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Immunofluorescent Identification of α1 Isoform Subunits of Voltage-Gated Ca^{2+}-Channels of Ca_V1, Ca_V2, and Ca_V3 Families in Areas of Cholinergic Synapses of Somatic Muscles in Earthworm *Lumbricus terrestris*

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Abstract—The α1 subunits of the voltage-gated Ca^{2+}-channels α1S, α1C, α1D, and α1F in channels of the $Ca_V1.1$–1.4 type; α1A in a channel of $Ca_V2.1$ type; α1E in a channel of $Ca_V2.3$ type; and α1G, α1H, and α1I in a channel of $Ca_V3.1$–3.3 type, as well as the synaptic vesicle exo-endocytotic protein synaptophysin, were identified using fluorescence and confocal microscopy in the somatic muscle of the earthworm *Lumbricus terrestris*. The presynaptic membrane of cholinergic synapses contains voltage-gated Ca^{2+}-channels of $Ca_V1.1$ and $Ca_V1.2$ (including α1S and α1C subunits), $Ca_V2.1$ (with α1A subunit), $Ca_V2.3$ (with α1E subunit), and $Ca_V3.2$ and $Ca_V3.3$ (with subunits α1H, α1I) types, while $Ca_V1.3$ and $Ca_V1.4$ Ca2+-channels with subunits α1D and α1F and $Ca_V3.1$ channels with subunit α1G are predominantly parts of the muscle membranes.

Keywords: voltage-gated Ca^{2+}-channels, isoforms of the α1 subunit, somatic muscle, cholinergic synapses, annelids

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INTRODUCTION

The key event in triggering of the vesicular cycle of synaptic vesicles regulating a mediator secretion is the entry of Ca^{2+} into the motor nerve terminals through voltage-gated Ca^{2+}-channels. Voltage-gated Ca^{2+}-channels are composed of the pore-forming subunit α1 bound with three auxiliary subunits α2/δ, β, and γ (Catterall, 2000). The subunit α1 in the ion channel can be represented by various isoforms. Each isoform is controlled by a separate gene. It is currently known that the genome of both vertebrate and invertebrate animals can have up to ten such genes (Catteral et al., 2005). Isoforms α1S, α1C, α1D, and α1F of the α1 subunit are typical for $Ca_V1.1$–1.4 channels, α1A for $Ca_V2.1$, α1B for $Ca_V2.2$, α1E for $Ca_V2.3$, and α1G, α1H, and α1I for $Ca_V3.1$–3.3 (Catterall et al., 2005).

Ca^{2+}-channels with different isoforms of the α1 subunit have different sensitivities to depolarization. According to this criterion and a number of pharmacological differences they are combined into a number of families, namely, high-threshold channels $Ca_V1.1$–1.4 (L-type), $Ca_V2.1$ (P/Q-type), and $Ca_V2.2$ (N-type) and low-threshold channels $Ca_V3.1$–3.3 (T-type) (Catterall, 2000; Nurullin et al., 2011; Nurullin et al., 2013). Between high- and low-threshold channels are $Ca_V2.3$ (R-type) channels (Pardo el., 2006; Wormuth et al., 2016). The synchronicity of the induced and spontaneous release of the mediator in the neuromuscular synapses in the exo-endo vesicular cycle are largely determined by the functioning of the Ca^{2+}-channels of the presynaptic membrane (Smith et al., 2012; Kaeser and Regehr, 2014). It was found that the somatic muscle of the earthworm is innervated by cholinergic synapses (Walker et al., 1993; Volkov et al., 2012), in which the vesicular cycle has obvious Ca^{2+} dependence (Volkov, 2012). A number of key Ca^{2+} sensory proteins of the vesicular cycle, as well as Ca^{2+}-channels of the $Ca_V2.2$ type (Volkov et al., 2012), are revealed in presynaptic formations. The presence of the other types of Ca^{2+}-channels described above in cholinergic neuromuscular synapses in the evolution-

Abbreviations: ACh—acetylcholine, TMR-B—tetramethylrhodamine-α-bungarotoxin.

ary primary striated muscles of annelids remains elusive.

