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Immunofluorescent Identification of GABAergic Structures in the Somatic Muscle of the Earthworm *Lumbricus terrestris*

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Abstract—Using the immunofluorescence confocal microscopy, we detected the following GABAergic structures in the somatic muscle of the body wall of the earthworm: neurotransmitter gamma-aminobutyric acid (GABA); the enzyme responsible for synthesis of GABA, glutamate decarboxylase; type 1, 2, and 3 membrane transporters of GABA providing its reuptake; pre- and postsynaptic type A (ionotropic) and type B (metabotropic) GABA receptors. These structures are localized in the areas of cholinergic neuromuscular synapses. We assume that GABA can participate in modulation of motor activity of the earthworm somatic muscles both at pre- and postsynaptic levels of cholinergic neuromuscular synapses.

Keywords: GABA, glutamate decarboxylase, membrane GABA transporters, GABA receptors of type A and type B, cholinergic neuromuscular synapses, earthworm *Lumbricus terrestris*

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INTRODUCTION

The somatic muscle of the earthworm has cholinergic innervation [1]. The muscle cell membrane, in addition to acetylcholine receptors (AChRs), contains receptors for gamma-aminobutyric acid (GABA), functionally similar to GABA receptors of A and B type, selective activation of which leads to hyperpolarization of the muscle membrane [2, 3]. It is assumed that along with cholinergic innervation, the somatic muscle of the earthworm has GABAergic innervation, which can participate in modulation of the motor activity. The mechanism of such influence at presynaptic level may be the regulation of quantal secretion of acetylcholine [4], and at postsynaptic level it may be changes in the membrane potential of muscle cells [2, 3].

However, there is no direct confirmation of the presence of functioning GABAergic structures in the somatic muscle of the earthworm. Their spatial relationship with cholinergic neuromuscular synapses has not been established either. The aim of the present study was the immunofluorescent identification of the elements of the GABAergic system in the cholinergic synapses of the somatic muscle of the earthworm *Lumbricus terrestris*. Among these elements were neurotransmitter GABA; the enzyme of GABA synthesis glutamate decarboxylase (GAD); three GABA membrane transporters (GAT-1, -2, and -3), as well as pre- and postsynaptic membrane GABA receptors. Detection of such elements may serve as evidence of coupling of GABAergic system with cholinergic innervation. It should be emphasized that the *Annelida* taxonomic type, to which the earthworm belongs, is the oldest group of animals [5]. The representatives of this type evolved the abilities to actively control the movement of somatic musculature [6]. In this regard, this study is important from the fundamental point of view, because it will allow us to expand the ideas about the formation of the neuromuscular system at the earliest stages of the evolutionary development of the animal world.

Abbreviations: GABA, gamma-aminobutyric acid; AChRs, acetylcholine receptors; GAD, glutamate decarboxylase; PBS, phosphate-buffered saline; BSA, bovine serum albumin; TMR-α-B, tetramethylrhodamine-α-bungarotoxin.

MATERIALS AND METHODS

Object and preparations. Isolated preparations of body wall fragments of the earthworm *Lumbricus terrestris* were attached with needles at the bottom of Petri dishes filled with Sylgard resin and perfused with Dreves–Pax solution (composition in mM: 77 NaCl, 4 KCl, 43 Na_2SO_4, 6 $CaCl_2$, 2 Tris, and 167 sucrose; pH 7.4) for about 30 min at room temperature (22 ± 1°C). The preparations were then fixed in 2% *p*-formalde-

hyde solution for 30 min, washed 3 times for 30 min each in phosphate-buffered saline (PBS, composition in mM: 137 NaCl, 2.7 KCl, 4.3 Na_2SO_4, 1.4 KH_2PO_4, pH 7.2). Muscles were incubated sequentially: 30 min in 0.5% Triton X-100 solution; 15 min in a solution containing 5% normal goat serum, 1% bovine serum albumin (BSA), and 0.5% Triton X-100; 15 min in a solution of 1% BSA and 0.5% Triton X-100 (solution A). All these solutions were prepared in PBS.

