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Correlative morphometric and electrochemical measurements of serotonin content in earthworm muscles

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Abstract

Distribution of serotonin (5-HT) content of nervous fibers in both the somatic and the visceral muscle of *Eisenia fetida* have been investigated using immunocytochemical staining and voltammetric measurements. The somatic muscles in the body wall are richer innervated with serotoninergic fibers than the visceral ones in the pharynx and gizzard. The relative density of immunopositive fibers in the circular muscle layer of the body wall was found to be 2.73% while in the prostomium it was 1.02%. In the case of the muscle in pharynx 1.12% and in gizzard 1.28% density values were found. Differential Pulse Voltammetric (DPV) measurements with carbon fiber electrodes in the above mentioned muscle layers gave 272.5 nA, 135.0 nA, 122.5 nA, 137.5 nA peak heights, respectively. In the statistical analysis *T*-test was used at a confidence level of 95% ($p<0.05$). DPV current peak (i_p) values reflect clearly the 5-HT concentration differences.

Significant correlation was found between the innervation density and the i_p values recorded in different areas. The i_p values recorded at different times in different locations are determined by instantaneous serotonin concentration of the living tissue. As far as we know this is the first report using *in vivo* voltammetry investigating serotonin content in earthworm, *E. fetida*.

Keywords: Immunocytochemistry; Differential Pulse Voltammetry; *In vivo* voltammetry; Serotonin; Earthworm

1. Introduction

Up till now several methods have been applied for determination of signal molecules in the nervous system of different species. Over the last years these methods have been refined considerably.

Though immunohistological studies reveal the distribution and neurochemical characters of neurons and fibers, physiological, pharmacological and analytical studies are required to establish the precise mode of chemical communications, the quantity and local concentration of signal molecules.

Serotonin (5-HT) is accepted as one of the most important signal molecules in earthworms. Detailed literature deals with the distribution of 5-HT positive elements in the central and peripheral nervous system after histofluorescence [1,2] and immunohistological staining [3,4]. In our earlier papers the occurrence of 5-HTergic nervous elements was reported in the body wall [5] as well as in the digestive tract [6] of *Eisenia fetida*.

Earlier Welsh and Moorhead [7] demonstrated spectrofluorimetrically the presence of 5-HT in the central nervous system (10.4 μg/g fresh tissue) and peripheral organs (e.g. body wall, 0.06–2.9 μg/g) of earthworm species. Similar values were obtained by Myhrberg [2].

5-HT containing neurons within the nerve cord send processes partly to the muscle layers of the body wall (somatic muscle) as well as the alimentary tract (visceral muscle). In the neuromuscular junctions 5-HT has an important role [8,9]. Dual action (inhibitory: [10] and excitatory: [11]) of this signal molecule on the visceral muscle has been verified by literature data. According to the pharmacological examinations [6] the actual effect of 5-HT depends on its concentration.

After the pioneering work of Adams [12], Gonon [13], Buda [14], Cespuglio [15,16], Wightman [17] and many others, *in vivo* voltammetric methods have been utilized for following the local concentration changes of electroactive neurotransmitter/

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modulator-related molecules in extracellular fluid of intact animals, without the use of complicated radioactive labelling techniques, any kind of histological methods or any sample preparations and chromatographic separations. In these experiments carbon fiber microelectrodes and different voltammetric measuring techniques such as chronocoulometry [12], fast cyclic voltammetry [17], Differential Pulse Voltammetry [14–16] have been most often employed.

5-HT is an electroactive molecule. *In vivo* voltammetric 5-HT measurements have been employed by several research groups [18–20]. Our earlier immunohistochemical studies revealed many interesting features at 5-HT distribution in earthworms. In order to further investigate the role of 5-HT earthworm physiology, we became interested using electrochemistry to measure and follow 5-HT concentration in the extracellular space among muscle cells. In our work carbon fiber electrodes were prepared and used. Voltammetric 5-HT measurements were carried out in living sedated earthworms.

In this paper we describe our recent results obtained by *in vivo* voltammetric investigation of the 5-HT distribution in earthworms. Immunohistological studies have also been carried out and the results obtained by the different methods have been compared.

