygdaloid nucleus, anterior part; CA3, Cornu ammonis area 3 of the hippocampus; CeC, central amygdaloid nucleus, capsular division; CeL, central amygdaloid nucleus, lateral division; CeM, central amygdaloid nucleus, medial division; CPu, caudate putamen; DTgP, dorsal tegmental nucleus, pericentral part; IC, inferior colliculus; LDT, laterodorsal tegmental nucleus; LGP, lateral globus pallidus; LH, lateral hypothalamic area; LL, lateral lemniscus; LSO, lateral superior olivary nucleus; Me5, mesencephalic trigeminal tract; Me, medial amygdaloid nucleus; ml, medial lemniscus; Mo5, motor trigeminal nucleus; NPO, nucleus pontis oralis; opt, optic tract; P, pyramidal tract; Pir, piriform cortex; PPT, pedunculopontine tegmental nucleus; scp, superior cerebellar peduncle; tz, trapezoid body; Tz, nucleus of the trapezoid body; *VA*, ventral anterior nucleus of the thalamus.

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the NPO and the pattern of innervations of NPO cells by the terminals of projections from the amygdala have not been studied. Accordingly, in the present experiment, we employed Phaseolus vulgaris-leucoagglutinin (PHA-L), an anterograde tracer, to examine the fibers and terminals of CNA neurons that project directly to the NPO. In addition, we explored that phenotypic nature of these projections to the NPO by double-immunostaining PHA-L-labeled fibers with anti-VGluT2 antibodies.

2. Materials and methods

Six adult male Sprague-Dawley rats (290–320 g weight) were employed. All rats were determined to be in good health by veterinarians of the VA Greater Los Angeles Healthcare System (VAGLAHS). Animal treatment and handling in these experiments were in accordance with the *Guide for the Care and Use of laboratory Animals* (7th ed., National Academy Press, Washington, DC, 1996), and all experimental protocols were approved by the Institutional Animal Care and Use Committee of the VAGLAHS.

The animals were anesthetized with isoflurane (4% induction, 2.5% maintenance) and placed in a stereotaxic apparatus. Phaseolus vulgaris-leucoagglutinin (PHA-L, Vector Laboratories, Burlingame, CA; Catalog No. L-1110; 0.5% in 0.1 M phosphate buffer, pH 6) was iontophoresed unilaterally (+6 μA, 7 s on and 7 s off for a duration of 20 min) into the CNA using a glass microelectrode.

After a survival time of 7–8 days, the rats were deeply anesthetized with sodium pentobarbital (45 mg/kg, i.p.) and perfused transcardially with 250 ml of ice-cold heparinized saline (containing 1000 units of heparin) followed by 500 ml of a fixative containing 4% paraformaldehyde, 15% saturated picric acid and 0.25% glutaraldehyde in 0.1 M phosphate buffer (PBS) (pH 7.4). Tissue blocks containing the amygdala and the brainstem were removed and postfixed overnight in fresh fixative at 4 °C. The following day, the tissue blocks were transferred into a solution containing 20% sucrose (w/v) in 0.1 M PBS which was kept at 4 °C for 48 h; they were then frozen with dry ice and 15 μm coronal sections were cut with a Reichert-Jung cryostat. All sections were stored in a solution of 0.1 M PBS containing 0.3% Triton X-100 and 0.1% sodium azide at 4 °C.

Immunohistochemical procedures for detecting PHA-L were similar to those previously published [30]. Free-floating sections were incubated overnight with a rabbit antibody against PHA-L (Phoenix Pharmaceuticals, Mountain View, CA; diluted 1:1500–1:8000) in PBST solution (0.1 M PBS with 0.3% Triton X-100). On the following day, the sections were incubated in PBST containing biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA; diluted at 1:300) for 90 min. They were then incubated in an ABC complex (Vector Laboratories, Burlingame, CA; diluted at 1:200) for another 90 min. Subsequently, the sections were treated with a color reaction solution that contained 0.02% 3,3′-diaminobenzidine (DAB) and 0.015% H_2O_2 in 50 mM Tris buffer (pH 7.5) for 15–30 min. After the DAB reaction, the sections were mounted on gelatin-coated glass slides, dehydrated and coverslipped with Permount. In order to identify structures within the amygdala and brainstem, at least one set of sections from each animal was counterstained by neutral red before dehydration.