In accordance with the goal of this study, the following tasks were set out: (1) determination of Ca^{2+}-channel types and their α1 subunit isoforms with immunofluorescence and (2) study of the localization of Ca^{2+}-channels from different families on the pre- and postsynaptic membranes of cholinergic motor neuromuscular synapses of the striated muscles in the earthworm *Lumbricus terrestris*.

MATERIALS AND METHODS

Isolated fragments of the body wall of the earthworm *Lumbricus terrestris* were fixed with needles on the bottom of the Petri dishes filled with Sylgard resin and perfused with Drewes–Packs solution (composition in mM: 77 NaCl, 4 KCl, 43 Na_2SO_4, 6 $CaCl_2$, 2 Tris, 167 sucrose, pH 7.4) for about 30 min at room temperature (22 ± 1°C). They were then fixed in 2% p-formaldehyde and washed three times for 30 min in phosphate buffer. The muscles were incubated sequentially in 0.5% Triton X-100 for 30 min; 5% normal goat serum, 1% bovine serum albumin, and 0.5% Triton X-100 for 15 min; and the next 15 min in 1% bovine serum albumin and 0.5% Triton X-100 (solution A). All of these solutions were prepared with phosphate buffer.

The samples were incubated for 12 h at 4°C in solution A with polyclonal antibodies to α1A, α1C, α1D, α1E, α1F, α1G, α1H, α1I, and α1S subunits of the voltage-gated Ca^{2+}-channels and synaptophysin (dilution 1 : 200), washed in solution A three times for 30 min and incubated for 1 h at room temperature with the corresponding secondary antibodies conjugated to Alexa 488 or 647 in solution A (dilution 1: 800). Staining of postsynaptic nicotinic acetylcholine (ACh) receptors was performed using tetramethylrhodamine-α-bungarotoxin (TMR-B, 20 μg/mL) for 30 min. Control experiments were performed to confirm the specificity of the binding of polyclonal antibodies to the corresponding proteins. For negative control, samples were incubated with secondary antibodies without prior incubation with primary antibodies. For positive control, the preparation was incubated with primary antibodies in the presence of an immunogenic peptide used for primary antibodies production. The absence of staining in the control experiments indicates the specificity of the binding of antibodies to the corresponding peptides.

The preparations washed in the phosphate buffer were placed on a glass slide in the solution of phosphate buffer and glycerin (1 : 1) for microscopic examination with Zeiss LSM 510 Meta laser scanning confocal microscope (Carl Zeiss, Germany) using a 63×/1.4 oil immersion lens. Fluorophore emission was exited with argon and helium-neon lasers. Excitation wavelengths: for fluorophores, Alexa 488 was 488 nm; for tetramethylrhodamine, 543 nm; and, for Alexa 647, 633 nm. Confocal images were processed with the ImageJ software (National Institutes of Health, United States).

The following reagents were used: p-formaldehyde, Tris, phosphate buffer (137 NaCl, 2.7 KCl, 4.3 Na_2SO_4, 1.4 KH_2PO_4, pH 7.2), Triton X-100, normal goat serum, bovine serum albumin, TMR-B, and glycerin (Sigma-Aldrich, United States); primary polyclonal antibodies and their corresponding immunogenic peptides (Santa Cruz Biotechnologies, United States); and secondary antibodies conjugated with Alexa 488 or Alexa 647 (Invitrogen, United States).

RESULTS AND DISCUSSION

The synaptophysin protein, a key component of the molecular machinery of the exo-endocytosis cycle of synaptic vesicles, was used as a marker of motor nerve terminals (Valtorta et al., 2004; Kwon and Chapman, 2011). TMR-B a specific blocker of nicotinic ACh-receptors (Krause and Wernig, 1985; Nurullin et al., 2011) was applied to determine the area of the postsynaptic membrane with cholinergic synapses. Immunohistochemical staining of muscles of body wall samples from the earthworm with antibodies to the α1S subunit of the voltage-gated Ca^{2+}-channel, $Ca_V1.1$ type, revealed an immunopositive reaction to this subunit (Figs. 1a, 1f). Moreover, staining for α1S was detected in all observation zones (Figs. 1a, 1f). Staining of the α1S subunit coincided with staining with antibodies to synaptophysin protein (Figs. 1a, 1b, 1d, 1f, 1g, 1i, arrows) and in some places with antibodies to TMR-B a specific blocker of nicotinic Ach-receptor (Figs. 1a, 1c, 1e, 1f, 1h, 1j, arrows). Supposedly, the number of Ca^{2+}-channels of $Ca_V1.1$ type having the α1S subunit increases in the area of motor nerve terminals.

