Neuroscience 224 (2012) 145–159

ULTRASTRUCTURAL ANALYSIS OF RAT VENTROLATERAL PERIAQUEDUCTAL GRAY PROJECTIONS TO THE A5 CELL GROUP

D. BAJIC, [a]* **E. J. VAN BOCKSTAELE** [b] **AND H. K. PROUDFIT** [a]

[a] *Department of Pharmacology, University of Illinois at Chicago, 835 S. Wolcott Avenue, Chicago, IL 60612, USA*

[b] *Department of Pathology, Anatomy and Cell Biology, Thomas Jefferson University, 1020 Locust Street, Philadelphia, PA 19107, USA*

Abstract—Stimulation of neurons in the ventrolateral periaqueductal gray (PAG) produces antinociception as well as cardiovascular depressor responses that are mediated in part by pontine noradrenergic neurons. A previous report using light microscopy has described a pathway from neurons in the ventrolateral PAG to noradrenergic neurons in the A5 cell group that may mediate these effects. The present study used anterograde tracing and electron microscopic analysis to provide more definitive evidence that neurons in the ventrolateral PAG form synapses with noradrenergic and non-catecholaminergic A5 neurons in Sasco Sprague–Dawley rats. Deposits of anterograde tracer, biotinylated dextran amine, into the rat ventrolateral PAG labeled a significant number of axons in the region of the rostral subdivision of the A5 cell group, and a relatively lower number in the caudal A5 cell group. Electron microscopic analysis of anterogradely-labeled terminals in both rostral (n = 127) and caudal (n = 70) regions of the A5 cell group indicated that approximately 10% of these form synapses with noradrenergic dendrites. In rostral sections, about 31% of these were symmetric synapses, 19% were asymmetric synapses, and 50% were membrane appositions without clear synaptic specializations. In caudal sections, about 22% were symmetric synapses, and the remaining 78% were appositions. In both rostral and caudal subdivisions of the A5, nearly 40% of the anterogradely-labeled terminals formed synapses with non-catecholaminergic dendrites, and about 45% formed axoaxonic synapses. These results provide direct evidence for a monosynaptic pathway from neurons in the ventrolateral PAG to noradrenergic and non-catecholaminergic neurons in the A5 cell group. Further studies should evaluate if this established monosynaptic pathway may contribute to the cardiovascular depressor effects or the analgesia produced by the activation of neurons in the ventrolateral PAG. Published by Elsevier Ltd. on behalf of IBRO.

Key words: anterograde tracer, biotinylated dextran amine, electron microscopy, synapse, tyrosine hydroxylase, ultrastructure.

INTRODUCTION

The periaqueductal gray (PAG) region, which extends throughout the midbrain surrounding the cerebral aqueduct (Paxinos and Watson, 1997), has been divided into several longitudinal zones or columns on the basis of cytoarchitecture, chemoarchitecture, and connections (Bandler et al., 1991; Bandler and Depaulis, 1991; Bandler and Shipley, 1994). The major autonomic connections of the PAG are within the ventrolateral zone that integrates a behavioral response characterized by quiescence, hyporeactivity, hypotension and bradycardia (Bandler and Depaulis, 1991). Excitation of neurons within this discrete ventrolateral region evokes a behavioral reaction that is seemingly identical to that evoked by pain arising from deep structures (Keay et al., 1994). Also, several studies have demonstrated that the ventrolateral PAG is selectively activated by deep pain (Keay et al., 1994; Clement et al., 1996, 2000) and hypotension (Murphy et al., 1995). Furthermore, the ventrolateral PAG is a major brainstem site involved in mediating antinociceptive effects (Fardin et al., 1984a,b), as well as a major site at which opioids act to produce analgesia (Yaksh and Rudy, 1978; Jones, 1992).

