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Research article

Anatomical evidence for lateral hypothalamic innervation of the pontine A7 catecholamine cell group in rat

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ABSTRACT

Substantial behavioral evidence exists to support the idea that the lateral hypothalamus (LH) makes axonal connection with spinally-projecting noradrenergic neurons of the A7 catecholamine cell group in the pons. Through this putative projection, the LH modulates nociception via α_1- and α_2-adrenoceptors in the dorsal horn. We used double-label immunocytochemistry to demonstrate that axons from the LH labeled with the anterograde tracer biotinylated dextran amine (BDA) appose tyrosine hydroxylase-immunoreactive (TH-ir) neuron profiles in the A7 area. Other pontine areas labeled with BDA included the dorsomedial tegmental area, the pontine reticular nucleus, oral part, the caudal aspect of the dorsal raphe, the periaqueductal grey and the A6 area. To confirm the findings of the brightfield experiment, we used confocal microscopy to identify axons from the LH labeled with the anterograde tracer Fluoro-Ruby co-localized with TH-ir dendrites and cell bodies in the A7 cell group. These findings provide an anatomical substrate for behavioral studies in which stimulation of the LH modifies nociception in the spinal cord via norepinephrine.

1. Introduction

It is well known that spinally-projecting noradrenergic neurons from the pontine A7 catecholamine cell group play a role in the descending modulation of pain [1–4]. Axons from A7 neurons innervate laminae I and II of the spinal cord dorsal horn, areas known to process nociceptive input from the periphery [5], and stimulation of the A7 cell group in rat produces antinociception mediated by α_2-adrenoceptors, but pronociception mediated by α_1-adrenoceptors in the dorsal horn [6–8]. The lateral hypothalamus (LH) is also recognized as part of the descending pain modulatory system [9–14]. Stimulation of the LH produces an opposing action of norepinephrine in the dorsal horn similar to that seen by direct stimulation of the A7 cell group; that is, antinociception mediated by α_2-adrenoceptors, but pronociception mediated by α_1-adrenoceptors in the dorsal horn, in both a model of inflammatory nociception [11] and a model of acute nociception [12]. Anatomical studies provide evidence of a direct LH to dorsal horn connection [15,16], but there is no evidence for spinally-projecting noradrenergic neurons in the LH. Taken together, the evidence is suggestive that the LH effects noradrenergic modulation in the dorsal horn through connections with discrete, spinally projecting monoamine cells in the brainstem, such as the A7 cell group.

To test this hypothesis, we used anterograde tract tracing combined with double label immunocytochemistry to demonstrate axonal connections from neurons in the LH with dendrites and cell bodies of tyrosine hydroxylase – immunoreactive (TH-ir) neurons in the A7 area.

2. Experimental procedures

This study was done at the University of Illinois at Chicago and the Institutional Animal Care Committee approved the experimental protocols. The experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996) and EC Directive 86/609/EEC. All efforts were made to minimize animal suffering, reduce the numbers of animals used, and use alternatives to in vivo experiments.

2.1. Animals

Female Sprague-Dawley rats (250–350 g, Charles River, Portage, MI, USA) were used. Rats were kept on a 12-h day/night schedule with free access to food and water.

2.2. Tracer iontophoresis

Each of twelve rats was deeply anesthetized with sodium pentobarbital (50 mg/kg) and surgically prepared using aseptic technique. A

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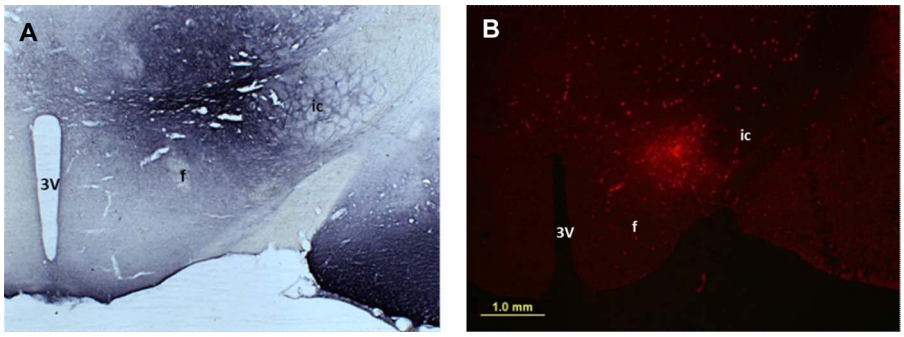

Fig. 1. Photomicrographs (2×) of BDA (A) and Fluoro-Ruby (B) injections in the LH that resulted in anterograde labeling of axons seen in Figs. 2–5. The core of compacted tracer at the center of the injection can be seen in both photos. A halo of neurons around the core represent local labeling of neurons. Abbreviations: 3 V, third ventricle; f, fornix, ic, internal capsule. Scale bar = 1 mm. Distance from bregma is approximately −3.36 mm.

glass micropipette with a tip diameter of 35–40 um was filled with either a 10% solution of BDA (Molecular Probes, Eugene, OR, USA; n = 6) or Fluoro-Ruby (Fluorochrome, Inc., Denver, CO, USA; n = 6) and lowered into the LH through a burr hole to a point defined by the following stereotaxic coordinates: −1.5 mm from bregma, lateral +1.7 mm, vertical +2.2 mm, incisor bar set at +2.5 mm. BDA or Fluoro-Ruby was iontophoretically deposited using 5–15 μA positive current pulses of 500 ms duration and delivered at a rate of 0.5 Hz for 20 min. The pipette was kept in place for an additional 60 s before removal to minimize diffusion of tracer into the pipette track. Two weeks later, each rat was deeply anesthetized with pentobarbital and the brains fixed by transcardial perfusion with the following solutions: 100 ml of normal saline followed by 200 ml of cold 4% paraformaldehyde in 0.1 M phosphate-buffer (pH 7.4), and 100 ml 10% sucrose in 0.1 M PB.