The structure of Ca^{2+}-channels of Ca_V1 type also includes subunits of α1C ($Ca_V1.2$), α1D ($Ca_V1.3$), and α1F ($Ca_V1.4$). It was found that staining for α1C and α1D subunits was irregular (Figs. 2a, 2f; 3a, 3f). Staining for α1C coincided with staining for ACh-receptors (Figs. 2a, 2c, 2e, 2f, 2h, 2j, arrows), but did not coincide with staining for synaptophysin (Figs. 2a, 2b, 2d, 2f, 2g, 2i). Staining for α1D did not coincide with staining for synaptophysin and ACh-receptors (Figs. 3a–3j). It is highly probable that $Ca_V1.2$ Ca^{2+}-channels containing the α1C subunit are more concentrated in the zone of cholinergic synapses in the postsynaptic membrane, while $Ca_V1.3$ channels with α1D are not concentrated in the area of neuromuscular synapse of the presynaptic and postsynaptic membrane. Staining for the α1F subunit of the Ca^{2+}-channel ($Ca_V1.4$ type) was visible in all zones (Figs. 4a, 4f) and did not coincide with simultaneous staining for

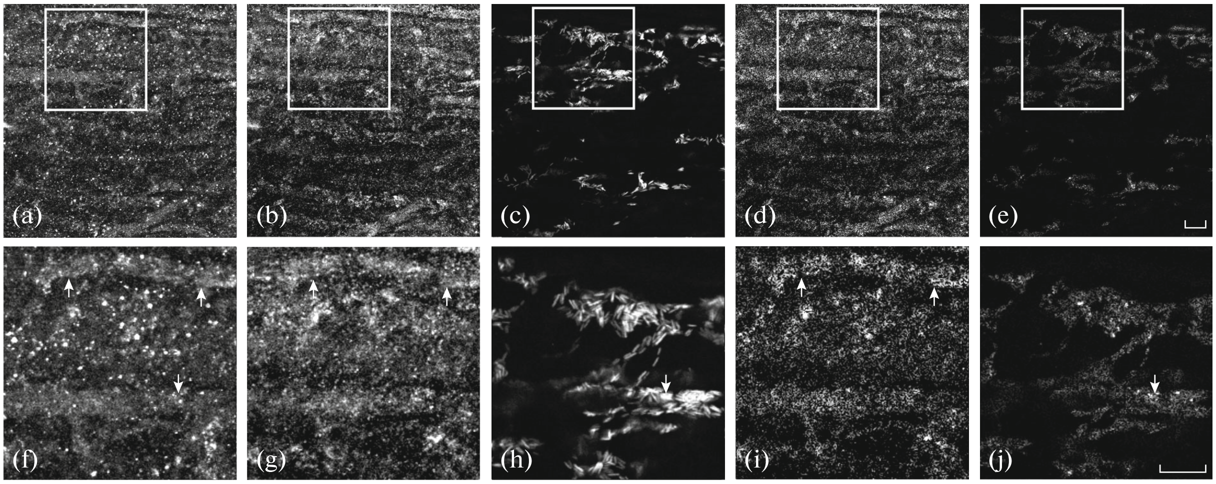

Fig. 1. Fluorescent triple staining of somatic muscle fibers of the earthworm with (a, f) antibodies to the α1S subunit of the voltage-gated Ca^{2+}-channel of $Ca_V1.1$ type, (b, g) antibodies to the presynaptic protein synaptophysin, and (c, h) and staining of nicotinic acetylcholine (ACh) receptors with tetramethylrhodamine-α-bungarotoxin (TMR-B). In all figures (Figs. 1–9): the lower panel shows enlarged areas corresponding to the highlighted squares on the upper panel; (d) processed image obtained by combining images a and b (subunit α1 and synaptophysin), showing only light pixels that coincide when overlapping images (i is enlarged image d); (e) processed image obtained by combining images a and c (α1 subunit and TMR-B), showing only light pixels that coincide when overlapping images (j is enlarged image e). When superimposing two images, bright pixels present in only one of the images were not taken into account in the resulting images. Taken together, the sites of staining coincidence for the markers are shown. Arrows on the lower panels indicate the coincidence of staining for the subunit α1, synaptophysin, and ACh-receptors. The scale bar is 10 μm.