The sympathetic inhibition and antinociception produced by stimulation of the ventrolateral PAG are mediated in part by activation of spinally-projecting neurons in the ventromedial medulla (Gebhart and Randich, 1990; Jones, 1992; Lovick, 1993). However, several anatomical and pharmacological studies suggest that projections from the ventrolateral PAG to the noradrenergic neurons in the A5 cell group may contribute to behavioral responses and antinociception produced by activating neurons in the ventrolateral PAG. Our previous anterograde tracing studies (Bajic and Proudfit, 1999) have demonstrated that neurons in the ventrolateral PAG provide a moderately dense innervation of noradrenergic neurons in the A5 cell group. Furthermore, noradrenergic neurons in the A5 cell group have been shown to innervate the intermediolateral cell column in thoracic spinal cord

*Corresponding author. Permanent address: Department of Anesthesiology, Perioperative and Pain Medicine, Boston Children's Hospital, Bader 3, 300 Longwood Avenue, Boston, MA 02115, USA. Tel: +1-617-355-7737; fax: +1-617-730-0894.

E-mail address: dusica.bajic@childrens.harvard.edu (D. Bajic).

Abbreviations: BDA, biotinylated dextran amine; BSA, bovine serum albumin; CMM, central medial medulla; CVLM, caudal ventrolateral medulla; GAD, glutamate decarboxylase; PAG, periaqueductal gray; PB, phosphate buffer; PBS, phosphate-buffered saline; PHA-L, *Phaseolus vulgaris* leucoagglutinin; RVLM, rostral ventrolateral medulla; TBS, Tris–buffered saline; TH, tyrosine hydroxylase; TH-ir, tyrosine hydroxylase-immunoreactivity.

0306-4522/12 $36.00 Published by Elsevier Ltd. on behalf of IBRO.
http://dx.doi.org/10.1016/j.neuroscience.2012.08.021

segments (Loewy et al., 1979; Byrum and Guyenet, 1987; Romagnano et al., 1991; Clark and Proudfit, 1993; Bruinstroop et al., 2012), as well as spinal cord dorsal horn (Clark and Proudfit, 1993; Bruinstroop et al., 2012), where numerous nociceptive neurons are located (Light, 1992). In addition, stimulation of A5 neurons with microinjection of glutamate produces a depressor effect (Stanek et al., 1984; Burnett and Gebhart, 1991). Furthermore, microelectrophoretically applied norepinephrine inhibits single sympathetic preganglionic neurons in the thoracic segment of the spinal cord activated by electrical stimulation of a brainstem excitatory region (Coote et al., 1981), implicating descending noradrenergic projection from the A5 cell group in the regulation of cardiovascular reflexes. Similarly, both electrical (Miller and Proudfit, 1990) and chemical (Burnett and Gebhart, 1991) stimulation of sites near the A5 cell group can produce antinociception that is reduced by intrathecal injection of alpha2-adrenoceptor antagonists. Finally, norepinephrine appears to directly modulate the activity of spinothalamic tract neurons, because noradrenergic axon terminals form synapses with identified spinothalamic tract neurons (Westlund et al., 1990) and unidentified dorsal horn neurons (Hagihira et al., 1990; Doyle and Maxwell, 1991a,b), suggesting a role of A5 cell group in modulating nociception.

Although previous anterograde tracing studies (Bajic and Proudfit, 1999) provided anatomical evidence that neurons in the ventrolateral PAG project to the region containing noradrenergic neurons of the A5 cell group, these studies did not demonstrate synapses formed by ventrolateral PAG neurons with noradrenergic and non-catecholaminergic A5 neurons. In this study, we used the anterograde tracing technique combined with tyrosine hydroxylase (TH)-immunoreactivity, to evaluate the existence and extent of ventrolateral PAG projections to the A5 cell group. Therefore, the major goal of the present study was to provide ultrastructural evidence that neurons in the ventrolateral PAG form direct synaptic contacts with noradrenergic neurons of the A5 cell group. Also, the catecholamine neurons that comprise the A5 cell group are divided into two distinctive clusters (Dahlstroem and Fuxe, 1964; Lyons and Grzanna, 1988; Clark and Proudfit, 1993). A rostral subdivision is located caudal to the ventral nucleus of the lateral lemniscus at the level of the trigeminal motor nucleus, whereas a caudal subdivision is located lateral and dorsal to the superior olivary complex. Therefore, to evaluate the existence and extent of projections of neurons in the ventrolateral PAG to the A5 cell group, both rostral and caudal subdivisions of the A5 cell group were analyzed separately. The anterograde tracer biotinylated dextran amine (BDA) was iontophoretically deposited into sites in the ventrolateral PAG and electron microscopic analysis was used to visualize synapses formed by anterogradely-labeled terminals and profiles that exhibited immunoreactivity for the catecholamine synthesizing enzyme, TH. The results of this study provide direct ultrastructural evidence for a monosynaptic pathway from neurons in the ventrolateral PAG to noradrenergic as well as non-catecholaminergic dendrites in the A5 cell group.