2.3. Tissue processing

Forebrain tissue blocks containing the LH and brainstem blocks containing the dorsolateral pontine tegmentum were frozen, 40-μm transverse sections were cut on a cryostat microtome, and free-floating sections were processed for visualization of BDA and tyrosine hydroxylase-immunoreactivity as previously described [17,18]. Briefly, sections were first processed to visualize BDA using the avidin–biotin complex (Elite Vectastain ABC, Vector Laboratories, Inc., Burlingame, CA, USA) and the nickel-enhanced 3,3′-Diaminobenzidine (DAB) chromogen reaction. Following rinses, sections were incubated for 60–90 min in a solution containing the avidin–biotin complex. The blue–black nickel-enhanced DAB chromogen was developed by incubating sections in a solution containing 0.05 M Tris–HCl (pH 7.6), 0.67% nickel ammonium sulfate, filtered 0.013% DAB and 0.005% hydrogen peroxide. Sections from the pons were incubated overnight in a solution containing mouse antisera directed against tyrosine hydroxylase (TH; IncStar Co., Stillwater, MN, USA) diluted 1:2000 with phosphate buffered saline (PBS) and 0.1% Triton X-100. Following rinses, sections were incubated for 60 min in a solution containing rabbit anti-mouse secondary antibody (Jackson ImmunoResearch Inc., West Grove, PA, USA) diluted 1:80 with PBS and then rinsed. Sections were then incubated for 60 min in a solution containing mouse PAP complex (Cappel Laboratories Inc., Cochranville, PA, USA) diluted 1:150 with PBS, and rinsed. The brown DAB chromogen was developed by incubating the sections for 6 min in a solution that contained 0.05% filtered DAB and 0.005% hydrogen peroxide in Tris–HCl buffer at pH 7.6.

Fluoro-Ruby brains were perfused as previously described, removed and placed in a 4% paraformaldehyde solution for 4 h, and then transferred to a 20% sucrose solution for 48 h. Tissue blocks containing the LH and the A7 cell group were then sliced as described. Sections containing the A7 area were incubated in blocking solution (75 ml PBS, 0.6 g of 0.8% bovine albumin, Sigma, St. Louis, MO, USA); 0.74 ml of 0.2% Amersham gelatin (GE Healthcare Life Sciences, Piscataway NJ, USA) and then immediately incubated for 48 h in primary antiserum made in mouse against TH diluted 1:2000 with PBS and containing 0.5% Triton X-100. After rinses, the tissues were incubated for 60 min in a secondary antibody made against rabbit and labeled with cyanine2 (Cy2; 1:200; Invitrogen, Austin, TX, USA) in 0.5% Triton X-100 in PBS. All sections were mounted on gel-coated slides, processed through alcohols and xylenes, and coverslipped.

2.4. Microscopy

BDA photomicrographs were made using a Zeiss Ax10 Imager M1. The low powered pontine section was photographed on Nikon E800 light microscope, as was the Fluoro-Ruby injection site, but with a fluorescence TRITC filter. Sections containing the A7 cell group were photographed using a Zeiss LSM 510 META confocal microscope. Stack images were taken at 100X magnification. No image corrections were made for contrast or color.

3. Results

This is a descriptive study in which anterograde transport of BDA or Fluoro-Ruby was used to identify the locations of axons in apposition to, and co-localized with, TH-ir soma and dendrites in the A7 area. Both of the injection sites for BDA and Fluoro-Ruby were located in the tuberal aspect of the LH, between the fornix and the internal capsule. Fig. 1 shows a core of compacted tracer for BDA (A) and Fluoro-Ruby (B) in the LH with some labeling of neuron cell bodies and processes in the area surrounding the compaction.

3.1. Brightfield microscopy

Generally, the most densely labeled axons were seen in the ipsilateral dorsomedial tegmentum medial to the A7 area (Fig. 2C) and in the pontine reticular nucleus oralis (Fig. 2E). This labeling continued through the rostro-caudal extent of the pons, although the labeling was much less in the caudal aspect of the pons. Contralateral projections were much more limited (Figs. 2D and F). Also noted were a number of BDA labeled axons in the central aspect of the caudal dorsal raphe nucleus (Fig. 2G) and in apposition to TH-ir neuron profiles in the A6 area (Fig. 2H). BDA labeled axons were also seen in the ventrolateral periaqueductal gray (Fig. 2I). In the dorsal raphe, two large multipolar neuron profiles were labeled purple-black, and in the A7 area, a few neuron profiles were lightly labeled with BDA-ir.

In the A7 area, TH-ir neuron profiles were found in the dorsolateral pontine tegmentum below the superior cerebellar peduncle and adjacent to the Kölliker-Füse nucleus. These profiles were multipolar, approximately 30–40 μ in diameter, and with some dendritic processes visible. BDA labeled axons were primarily ipsilateral to the LH injection site. Varicosities were clearly seen on a number of the labeled axons. Fig. 3A and B are 40× magnifications of TH-ir neuron profiles in the A7 area with BDA-labeled axons in the same plane of focus.