Occlusion of the Femoral Artery Induced Fos-like Immunoreactive Neurons in the Lateral Habenular Nucleus Projecting to the Midbrain Periaqueductal Gray in the Rat

By

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Summary: Wheat germ agglutinin conjugated horseradish peroxidase (WGA-HRP) injection into the midbrain periaqueductal gray (PAG) resulted in heavy accumulation of retrogradely labeled neurons in the lateral habenular nucleus (LHb) bilaterally. When the left femoral artery was persistently ligated for 2 h, the expression of Fos-like immunoreactivity (FOS-LI) was also found bilaterally in the LHb. In the present study, by combining the retrograde labeling method of injecting WGA-HRP into the PAG and the immunohistochemical staining of the FOS-LI neurons in the LHb induced by occlusion of the femoral artery, it was demonstrated that there are neurons containing both HRP labeling and FOS-LI. These neurons appeared to be located mainly in the medial part and posterior half of the LHb, and constitute about 24% of the total number of all labeled cells.

It was pointed out that the habenular nucleus (Hb) plays an important role as a link between the limbic forebrain structures and midbrain areas^{5,16,17}. In particular, the LHb has been well known to constitute a relay in the pathway from the nucleus accumbens to the PAG⁹, and to participate in subserving antinociception¹⁸. Recently, it has been demonstrated that the LHb neurons respond to noxious input from the peripheral nerve⁴ and noxious stimulation induces the expression of FOS-LI in the LHb¹¹. With respect to noxious stimulation of the hindlimb, it is of particular interest that a recent immunohistochemical study also has indicated the expression of FOS-LI in the LHb induced by formalin injection into the unilateral hindpad¹³. There remain, however, significant lacunae in our knowledge of the effects of ischemia of the hindlimb upon the Hb.

Therefore, the present study was done to elucidate in detail the relationship between LHb neurons having descending projections to the midbrain PAG and FOS-LI neurons in the LHb induced by unilateral occlusion of the femoral artery in the rat.

Materials and Methods

Twenty-two Wistar rats weighting 200–230 g were used in this study. All surgical procedures were performed under general anesthesia with sodium pentobarbital (40 mg/kg i.p.).

Six rats received a stereotaxic injection of 0.02–0.03 µl of 5–10% WGA-HRP (Toyobo) into the midbrain PAG using a 1.0 µl Hamilton microsyringe attached to a glass micropipette (tip diameter: 30–40 µm). After a survival period of 48 h, the animals were anesthetized and perfused with a fixative containing 1% paraformaldehyde and 1.25% glutaraldehyde in 0.1 M PB (pH 7.4). The brains were removed from the skull immediately and placed into 30% sucrose in 0.1 M PB at 4°C. Serial 50 µm-thick frozen sections were cut transversely on a microtome and processed for the demonstration of HRP reaction product according to the tetramethyl benzidine (TMB) protocol¹².

Six rats were housed individually in a temperature-controlled room. After being anesthetized, the left femoral artery was exposed and the rostral

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portion of the artery was ligated by nylon thread. After persistent ischemia for 2 h, the animals were perfused with 4% paraformaldehyde in 0.1 M PB through the ascending aorta. Serial 50 μ m-thick frozen sections were cut through the Hb and processed for Fos immunohistochemistry according to the method of Hisano *et al.*⁷⁾. Briefly, sections were firstly incubated in 10% normal goat serum (in 0.02 M phosphate buffer saline with 0.2% Triton X-100) for 30 min at room temperature, and then incubated for overnight (at 4°C) in polyclonal rabbit antiserum directed against c-Fos protein (Calbiochem.), diluted 1:1000. The sections were washed, exposed to a secondary biotinylated rabbit anti-goat IgG (containing 0.5% skim milk) for 1 h at 35°C, and then processed using the avidin-biotin-complex (ABC) method⁸⁾.

In six rats, 0.02–0.03 μ l of 5–10% WGA-HRP were injected into the PAG. After a survival period of 48 h, the animals were anesthetized again and the left femoral artery was ligated following the same method mentioned above. After 2 h, the animals were perfused with 4% paraformaldehyde in 0.1 M PB. Sections through the diencephalon were subjected to HRP histochemical reaction by using TMB method, treated with CoCl_2 and diaminobenzidine (DAB) to intensify HRP reaction product¹⁵⁾. Then, the sections were processed for Fos immunohistochemistry.