Staining of preparations. The preparations were incubated for 12 h at 4°C in solution A with polyclonal antibodies. Antibodies to GABA were used, as well as antibodies to enzyme GAD; GABA transporters GAT-1, -2, and -3; α1, β2, γ2 subunits of $GABA_A$ receptor; R1 and R2 subunits of $GABA_B$ receptor, and synaptophysin. The preparations were washed in solution A 3 times 30 min each and incubated for 1 h at 20°C in solution A containing the appropriate secondary antibodies conjugated to Alexa 488 or 647 (1 : 800). Post-synaptic nicotinic AChRs were stained with tetramethylrhodamine-α-bungarotoxin (TMR-α-B, 20 μg/mL; incubation time 30 min). To confirm the specificity of polyclonal antibody binding to the corresponding proteins, control experiments were performed. For negative controls, the preparation was incubated with secondary antibodies without prior incubation with primary antibodies. For positive controls, incubation of the preparation with primary antibodies in the presence of the immunogenic peptide to which the primary antibodies were produced was performed. The absence of staining in the control experiments indicates the specificity of antibody binding to the corresponding peptides.

Microscopy. After washing in PBS, the preparations were placed in glycerol/PBS solution (1 : 1) and placed on a slide for examination on a Leica TCS SP5 MP laser scanning confocal microscope (Leica Microsystems, USA). An oil immersion objective 63×/1.4 was used. Argon and helium–neon lasers were used to excite the emission of fluorophores. Excitation wavelengths for the fluorophores were as follows: 488 nm for Alexa 488; 543 nm for TMR, and 633 nm for Alexa 647. Confocal images were analyzed using ImageJ software (NIH, USA).

Reagents. We used *p*-formaldehyde, Tris, Triton X-100, normal goat serum, bovine serum albumin (BSA), fluorescently labelled α-bungarotoxin (TMR-α-B), glycerol (Sigma-Aldrich); primary polyclonal antibodies and their corresponding immunogenic peptides (Santa Cruz Biotechnologies, USA); secondary antibodies carrying fluorophores Alexa 488 and Alexa 647 (Invitrogen, USA).

RESULTS AND DISCUSSION

Nerve endings in the somatic muscles of the earthworm were stained with antibodies to the membrane glycoprotein synaptophysin, which is well represented in synaptic vesicles [7, 8]. Postsynaptic nicotinic AChRs were detected by staining samples with fluorescently labelled α-bungarotoxin (TMR-α-B) [9]. The immunohistochemical staining of body wall fragments revealed that fluorescent signal from antibodies to GABA (Fig. 1a, panel *1*) colocalized with fluorescently labelled markers of synaptophysin and postsynaptic AChRs (Figs. 1a–1e, panel *1*). This experimental fact indicates the presence of GABA in the local area of cholinergic neuromuscular synapses. Staining for enzyme GAD also coincided with the zone of the endplate of the cholinergic synapse (Figs. 1a–1e, panel *2*). This observation suggests that the zone of the cholinergic synapse contains structures capable of both synthesizing and secreting GABA.

Immunohistochemical staining of body wall fragments of the earthworm with antibodies specific for membrane GABA transporters, GAT-1 (Fig. 2, panel *1*), GAT-2 (Fig. 2, panel 2), GAT-3 (Fig. 2, panel *3*) type, showed positive staining for all three types of membrane transporters, which overlapped with fluorescent labelling for synaptophysin (Figs. 2a, 2b, 2d, panels *1–3*) and postsynaptic nicotinic AChRs (Figs. 2a, 2c, 2e, panels *1–3*). These data indicate the presence of all three types of membrane transporters in the zone of cholinergic neuromuscular synapses of the earthworm somatic muscle, which provide GABA reuptake from the perimembrane cell areas [10], which is the most important mechanism of GABA concentration regulation in the intercellular space. Immunohistochemical experiments also revealed the presence of the $GABA_A$-receptor subunits α1 (Fig. 3, panel *1*), β2 (Fig. 3, panel *2*), and γ2 (Fig. 3, panel *3*) in the synaptic cholinergic contact zone, as their fluorescent signal co-localized with that of synaptophysin (Figs. 3a, 3b, 3d, panels *1–3*) and nicotinic AChRs (Figs. 3a, 3c, 3e, panels *1–3*). Similar results were obtained for subunits R1 (Fig. 4, panel *1*) and R2 (Fig. 4, panel *2*) of the $GABA_B$ receptor. Localization of these fluorescently stained subunits also coincided with that of synaptophysin (Figs. 4a, 4b, 4d) and nicotinic AChRs (Figs. 4a, 4c, 4e). Thus, two types of GABA receptors are present in the cholinergic myoneural synapse zone of the earthworm somatic muscle: GABA receptors of type A, ionotropic [11], and type B, metabotropic [12].