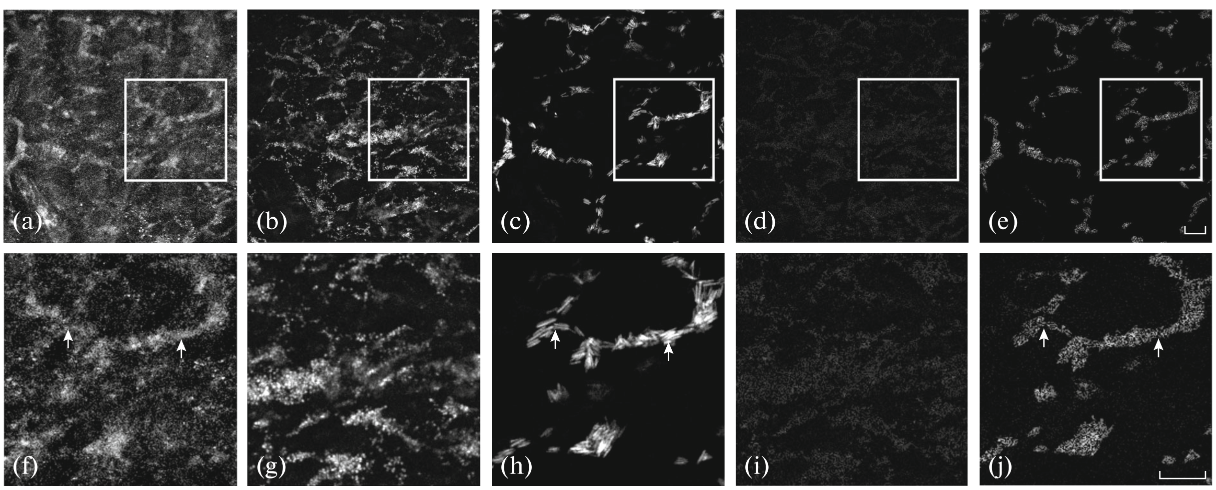

Fig. 2. Triple fluorescence staining of somatic muscle fibers of the earthworm with (a, f) antibodies to the α1C subunit of the voltage-gated Ca^{2+}-channel of $Ca_V1.2$ type, (b, g) antibodies to the presynaptic protein synaptophysin, and (c, h) staining of ACh-receptor with TMR-B. Here and in Figs. 3–9, for d, e, i, and j, see the explanations in Fig. 1.

synaptophysin and ACh-receptors (Figs. 4a–4j). Most likely, the Ca^{2+}-channels of $Ca_V1.4$, which contain the α1F subunit, are concentrated mainly in the cell membranes of muscle tissue.

The staining with antibodies to the α1A subunit of the Ca^{2+}-channel of the $Ca_V2.1$ type was not uniform (Figs. 5a, 5f). The most intense staining for this subunit coincided with staining with antibodies to synaptophysin and TMR-B staining of the Ach-receptors (Figs. 5a–5j, arrows). It can be supposed that the presynaptic membrane of the cholinergic neuromuscular synapses of the somatic muscle in the earthworm contains Ca^{2+}-channels of $Ca_V2.1$ type.

The staining for the α1E subunit of the Ca^{2+}-channel, type $Ca_V2.3$, was also not uniform (Figs. 6a, 6f). However, some areas of intense staining for the α1E subunit coincided with staining for ACh-receptors (Figs. 6a, 6c, 6e, 6f, 6h, 6j, arrows), but not with stain-