EXPERIMENTAL PROCEDURES

Animal care and use

The Animal Care and Use Committees of the University of Illinois and the Thomas Jefferson University where the studies were performed approved the experimental protocols for the use of vertebrate animals in this study. The experiments were conducted according to 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 the number of animals used and their suffering.

Anterograde tracer iontophoresis and tissue fixation

Ten female and six male Sprague–Dawley rats (250–300 g; Sasco, Madison, WI) were injected with anterograde tracer BDA, and two female animals were selected for ultrastructural analysis. Animals were deeply anesthetized with pentobarbital (50 mg/kg) and surgically prepared using aseptic techniques. A glass micropipette (1.2 mm outer diameter, with filament; World Precision Instruments, Inc., Sarasota, FL) with a tip diameter of 15–20 μm was filled with a 10% solution of the anterograde tracer BDA (10,000 MW; D-1956, Molecular Probes, Eugene, OR) in saline and lowered to the appropriate target site in the ventrolateral PAG using the following stereotaxic coordinates relative to lambda: anterior, 0.0 mm; lateral, 0.5 mm; and ventral, 5.7 mm to the surface of the brain. The incisor bar was set at −2.5 mm. BDA was iontophoretically deposited using 5- μA positive current pulses of 500-ms duration at a rate of 0.5 Hz for 30 min. The pipette remained in place for 60 s after the injection to minimize diffusion of the tracer along the electrode track. A period of 12–18 days was allowed to tracer transport. Animals were then deeply anesthetized with pentobarbital (70 mg/kg, intraperitoneally) and transcardially perfused with: (1) 10 ml of heparinized saline (1000 units/ml; Elkins-Sinn, Inc., Cherry Hill, NH) at a speed of 100 ml/min, followed by (2) 100 ml of 3.75% acrolein (Electron Microscopy Sciences, Fort Washington, PA) and 2% paraformaldehyde in 0.1 M phosphate buffer (PB) adjusted to pH 7.4 and perfused at the same speed, and (3) 100 ml of 2% paraformaldehyde in 0.1 M PB adjusted to pH 7.4 and perfused at a speed of 100 ml/min; an additional 100 ml of a latter solution was perfused at a speed of 60 ml/min. Following perfusion, fixed brains were removed, cut into blocks and post-fixed in 2% paraformaldehyde overnight. Finally, brain blocks were immersed in cold 0.1 M PB and 40-μm transverse sections were cut on a Vibratome. Freely floating sections were then processed for immunocytochemistry.

Immunocytochemical tissue processing

Tissue sections were processed for visualization of BDA using methods described in previous reports (Bajic et al., 2000, 2001). Tissue sections were rinsed for 5 min in 0.1 M PB (pH 7.4), 30 min in 1.5% sodium-borohydride in 0.1 M PB solution, and 10 min in 0.1 M Tris–buffered saline (TBS, pH 7.6). Sections were then incubated for 1.5–2 h in a solution containing avidin–biotin complex (Elite Standard Vectastain ABC Kit, PK-6100, Vector Laboratories, Inc.) at room temperature, followed by two 10-min rinses in 0.1 M TBS (pH 7.6). Brown peroxidase reaction product was produced by the