The obtained data suggest the presence of full-fledged GABAergic structures in the zone of cholinergic myoneural synapses in the somatic muscle of the earthworm. These GABAergic structures include all obligatory components, such as neurotransmitter GABA; GABA synthesizing enzyme GAD; membrane transporters of all three types providing GABA reuptake, as well as pre- and postsynaptic GABA receptors of type A and B. A legitimate question arises, what structures are the producers of GABA? We can put forward three hypotheses. First, there are nerve terminals of GABAergic neurons in the area of endplates of cholinergic myoneural synapses. However, to

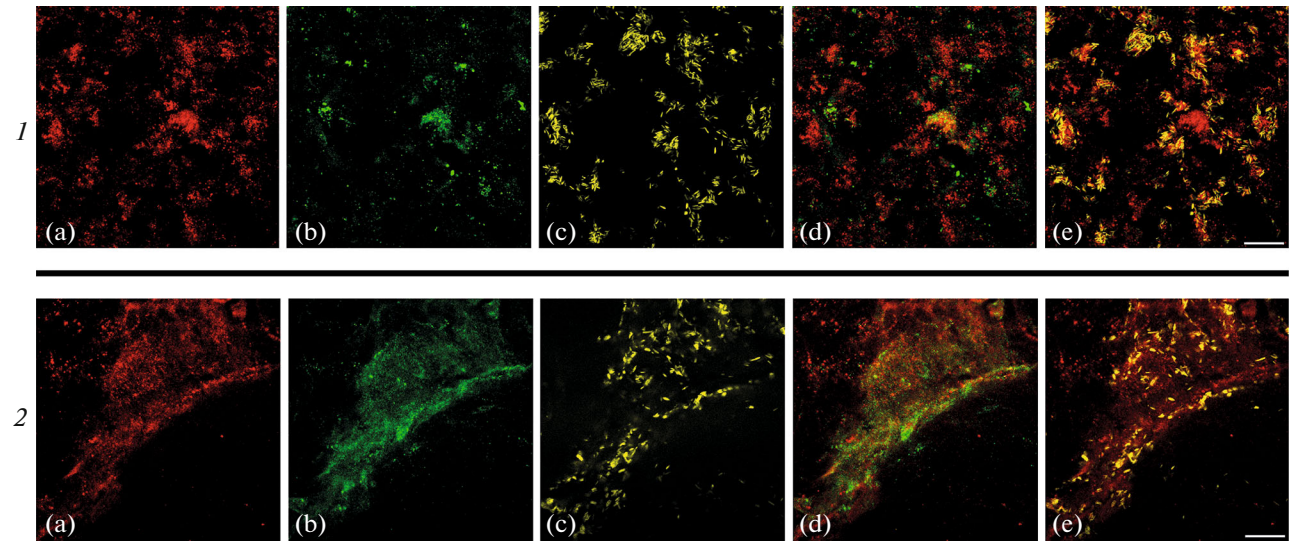

Fig. 1. Triple fluorescent immunohistochemical staining of the preparation of somatic muscle fibers of the earthworm *Lumbricus terrestris*. (a) Staining with antibodies to GABA (*red*; panel *1*) and to enzyme GAD (*red*; panel *2*). (b) Staining with antibodies to presynaptic protein synaptophysin (*green*). (c) Staining with TMR-α-B of postsynaptic nicotinic AChRs (*yellow*). (d) Superposition of images (a) and (b). (e) Superposition of images (a) and (c). Scale bar, 10 μm.