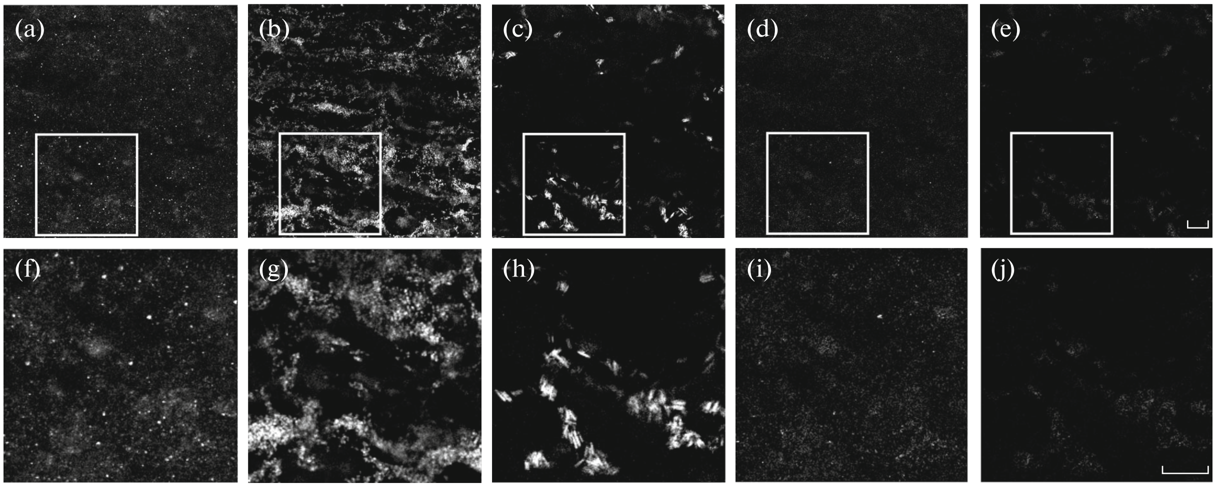

Fig. 3. Triple fluorescence staining of somatic muscle fibers of the earthworm with (a, f) antibodies to the α1D subunit of the voltage-gated Ca^{2+}-channel of $Ca_V1.3$ type, (b, g) antibodies to the presynaptic protein synaptophysin, and (c, h) staining of ACh-receptor with TMR-B.

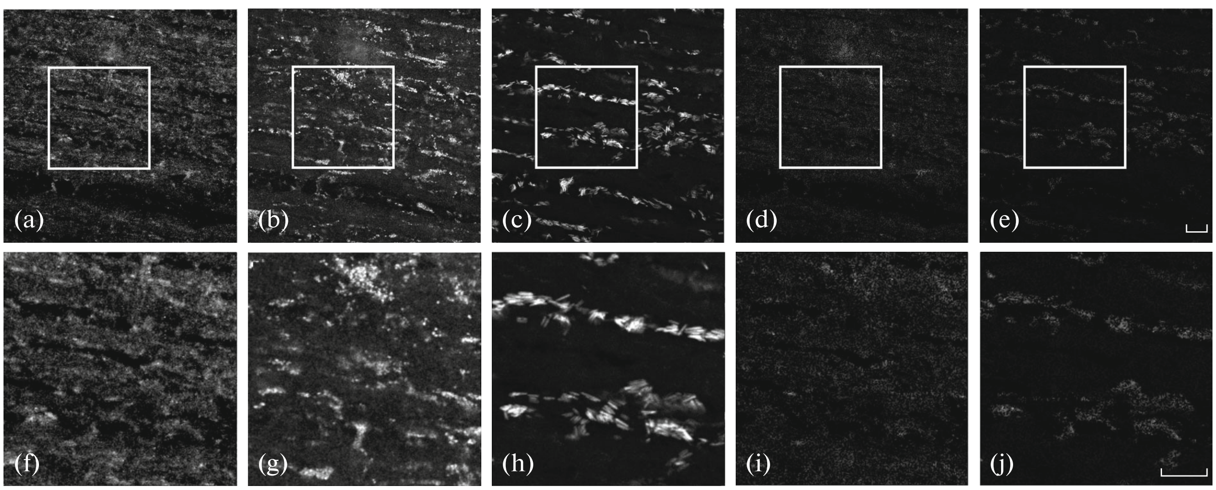

Fig. 4. Triple fluorescence staining of somatic muscle fibers of the earthworm with (a, f) antibodies to the α1F subunit of the voltage-gated Ca^{2+}-channel of $Ca_V1.4$ type, (b, g) antibodies to the presynaptic protein synaptophysin, and (c, h) staining of ACh-receptor with TMR-B.

ing for synaptophysin (Figs. 6a, 6b, 6d, 6f, 6g, 6i, arrows). These findings suggest the presence of Ca^{2+}-channels of the $Ca_V2.3$ type in the zone of cholinergic synapses of the postsynaptic membrane in the somatic muscle of the earthworm.