In order to identify the phenotype of PHA-L-labeled fibers and terminals in the NPO, a double immunofluorescent technique was employed with antibodies against PHA-L and the vesicular glutamate transporter type 2 (VGluT2). The procedure for double immunohistochemical staining was based on that employed in a previous publication [29]. In brief, free-floating sections were incubated simultaneously with a rabbit anti-PHA-L (Phoenix Pharmaceuticals, Mountain View, CA; diluted 1:1500–1:8000) antibody and a mouse monoclonal antibody directed against VGluT2 (MAB5504, 1:350; Millipore, Temecula, CA) in 0.3% normal donkey serum, PBST and 0.1% sodium azide first, at room temperature overnight, and then at 4 °C for 48 h. The sections were then incubated in PBST containing donkey anti-mouse IgG conjugated with Rhodamine (Vector Laboratories, Burlingame, CA; diluted at 1:300) for 90 min. After rinsing for 20 min with PBST, the sections were incubated for another 90 min with donkey anti-rabbit IgG conjugated with fluorescein isothiocyanate (FITC) in the PBST (Vector Laboratories, Burlingame, CA; diluted at 1:200). Finally, the sections were coverslipped with an aqueous solution.

To verify the specificity of the primary antibodies, i.e. anti-PHA-L and anti-VGlut2, selected sections were processed according to the following double-staining procedures: (1) omission of the anti-PHA-L antibody; (2) omission of the anti-VGlut2 antibody; (3) omission of both anti-PHA-L and anti-VGLut2 antibodies.

A Nikon microscope (Eclipse 80*i*) equipped with a CCD camera was used to determine the location of PHA-L labeled fibers and terminals in the NPO under bright-field microscopy. Double florescent staining was examined under the same microscope with a fluorescence generator. Delineation of the boundary of subnuclei within the amygdala and brainstem was determined based upon examination of sections stained with neutral red according to the Paxinos atlas of the rat brain [12]. Other nomenclature for the nucleus pontis oralis (NPO) in the brainstem is the perilocus coeruleus alpha, the sublaterodorsal nucleus, subcoeruleus, etc. [3,9].

3. Results

Under light microscopy, numerous PHA-L labeled neurons were observed within the injection sites in the rostral portion of the CNA; these neurons contained deposits of intense black granules. As shown in Fig. 1B and B′, PHA-L-labeled neurons were confined within the boundary of the CNA.

In all cases, in conjunction with the identification of PHA-L immunoreactive neurons in the CNA, anti-PHA-L-labeled fibers were found in the ipsilateral NPO (Fig. 2A–D). Very few PHA-L-labeled fibers were observed in the NPO on the side that was contralateral to the injection site. PHA-L positive fibers in the NPO exhibited numerous arborizations and en passant buttons that constitute the projection fields of axons of PHA-L-labeled neurons in the CNA. These axons terminate either in the neuropil (Fig. 2B and D) or on small and medium-sized neurons in the NPO (Fig. 2b and d). At the rostral level of the NPO, these terminal fields were found in the dorsolateral portion of the nucleus (Fig. 2A), while at the caudal level, terminal fields were observed in the lateral portion of the NPO (Fig. 2C). In addition, the intensity of PHA-L immunoreactivity in the terminal fields at the rostral level was weaker than that at the caudal level of the NPO. Under high magnification, in the terminal fields at the rostral levels, fragments of PHA-L-labeled fibers were observed that were approximately 200–300 μm in length (Fig. 2B). In contrast, fragments of the same types of fibers at caudal levels were only about 25–50 μm in length (Fig. 2D). However, the number of en passant buttons per unit length of the fibers was similar at both the caudal and rostral levels (~1 buttons/50 μm) (Fig. 2B and D). At the rostral level, the majority of the PHA-L-labeled fibers were oriented in a dorsolateral to ventromedial direction; at the caudal level of the NPO similar fibers were predominantly oriented in the vertical plane (Fig. 2B and D).

In order to characterize the neurochemical phenotype of the CNA neurons that project to the NPO, double immunostaining techniques were employed to determine the expression of the vesicular glutamate transporter 2 (VGluT2), a glutamatergic marker, by PHA-L-labeled fibers in the NPO. Under immunofluorescent microscopy at high magnification (100× objective lens), a substantial proportion (26%) of PHA-L positive fibers with en passant buttons in the