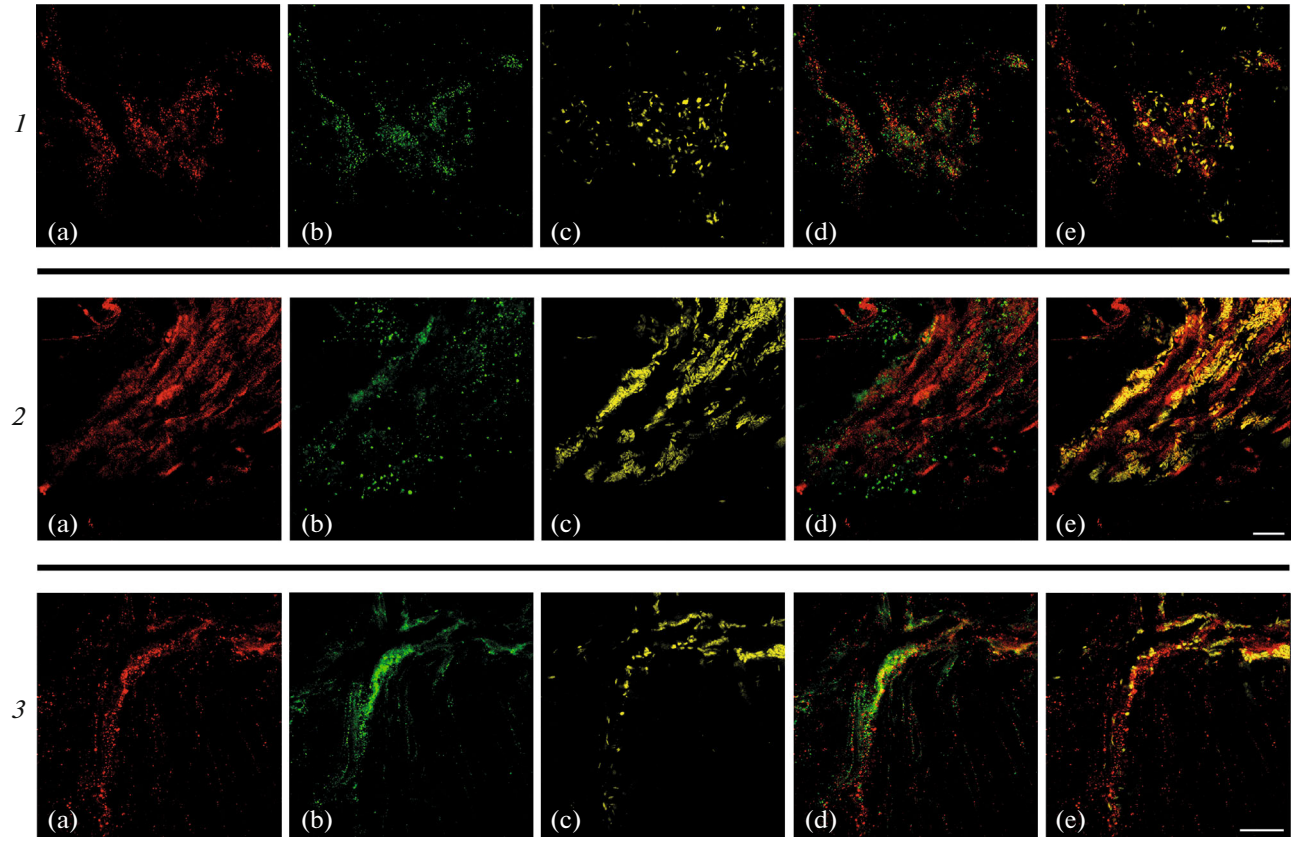

Fig. 2. Detection of GABA transporters (GAT-1, 2, 3) by triple immunofluorescent staining of the preparation of somatic muscle fibers of the earthworm. (a) Staining with antibodies to GAT-1 (*red*; panel *1*), GAT-2 (*red*; panel *2*), GAT-3 (*red*; panel *3*). (b) Staining with antibodies to presynaptic protein synaptophysin (*green*). (c) Staining of nicotinic AChRs using TMR-α-B (*yellow*). (d) Superposition of images (a) and (b). (e) Superposition of images (a) and (c). Scale bar, 10 μm.