As a control group, in four rats the left femoral artery was exposed without ligation for 2 h. After perfusion, sections through the Hb were processed for Fos immunohistochemistry.

Results

WGA-HRP injection was made into the mid-brain PAG. The site of WGA-HRP injection was confirmed as a black mass which was located in the ventral PAG with some diffusion into the dorsal raphe nucleus (DR) as well as the injection shown in Fig. 3B. This injection resulted in retrograde labeling of the LHB bilaterally, while there was no labeling in the medial Hb (MHb). HRP-labeled neurons, medium in size and oval or triangular in shape, seemed to be confined mainly to the medial part of the LHB with a few distributed to the lateral part (Figs. 1A and 2A).

Occlusion of the left femoral artery resulted in strong bilateral expression of FOS-LI only in the LHB. The number of immunoreactive neurons was much higher than HRP labeling. In addition, it was of interest that these neurons were heavily accumulated in the medial part throughout the LHB although there are some distributed to the lateral

part (Figs. 1B and 2B).

WGA-HRP injection into the PAG and ligation of the left femoral artery were made in the same animal. The tracer of enzyme injected into the PAG including the DR was somewhat located eccentrically (Fig. 3B). Then the number of labeled neurons was counted in one rat of the experimental group. This experiment revealed the HRP labeling ($n = 349$) and expression of FOS-LI ($n = 509$) in the LHB. However, the most prominent morphological feature in the present study was that there were neurons containing fine black granules in the cytoplasm with brown-colored nucleus. These neurons labeled with both HRP and Fos protein appeared to be located mainly in the medial part and posterior half of the LHB although there were a few neurons in the lateral part (Figs. 1C and 3A). These neurons were observed to constitute about 24% ($n = 274$) of the total number of all labeled cells.

As a control, the left femoral artery was exposed without ligation. This experiment showed very weak expression of FOS-LI in the medial part of the LHB.

Discussion

The present study demonstrates in the rat that FOS-LI neurons in the LHB, induced by occlusion of the left femoral artery, also project to the mid-brain PAG/DR. HRP/FOS double-labeled neurons are characterized by predominant distribution to the medial part of the LHB with a few distributed to the lateral part. The medial part of the LHB is regarded as a major relay in the pathway from the limbic forebrain structures to the PAG, raphe nuclei, ventral tegmental area and substantia nigra pars compacta^{1,9,14)}. With respect to the functional significance of the LHB, it is reported that the LHB contains FOS-LI neurons induced by formalin injection into the hindpad¹³⁾ and electrical stimulation of the LHB produces behavioural analgesia^{3,10)}. Additionally, the PAG/RD is indicated to be the major site for the endogenous pain control system^{2,6)}. Considering these anatomical and physiological findings of the LHB and the PAG/RD, the present study would indicate the LHB to responds to acute ischemia of the hindlimb and subsequently may exert pain modulatory influences on the PAG/DR.

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Explanation of Figures

Plate I

Fig. 1. Schematic drawings showing the distribution of three kinds of labeled neurons in the LHb. Filled circle, small dots and black triangles indicate HRP-labeled (●), FOS-LI (●) and HRP/FOS double-labeled neurons (▲), respectively. Note that double-labeled neurons are confined mainly to the medial part and posterior half of the LHb. Each mark represents two labeled neurons. LHb, Lateral habenular nucleus; MHb, medial habenular nucleus; SM, stria medullaris thalami; III, third ventricle.

Plate II

Fig. 2. Photomicrographs showing HRP-labeled (A) and FOS-LI neurons (B) in the LHb. Note that neurons labeled with HRP and fos protein are also found mainly in the medial part of the LHb. LHb, lateral habenular nucleus; MHb, medial habenular nucleus; SM, stria medullaris thalami; III, third ventricle. Scale bars = 200 μ m in A and B.

Plate III

Fig. 3. Photomicrographs showing neurons labeled with both HRP and Fos protein in the medial part of the LHb (A) and the site of WGA-HRP injection into the PAG (B). Solid and open arrows and arrowheads indicate HRP/FOS double-labeled, HRP-labeled and FOS-LI neurons, respectively. Aq, aqueductus cerebri. Scale bars = 50 μ m in A and 1 mm in B.