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Projections from the rat cuneiform nucleus to the A7, A6 (locus coeruleus), and A5 pontine noradrenergic cell groups

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ABSTRACT

Stimulation of neurons in the cuneiform nucleus (CnF) produces antinociception and cardiovascular responses that could be mediated, in part, by noradrenergic neurons that innervate the spinal cord dorsal horn. The present study determined the projections of neurons in the CnF to the pontine noradrenergic neurons in the A5, A6 (locus coeruleus), and A7 cell groups that are known to project to the spinal cord. Injections of the anterograde tracer, biotinylated dextran amine in the CnF of Sasco Sprague-Dawley rats labeled axons located near noradrenergic neurons that were visualized by processing tissue sections for tyrosine hydroxylase-immunoreactivity. Anterogradely labeled axons were more dense on the side ipsilateral to the BDA deposit. Both A7 and A5 cell groups received dense projections from neurons in the CnF, whereas locus coeruleus received only a sparse projection. Highly varicose anterogradely labeled axons from the CnF were found in close apposition to dendrites and somata of tyrosine hydroxylase-immunoreactive neurons in pontine tegmentum. Although definitive evidence for direct pathways from CnF neurons to the pontine noradrenergic cell groups requires ultrastructural analysis, the results of the present studies provide presumptive evidence of direct projections from neurons in the CnF to the pontine noradrenergic neurons of the A7, locus coeruleus, and A5 cell groups. These results support the suggestion that the analgesia and cardiovascular responses produced by stimulation of neurons in the CnF may be mediated, in part, by pontine noradrenergic neurons.

1. Introduction

The cuneiform nucleus (CnF) is a part of midbrain reticular formation (Jones, 1995) located ventral to the inferior colliculus, lateral to the mesencephalic trigeminal nucleus, medial to the lateral lemniscus, and dorsal to the pedunculopontine tegmental nucleus and the parabrachial region (Paxinos and Watson, 1998). The CnF receives extensive afferent projections from ascending spinomesencephalic tract neurons in laminae I (Lima and Coimbra, 1989; Menetrey et al., 1982; Swett et al., 1985; Veazey and Severin, 1982), V (Leah et al., 1988; Menetrey et al., 1982), IX (Menetrey et al., 1982; Leah et al., 1988), and its neurons are responsive to nociceptive stimuli (Carlson et al., 2004; Haghparast et al., 2010; Lanteri-Minet et al., 1994). Activation of CnF is associated with bradycardia, increased blood pressure, as well as antinociception. Specifically, electrical stimulation of sites in the CnF increases blood pressure (Lam and Verberne, 1997; Verberne, 1995; Verberne et al., 1997) and produces bradycardia (Korte et al., 1992). Bradycardia is also produced by microinjection of the excitatory amino acid glutamate or homocysteic acid into the CnF (Lin et al., 1987). Furthermore, both electrical (Guinan et al., 1989; Sandkuhler and Gebhart, 1984; Zemlan and Behbehani, 1988) and chemical stimulation of the CnF by microinjection of morphine (Haigler, 1976; Haigler and Mittleman, 1978) or glutamate (Carstens, 1988; Carstens et al., 1990) produces antinociception. Finally, microinjections of the nicotinic cholinergic agonist N-methylcarbachol in the CnF produces antinociception that can be blocked by intrathecal injection of alpha$_2$-adrenoceptor

Abbreviations: 4V, fourth ventricle; 7n, facial nerve; A5, A5 cell group; A6, A6 cell group (locus coeruleus); A7, A7 cell group; Aq, cerebral aqueduct (Sylvius); BDA, biotinylated dextran amine; cA5, caudal A5 cell group; CG, central gray; cLC, caudal locus coeruleus; CnF, cuneiform nucleus; g7, genu facial nerve; Gi, gigantocellular reticular nucleus; GiA, gigantocellular reticular nucleus pars alpha; IC, inferior colliculus; LC, locus coeruleus (A6 cell group); lfp, longitudinal fasciculus of the pons; ll, lateral lemniscus; me5, mesencephalic trigeminal tract; ml, medial lemniscus; mlf, medial longitudinal fasciculus; Mo5, motor trigeminal nucleus; PAG, periaqueductal gray; PBS, phosphate-buffered saline; PGi, paragigantocellular reticular nucleus; PHA-L, *Phaseolus vulgaris* leucoagglutinin; Pn, pontine nuclei; PnC, pontine reticular nucleus caudal part; PnO, pontine reticular nucleus oral part; py, pyramidal tract; rA5, rostral A5 cell group; rLC, rostral locus coeruleus; RMg, nucleus raphe magnus; RtTg, pontine reticulotegmental nuclei; scp, superior cerebellar peduncle; SO, superior olive; SubC, subcoeruleus nucleus; TBS, TRIS-buffered saline; TH, tyrosine hydroxylase; TH-ir, tyrosine hydroxylase-immunoreactive; tth, trigeminothalamic tract; WGA-HRP, germ agglutinin conjugated to horseradish peroxidase.