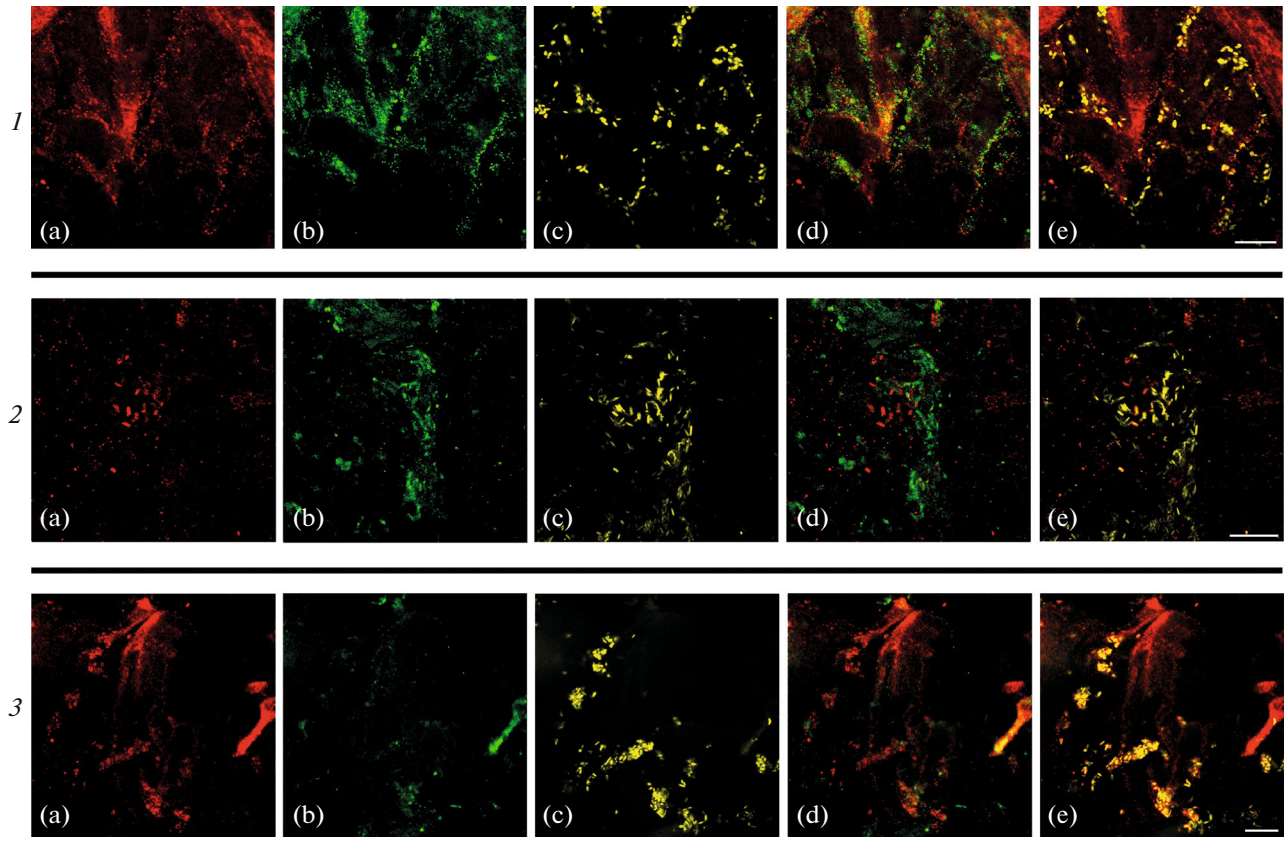

Fig. 3. Presence of α1, β2, γ2 subunits of $GABA_A$ receptor revealed by triple immunofluorescent staining of the preparation of somatic muscle fibers of the earthworm. (a) Staining with antibodies to α1 (*red*; panel *1*), β2 (*red*; panel *2*), γ2 (*red*; panel *3*) subunits of the $GABA_A$ receptor. (b) Staining with antibodies to the presynaptic protein synaptophysin (*green*). (c) Staining of nicotinic AChRs with TMR-α-B (*yellow*). (d) Superposition of images (a) and (b). (e) Superposition of images (a) and (c). Scale bar, 10 μm.