The staining for the α1G subunit of the Ca^{2+}-channel ($Ca_V3.1$ type) was visible in all zones (Figs. 7a, 7f) and did not coincide with the simultaneous staining for synaptophysin and ACh-receptors (Figs. 7a–7j). This suggests that Ca^{2+}-channels, type $Ca_V3.1$, are distributed over the membrane of somatic muscle cells of the earthworm. This type of Ca^{2+}-channel is known to be involved in spontaneous contractile activity (Catterall, 2000). It is also known that the somatic muscle of the earthworm is capable for spontaneous contractile activity by the type of cardiac muscle of the vertebrate (David, 1990). It is very likely that this type of muscle activity in the annelid muscle is linked with the corresponding type of Ca^{2+}-channels. Staining for the α1H subunit of the Ca^{2+}-channel $Ca_V3.2$ was not uniform (Figs. 8a, 8f) and was of different intensity. It frequently coincided with staining for the ACh-receptors (Figs. 8a, 8c, 8e, 8f, 8h, 8j, arrows) or synaptophysin (Figs. 8a, 8b, 8d, 8f, 8g, 8i). Staining for synaptophysin looked like bundles (Figs. 8b, 8g, arrows) visually connected with the fluorescence of the α1H subunit (Figs. 8a, 8f) and ACh-receptors (Figs. 8c, 8h).

Staining on the α1I subunit of the Ca^{2+}-channel of $Ca_V3.3$ type was also irregular (Figs. 9a, 9f). In areas where the fluorescence intensity was higher, staining

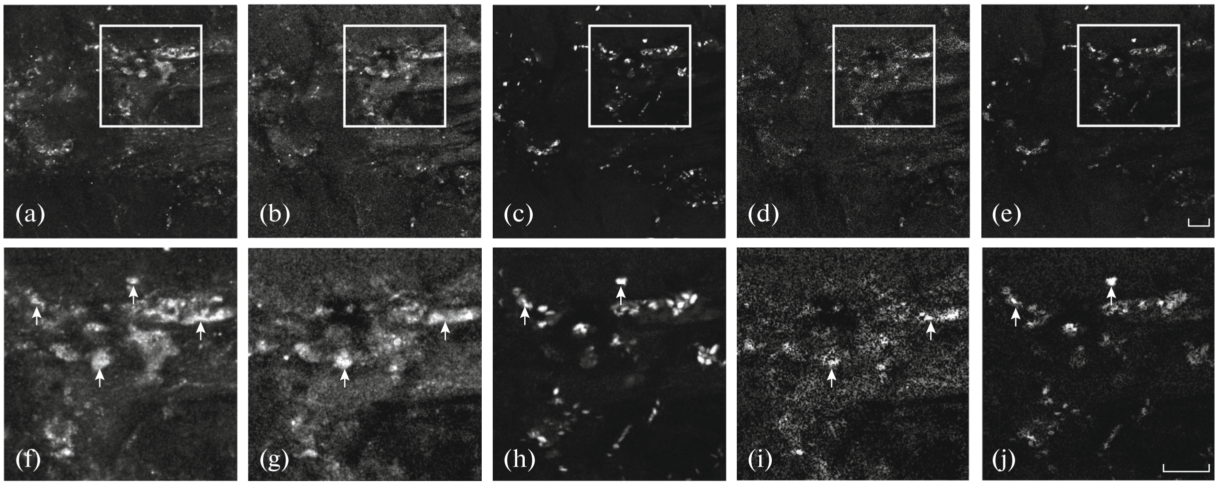

Fig. 5. Triple fluorescence staining of somatic muscle fibers of the earthworm with (a, f) antibodies to the α1A subunit of the voltage-gated Ca^{2+}-channel of $Ca_V2.1$ type, (b, g) antibodies to the presynaptic protein synaptophysin, and (c, h) and staining of ACh-receptor with TMR-B.

