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Acute restraint increases varicosity density and reduces the inter-varicosity distance in NADPH diaphorase-containing neurons in the rat dorsolateral periaqueductal gray matter

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ABSTRACT

The periaqueductal gray (PAG) is important for the organization of organismal response to different types of stress and painful stimuli. Its dorsolateral (dlPAG) column is distinctly characterized by the presence of nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d), which in many brain regions, is an indication of constitutive nitric oxide (NO) synthase (NOS)-containing neurons. Different stress paradigms activate the dlPAG NOS machinery presumably by a presynaptic influence of NO on dlPAG neurons to modulate the nuclear dynamics to elicit an appropriate response. Since presynaptic components of synapses reside in axonal varicosities, this study assessed the number of varicosities and inter-varicosity spacing of NADPH-d neurons in the dlPAG of free-behaving (control) and acutely restrained male rats. The study tested the hypothesis that stress-induced increase in endogenous NO synthesis involved changes in synaptic density and inter-varicosity spacing and therefore, a non-synaptic component of NO involvement in the dlPAG response to stress. Compared with control, the number of NADPH-d-positive cells, the staining intensity and the number of varicosities per microgram tissue were significantly higher in restrained animals. Also, the inter-varicosity spacing was significantly higher in control than restrained rats, presumably due to the increase in varicosities induced by restraint. Since neural connectivity and synaptogenesis depend on mean varicosity spacing and pattern of varicosity, respectively, the present observations suggest a mechanism whereby restraint stress induces increased activity via synaptic and non-synaptic NO-mediated neurotransmission within the dlPAG.

1. Introduction

The midbrain periaqueductal gray (PAG) which mediates the emotional coping response to different stressful paradigms is composed of distinct columns [33]. The PAG is involved in processing many aspects of organismal stress coping emotional response with preferential activation of a specific PAG column reflecting the type of emotional coping reaction triggered, and whether the stimulus involved physical or psychological stress [21]. Recent studies have shown that the dorsolateral PAG (dlPAG) can be activated by acute or repeated restraint [10] and capsaicin [29] – examples of psychological and physical stressors, respectively. The dlPAG is clearly distinguishable from other PAG columns because of a robust population of nicotinade adenine dinucleotide phosphate diaphorase (NADPH-d) [4,18,23]- and nitric oxide (NO) synthase (NOS)-containing neurons [27,32]. Different models of stress increase NOS expression in the PAG [2] with immobilization significantly increasing the number of NADPH-d neurons in the dlPAG [12]. Within the nervous system, the NADPH-d reaction is a potent index of neuronal profiles containing constitutive NOS [19]. While there are reports to support a role for NO in dlPAG function [9,10,15,20,29], exactly how NO influences dlPAG function remains to be clearly understood. For instance, contradictory reports indicate that NO may increase the rate of both excitatory and inhibitory dlPAG synaptic events [10,16,25,26]. Also, different intracellular mechanisms involving NO and activation of at least one other transmitter substance underlie its action in the dlPAG [29]. Furthermore, unlike in many other brain regions, neuronal NOS is unlikely to account for all NADP-d activity in the dlPAG [29]. Overall, these observations in the dlPAG function highlight the unique ability of NO to exert apparently paradoxical effects within discrete systems depending on the stimulus, and underscore the possibility of different mechanisms of action distinct from those traditionally associated with synaptic signal processing.

In the past two decades, a number of substances such as carbon monoxide and NO have been discovered which do not fulfill the

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criteria of classical neurotransmitters since they are not stored but 'released' immediately from the synthesizing enzyme and being freely diffusible, are able to penetrate biological membranes and influence functional plasticity through both synaptic and non-synaptic interactions [39]. Accumulating evidence indicate that the existence of non-synaptic varicosities is a general feature of the nervous system [3,11,39], with presynaptic components of synapses residing in axonal varicosities (synaptic boutons) that are commonly distributed in an *en passant*, 'beaded-string' manner along axonal profiles [37]. Transmitters can be released from these non-synaptic varicosities without being coupled to frequency-coded neuronal activity and can diffuse over large distances to complement classical neurotransmission [39]. The distribution pattern of varicosities is of interest because the mean spacing is a reflection of the synaptic density and also provides information about the mechanism of synaptogenesis [37].

On the basis of its physico-chemical properties, NO is an ideal candidate for nonsynaptic transmission in the nervous system [22,38] since, for example, it can alter the function of uptake carrier systems [7] as a possible regulatory mechanism of concentration of different transmitters in the extracellular space [13]. Hence an understanding of how varicosity density and spacing of nitrergic profiles within the dlPAG are possibly altered in different functional states may provide important clues regarding the possibility of a non-synaptic dimension to signal processing that complements regular synaptic transmission. A determination of varicosities in the dlPAG merits attention on the basis that varicosity spacing is an important aspect of axodendritic interactions and may hold critical clues about mechanisms of function-dependent synaptogenesis and development [37]. In this study therefore, the main goals were to determine: (i) the density and inter-varicosity spacing in dlPAG NADPH-d neuronal profiles and (ii) whether these indices of non-synaptic transmission are altered in any specific function-dependent manner. Together, these goals were based on the hypothesis that NO action in the dlPAG function involves non-synaptic transmission and is due, at least in part, to a change in the general and variable features of varicosity spacing profile. This hypothesis was tested by determining the effect of acute restraint (a potent activator of dlPAG nitrergic system) on the density and inter-varicosity spacing of NADPH-d profiles compared to that in control, free behaving animals.