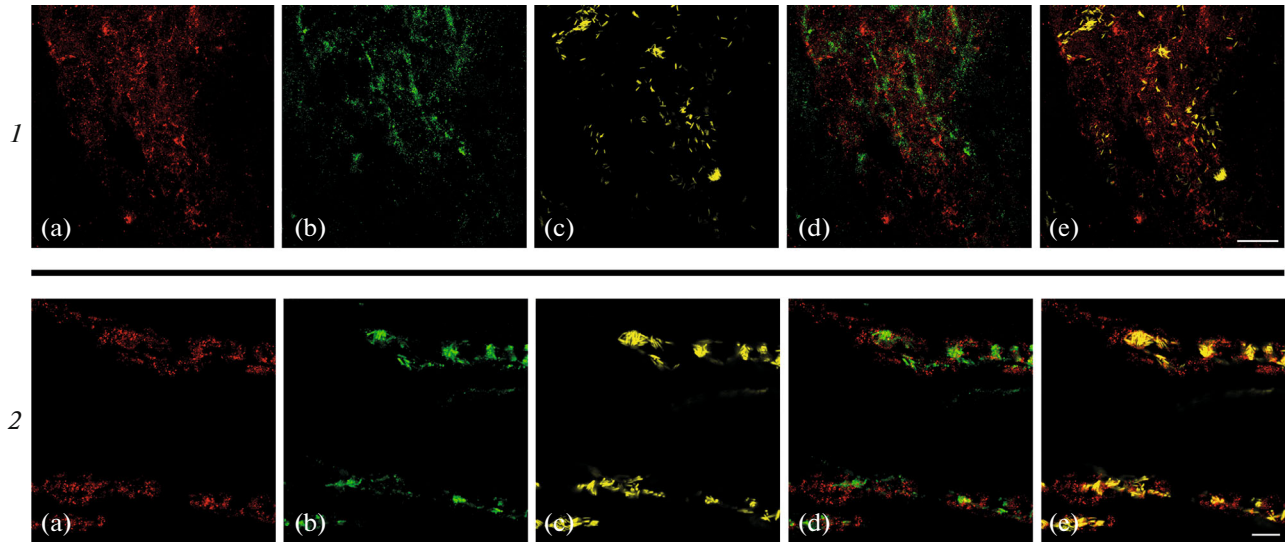

Fig. 4. Detection of R1 and R2 subunits of the $GABA_B$ receptor by triple immunofluorescent staining of the preparation of somatic muscle fibers of the earthworm. (a) Staining with antibodies to R1 (*red*; panel *1*) and R2 (*red*; panel *2*) of the $GABA_B$ receptor. (b) Staining with antibodies to the presynaptic protein synaptophysin (*green*). (c) Staining of nicotinic AChRs using TMR-α-B (*yellow*). (d) Superposition of images (a) and (b). (e) Superposition of images (a) and (c). Scale bar, 10 μm.

confirm this hypothesis, additional morphological ultrastructural studies are needed to show the presence of two types of nerve terminals in the local area of the neuromuscular contact. Second, these are glial cells of the nerve tissue. This hypothesis corresponds to the literature data [13, 14]. In this case GABA may act as a gliotransmitter [15, 16]. The third hypothesis assumes that GABA acts as a co-mediator in cholinergic synapses [17, 18]. This does not contradict the second hypothesis. Nevertheless, available data do not allow us to make a definitive conclusion in favour of one of the three hypotheses or their combination. This question should apparently be left open at this stage of the research.

Our studies allow us to draw the following conclusion. In the zone of cholinergic neuromuscular synapses of the earthworm somatic muscle, there are full-fledged GABAergic structures capable of synthesis, mediator secretion and reuptake, and interaction with pre- and postsynaptic ionotropic and metabotropic receptors. It is known that in cholinergic synapses of vertebrates GABA can modulate both quantal and non-quantal secretion of mediators through activation of metabotropic B-type receptors [4]. On the other hand, application of GABA to somatic muscle cells of the earthworm causes hyperpolarization of muscle membranes through selective activation of GABA A- and B-type receptors. The latter exert their action by increasing the "amperogenic pump component" of the Na^+/K^+-pump and active Cl^- symport in the integral value of the resting membrane potential [2, 3]. Besides, functional coupling of voltage dependent Ca^{2+} channels and metabotropic $GABA_B$ receptors through G-proteins has been reported [12, 19]. In neurons, activation of $GABA_B$ receptors modulates the activity of voltage dependent Ca^{2+} channels of L, N, P/Q, R, T types [20–22]. Calcium entry through these channels triggers exocytosis of synaptic vesicles [23–25], and temporary incorporation of voltage dependent Ca^{2+} channels into vesicular membranes during exocytosis triggers fast and slow endocytosis, which ensures the binding of exocytosis and vesicle endocytosis [26]. As we have shown previously, voltage dependent Ca^{2+} channels are expressed in the neuromuscular contact zone of the somatic muscles of the earthworm [27, 28]. It is quite possible that in cholinergic neuromuscular synapses of the earthworm one of the calcium mechanisms of exo-/endocytosis regulation of vesicles is carried out with the participation of metabotropic $GABA_B$ receptors.

Thus, there is every reason to believe that GABAergic structures may participate both in the modulation of cholinergic secretion in neuromuscular synapses and in the regulation of muscle membrane excitability threshold and, ultimately, in the motor activity of the earthworm somatic muscle.

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COMPLIANCE WITH ETHICAL PRINCIPLES

The authors declare no obvious and potential conflicts of interest related to the publication of this article.

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