Up till now most of the experiments were carried out in the central nervous system of anesthetized mouse [21,22], rats [23], snails [24] or monkeys [25]. As far as we know voltammetric measurements have not been applied in studying signal molecule distribution of earthworms. Therefore we believe that this work is the first report on successful use of voltammetric measurement in living *E. fetida*.

2. Materials and methods

2.1. Animals

Experiments were carried out on adult specimens of *E. fetida* (Oligochaeta, Annelida). After collection, animals used for different investigations were kept in climate box under standard temperature (10 °C) and humidity (60%).

2.2. Immunocytochemical examinations

2.2.1. Fixation

The animals were anaesthetized in carbonated water. The anterior parts (20 segments) of the bodies were fixed in Zamboni solution [26] for 24 h.

After fixation, tissue samples were washed in 0.1 M phosphate buffer (PB, pH 7.4), dehydrated in a graded ethanol series and embedded in Paraplast. 7 μm thick serial sections were cut on a rotation microtome (Anglia Scientific Instruments LTD, Cambridge, England) and mounted on slices coated with chrome-alumn gelatine.

2.2.2. Light microscopic immunocytochemistry

After deparaffination and rehydration the immunochemistry protocol was the following: the sections were washed for 3 × 20 min in salinated phosphate buffer-Triton X-100 (PBS-TX). Preincubation was performed for 1 h in 10% normal goat serum diluted with PBS containing 0.25% bovine serum albumin (BSA), 0.025% TX and 0.01% Na-azide.

Incubations with the primary antibody [rabbit polyclonal anti 5-HT antisera (Sigma) diluted 1:500] were performed overnight. After being washed in PBS sections were incubated for 1 h with biotinylated goat anti-rabbit IgG (Sigma) diluted 1:20 and then washed again in PBS and incubated by ExtrAvidin Peroxidase complex (Sigma) diluted 1:20 for 1 h.

All antisera were diluted with PBS-TX-BSA and applied at room temperature. To develop the peroxidase reaction sections were incubated in 0.1 M Tris–HCl buffer containing 0.05% 3,3′-diaminobenzidine and 0.01% H_2O_2 for 15 min.

Sections were dehydrated in graded ethanol, cleared in xylene and coverslipped in DPX.

Control experiments were performed by omitting the primary antiserum from the first incubation and substituting it by 1% normal goat serum. No immunostaining could be observed after the experiments. As a further specificity test, preabsorption of the antibody with 5-HT/BSA complex has also been done [27].

2.2.3. Quantitative light microscopy

Preparations were examined in a Nikon Eclipse 80i light microscopy equipped with a cooled CCD camera. Images were taken with Spot 4.0 software package, processed with Adobe Photoshop version 7.0. The colored pictures were transformed to grey scale images and inverted. In this way, the selected area (nervous element containing 5-HT) is automatically highlighted. Two parts of the body [dorsal surface of the 1st (prostomium) and 2nd segment as well as two parts of the foregut (pharynx and gizzard=ventriculus)] have been chosen for innervation density measurements. The original contrast was altered to +65 in case of the foregut and to +40 in case of the circular muscle layer of the body wall. For evaluation of immunopositivity of the selected tissue areas the grey scale median (GSM range 0–255) was set in the range from 235–255 and 0–235 for other tissue structures. Immunopositivity values for immunopositive elements were determined using the 'Histogram' tool of the software. This display shows the number of pixels that are covered by the selected area. Since the number of pixels reflects an area on the image, the immunoreactive area can be expressed as percentage of the entire image or in μm^2.

2.3. Voltammetry

2.3.1. Microelectrodes and microelectrode preparation

Carbon fiber microelectrodes were made from carbon fiber monofilaments of 33 μm in diameter. The fibers were a generous gift of the Specialty Materials (Massachusetts, USA). A single fiber was placed inside a glass capillary (1.5 mm O.D., 1.1 mm I.D., Sutter Instrument Co.) and the glass-carbon fiber combination was pulled by a vertical electrode puller (P-30 Micropipette Puller, Sutter Instrument Co.). After pulling, a razor blade was used to cut the fiber. The electric contact was established with mercury and copper wire. Most of the microelectrodes used in our *in vivo* work