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antagonists (Iwamoto and Marion, 1993) suggesting that the antinociception produced by stimulation of neurons in the CnF is mediated, in part, by activating spinally projecting noradrenergic neurons.

Since neither the CnF nor the spinal cord dorsal horn contains noradrenergic neurons (Dahlstroem and Fuxe, 1964; Hokfelt et al., 1984), CnF neurons must either directly or indirectly activate spinally projecting noradrenergic neurons. Three noradrenergic cell groups, designated A5, A6 (locus coeruleus, LC), and A7 (Dahlstroem and Fuxe, 1964), are known to project to the spinal cord (Westlund and Coulter, 1980; Westlund et al., 1981, 1982, 1983). In Sasco Sprague-Dawley rats, the noradrenergic neurons in the A7 cell group are implicated in modulation of antinociception (Clark and Proudfit, 1991b; Holden et al., 1999; Nuseir and Proudfit, 2000; Yeomans et al., 1992; Yeomans and Proudfit, 1992). In addition, noradrenergic A5 neurons are implicated in mediation of cardiovascular responses, such as bradycardia (Byrum and Guyenet, 1987; Clark and Proudfit, 1993; Drye et al., 1990; Loewy et al., 1979a, 1979b, 1986), as well as antinociception (Burnett and Gebhart, 1991; Miller and Proudfit, 1990). Finally, descending neurons in the caudal LC innervate the spinal cord ventral horn (Bjorklund and Skagerberg, 1982, Clark and Proudfit, 1991a, Clark et al., 1991, Proudfit and Clark, 1991; Clark and Proudfit, 1992) and are implicated in control of motor reflexes (Fung et al., 1991). Thus, we hypothesized that CnF neurons project to the noradrenergic neurons of the A7 and A5 cell groups, but not the LC. Presented studies analyzed projections of neurons in the CnF to the A5, LC, and A7 cell groups in Sasco Sprague-Dawley rats by using anterograde tracer biotinylated dextran amine (BDA) combined with immunocytochemical detection of the catecholamine synthesizing enzyme tyrosine hydroxylase (TH) in the same tissue sections. Although definitive evidence for the existence of monosynaptic pathways requires ultrastructural analysis, the results of the present studies provide presumptive evidence for direct projections from neurons in the CnF to pontine noradrenergic cell groups.

2. Materials and methods

2.1. Animal care and use

The University of Illinois Animal Care and Use Committee approved the experimental protocols involving the use of vertebrate animals. Also, 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). All efforts were made to minimize animal suffering, reduce the number of animals used, and use alternatives to *in vivo* experiments.

2.2. Tracer iontophoresis

The anterograde tracer BDA was iontophoretically deposited into sites in the CnF of twelve female Sprague-Dawley rats (250–350 g; Sasco, Madison, MI) and six of these cases were selected for the analysis (Fig. 1). Animals were deeply anesthetized with pentobarbital (50 mg/kg) and surgically prepared using aseptic techniques. No estrous stage was determined at the time of sacrifice. A glass micropipette with a tip diameter of 15–20 μm that was filled with a 10% solution of BDA (10,000 MW; D-1956, Molecular Probes, Eugene, OR) in saline was lowered to the appropriate target site in the CnF using the following stereotaxic coordinates: anterior 0.7 mm, ventral 4.3 mm, and lateral 1.7 mm with the incisor bar set at −2.5 mm. Coordinates are relative to the interaural line. BDA was iontophoretically ejected using 5–10 μA positive current pulses of 500 msec duration at a rate of 0.5 Hz for 20 min. The pipette remained in place for 60 seconds after the injection to minimize diffusion of the tracer along the electrode track. A period of 12–18 days was allowed for tracer transport and the animals were then deeply anesthetized with pentobarbital (50 mg/kg) and transcardially perfused using the method of Brandt and Apkarian (Brandt and Apkarian, 1992) as follows: 80 ml of physiological saline, followed by 100 ml of 4% paraformaldehyde in 0.1 M acetate buffer (pH 4.5), 300 ml of chilled 4% paraformaldehyde in 0.05 M borate buffer with 0.05% glutaraldehyde (pH 9.5), and 300 ml of chilled 10% sucrose solution in 0.1 M phosphate buffer (pH 7.6). Fixed brains were removed, cut into blocks, and stored in a solution of 20% sucrose in phosphate buffer (pH 7.6; 4 °C) for several days.

2.3. Tissue processing

Tissue blocks were frozen, 40 μm transverse sections were cut on a cryostat microtome and free-floating sections were processed for visualization of BDA and TH-immunoreactivity using methods described in a previous report (Bajic and Proudfit, 1999). Briefly, sections were first processed for visualization of BDA by incubating tissue sections for 60–90 min in a solution containing the avidin–biotin complex (Elite Standard Vectastain ABC Kit, PK-6100, Vector Laboratories, Inc., Burlingame, CA) followed by two 10 min rinses in phosphate-buffered saline (PBS, pH 7.6, 21 °C) and a 2 min rinse in 0.1 M Tris-buffered saline (TBS, pH 7.6). Blue-black nickel-enhanced peroxidase reaction product was produced by incubating tissue sections for 4–5 min in a solution containing 0.4 g of nickel ammonium sulfate, 15 mg of 3,3′-diaminobenzidine (Aldrich, Milwaukee, WI) and 16 μl of 30% hydrogen peroxide in 100 ml of 0.1 M TBS.