2. Materials and methods

Eight weight- and age-matched male rats (Long Evans strain, Charles River, Wilmington, MA, USA) weighing 180–250 g and housed under standard animal facility conditions with ad libitum access to rodent chow and water were used for the study. The animals were treated and handled in conformity with all the ethical considerations and NIH and IACUC guidelines for the care and use of laboratory animals for experimental investigation. Restraint was effected by wrapping the animals ($n = 4$) with flexible metal gauze for 3 h while free-behaving animals ($n = 4$) served as controls. Acute restraint for 3 h was based on our previous studies [31] and the need to determine whether the restraint-induced change in NADPH-d system within the adjacent dorsal raphe nucleus is equivalently exhibited in the dlPAG. At the end of the experiments, the animals were terminally anesthetized with Nembutal (75 mg/kg, Abbott Laboratories, North Chicago, IL, USA) and transcardially perfused with cold 0.1 M phosphate buffer (PB) followed by 4% paraformaldehyde fixative in 0.1 M PB. The brain tissues were harvested and postfixed in fixative and then cryoprotected in 30% sucrose and then sectioned at 20 μm thickness through the coronal neuroaxis of the PAG (−5.80 mm to −8.30 mm relative to bregma [34]), washed in buffer and then incubated in a 10 ml solution containing 20 mM PB, 10 mg reduced NADPH-d (Sigma, St. Louis, MO, USA), 1 mg nitroblue tetrazolium and 0.03% Tritox X-100 for 120 min at 40 °C. The reaction was stopped by washing in buffer and the sections were mounted on gelatin-coated glass slides, air dried overnight, cleared in graded doses of alcohol and xylene and coverslipped.

2.1. Image acquisition and semi-quantification (analysis)

Photomicrophic images were acquired at 10-fold objective magnification (1280 × 1024 pixels, 0.65 μm/pixel) through the dlPAG for cell count using an NIS-Elements Advanced Research (AR) 3.1 imaging software and a NIKON Eclipse 80i microscope fitted with a NIKON digital sight camera. The number of NADPH-d-positive cells in the bilateral dlPAG was manually counted and also quantified using the imaging software and the mean were determined. In instances with more than a 10% difference between the manual and software values, the manually counted number was used for computation because the software could not distinguish staining debris/artifacts from actual cells in some sections. At least six bilateral dlPAG sections from each animal were counted and used for analyses. Images for semi-quantitation were taken at 40-fold objective magnification (12-bit, 1280 × 1024 pixels; 0.16 μm/pixel) and analyzed with the imaging software using the same settings for intensity of staining, density of staining per unit area and number of varicosities. The minimum criterion to be included in the assessment of varicosity spacing was for a nitrergic process to have at least five sequential varicosities and images were taken to intentionally capture segments of the tissue with greatest clusters of varicosities. Six bilateral dlPAG sections were analyzed for each animal and the values were individually processed for statistical significance with two-tailed Mann–Whitney *U*-test using GraphPad Prism ver.4 software. Significant observations were subjected to further analysis with the unpaired *t*-test and only data that showed statistical significance at $P < 0.05$ after both manipulations were judged to be of biological significance and to reject the Null hypothesis.

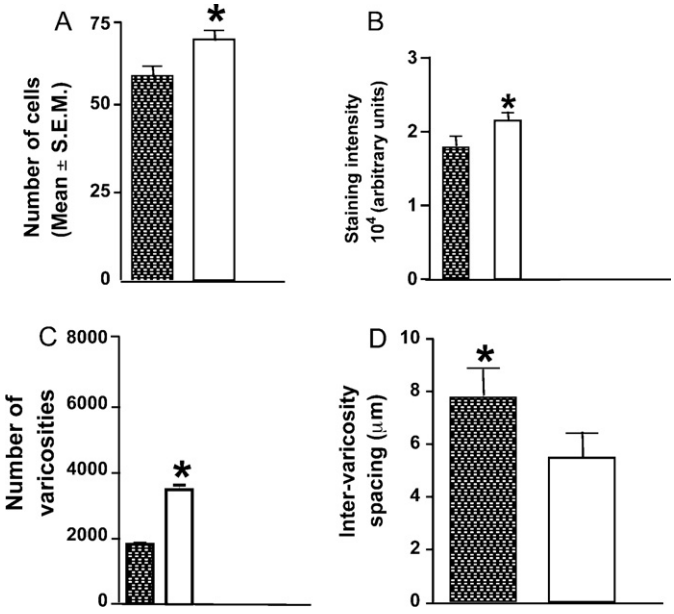

Fig. 1. (A) The mean number of NADPH-d-positive perikarya in the dorsolateral periaqueductal gray of control (hatched) versus restrained (unshaded) rats $^*P < 0.05$. (B) The staining intensity of NADPH diaphorase in the dorsolateral periaqueductal gray in control, freely behaving (hatched) and restrained (unshaded) rats $^*P < 0.05$. (C) The number of varicosities counted from six sections from each animal in freely, behaving control (hatched) and restrained (unshaded) rats $^*P < 0.01$. (D) The mean inter-varicosity distance in control (hatched) and restrained (unshaded) $^*P < 0.05$.