To visualize the location of tyrosine hydroxylase-immunoreative (TH-ir) neurons, brainstem sections were next incubated for 12 h in a solution containing mouse antisera directed against TH (Incstar Corp., Stillwater, MN) that was diluted 1:1000 with PBS and contained 0.5% Triton X-100. After two 10 min rinses in PBS, tissue sections were incubated for 50–60 min in a solution containing donkey anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, Inc., W. Grove, PA) diluted 1:100 with PBS that contained 0.5% Triton X-100, and were then rinsed twice in PBS. Finally, tissue sections were incubated for 50–60 min in a solution containing mouse peroxidase anti-peroxidase complex (ICN Pharmaceuticals, Inc., Costa Mesa, CA) diluted 1:150 with PBS that contained 0.5% Triton X-100, followed by two 10 min rinses in PBS and a 2 min rinse in 0.1 M TBS. Brown perioxidase reaction product was produced by incubating tissue sections for 3 min in a solution containing 22 mg of 3,3′-diaminobenzidine and 20 μl of 30% hydrogen peroxide in 100 ml of 0.1 M TBS. Sections were rinsed in PBS, mounted from 0.033 M phosphate buffer onto subbed slides, allowed to air-dry before dehydration in a series of ethanol, cleared in xylene, and coverslipped. This processing produced brown staining of TH-ir neurons in the noradrenergic cell groups that were clearly distinguishable from the blue-black staining of BDA-labeled axons that originate in the CnF.

2.4. Microscopic analysis

The drawings, counting of terminals, and density calculations were done using a 10×, 20×, as well as 60× oil immersion microscope objective and digital imaging software Neurolucida (MicroBrightField Inc., Colchester, VT). The numbers of anterogradely labeled axons with varicosities that were closely apposed to TH-ir profiles were determined by counting axons apposed to labeled somata and dendrites on both the ipsi- and contralateral sides of noradrenergic cell groups of interest. Being that the LC and A5 cell groups extend along the rostro-caudal axis, in comparison to the more compact nucleus of the A7 cell group, we have divided both the LC and A5 to rostral and caudal divisions. Specifically, the rostrocaudal extent of the A7 cell group corresponded to Plates 53–57 of Paxinos' Atlas (Paxinos and Watson, 1998), while A5 cell group area of quantification corresponded to Plates 56–62. The rostral A5 sections were located at the level of the motor nucleus of the trigeminal nerve while the caudal sections were located at the level of the superior olive. The rostrocaudal extend of the LC analysis corresponded to Plates 55–61 of Paxinos' Atlas (Paxinos and Watson, 1998). The rostral and caudal LC sections were located at the level of the motor nucleus of the trigeminal nerve and the genu of the seventh nerve, respectively. Thus, anterogradely labeled axons were counted in at least five transverse sections along the rostro-caudal axis through both rostral and caudal divisions of the LC and the A5 cell groups, as well as throughout the A7 cell group.

Quantitative analysis included all 6 cases shown in Fig. 1. Anterogradely labeled axons were considered 'closely apposed' to TH-ir profiles if: (1) the axonal varicosity was located immediately adjacent to a TH-ir profile and both structures were in the same focal plane or (2) the varicosity was located on the surface of a TH-ir profile and in the same focal plane as the labeled profile. The density of axons that were closely apposed to TH-ir somata and dendrites was determined by counting all axons with varicosities located within a circumscribed area that included all TH-ir profiles in each of the noradrenergic cell groups. Neurolucida software determined surface area of the initial circumscribed area of analysis. The surface area of regions of interest was in the range of 0.6–1.0 mm^2, 0.2–0.9 mm^2, and 0.6–1.2 mm^2 for A7, LC, and A5 cell groups respectively. Thus, density values were expressed as the average number of varicosities per mm^2/cell group/brain (Table 1). All anterogradely labeled axons were counted on both the ipsilateral and contralateral sides of each defined area of the noradrenergic cell groups. Comparison of average number of total ipsilateral varicosities closely apposed to noradrenergic profiles among different cell groups was done using one-way ANOVA analysis (*VassarStats: Website for Statistical Computation*); *p*-value less than 0.05 was considered statistically significant. Neurolucida drawings containing outlines of coronal sections, anterogradely labeled axons, and noradrenergic neurons were exported into the Corel Draw Graphic Suite using PC computer for final editing of presented schematic drawings. Specifically, although some of the drawings are shown at low magnification (Fig. 2), tracing was done at either 20× or 60× magnification. For Fig. 3, lines were edited for selected colors in the Neurolucida file prior to exporting it to Corel Draw software (Corel Inc., Mountain View, CA). The latter was used to assemble different panels and to add lettering. No scanning of drawings was done at any time. We did not digitally edit any of the presented photomicrographs.