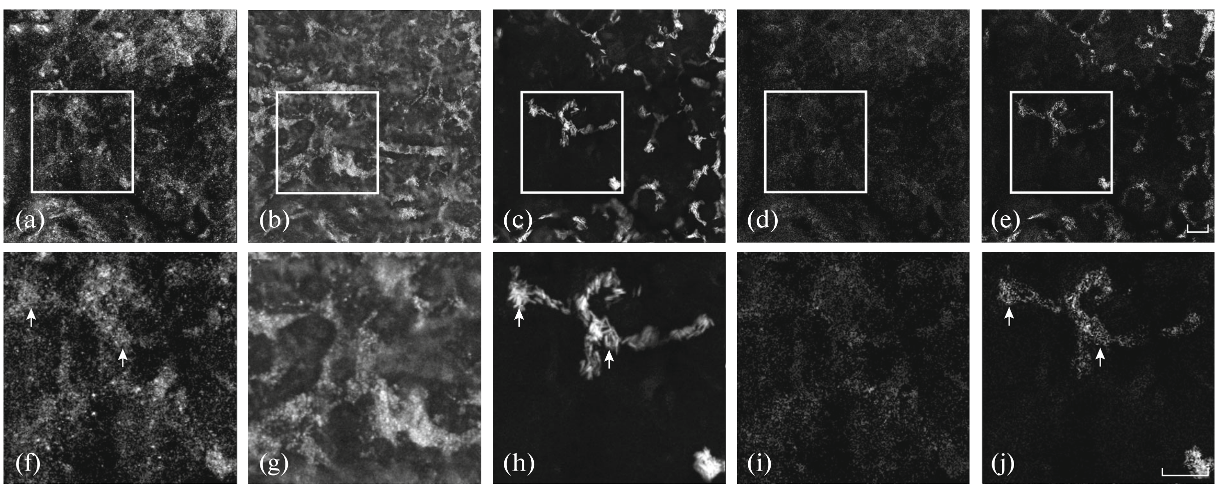

Fig. 6. Triple fluorescence staining of somatic muscle fibers of the earthworm with (a, f) antibodies to the α1E subunit of the voltage-gated Ca^{2+}-channel of $Ca_V2.3$ type, (b, g) antibodies to the presynaptic protein synaptophysin, and (c, h) staining of ACh-receptor with TMR-B.

on the α1I subunit almost completely coincided with staining for synaptophysin (Figs. 9a, 9b, 9d, 9f, 9g, 9i, arrows), and sometimes it coincided with staining for ACh-receptors (Figs. 9a, 9c, 9e, 9f, 9h, 9j, arrows). Thus, there is reason to believe that Ca^{2+}-channels of Ca_V3 type containing α1H ($Ca_V3.2$) and α1I ($Ca_V3.3$) subunits may be present on the membrane of the presynaptic terminals of cholinergic motor synapses in the earthworm muscle.

Thus, the genome of the earthworm *Lumbricus terrestris* contains genes encoding the peptide molecules α1S, α1C, α1D, and α1F (subunits) of Ca^{2+}-channels of $Ca_V1.1$–1.4 types, subunit of α1A of Ca^{2+}-channels of $Ca_V2.1$ type, α1E subunit of Ca^{2+}-channel of $Ca_V2.3$ type, and subunits of α1G, α1H, and α1I of Ca^{2+}-channels of $Ca_V3.1$–3.3 types.

We demonstrated earlier the expression of the α1B subunit of the Ca^{2+}-channel, $Ca_V2.2$ type, in somatic muscle fibers of the earthworm (Volkov et al., 2012). Thus, the neuromuscular synapses and cells of the somatic muscle annelids contain almost all known types of voltage-gated Ca^{2+}-channels common for vertebrate and invertebrate animals (Catterall, 2000; Jeziorski et al., 2000). The concentration of Ca^{2+}-channels Ca_V1 containing α1S ($Ca_V1.1$) and α1C ($Ca_V1.2$) subunits is higher in the area of cholinergic neuromuscular motor synapses, while Ca^{2+}-channels of this type having α1D subunits in their structure

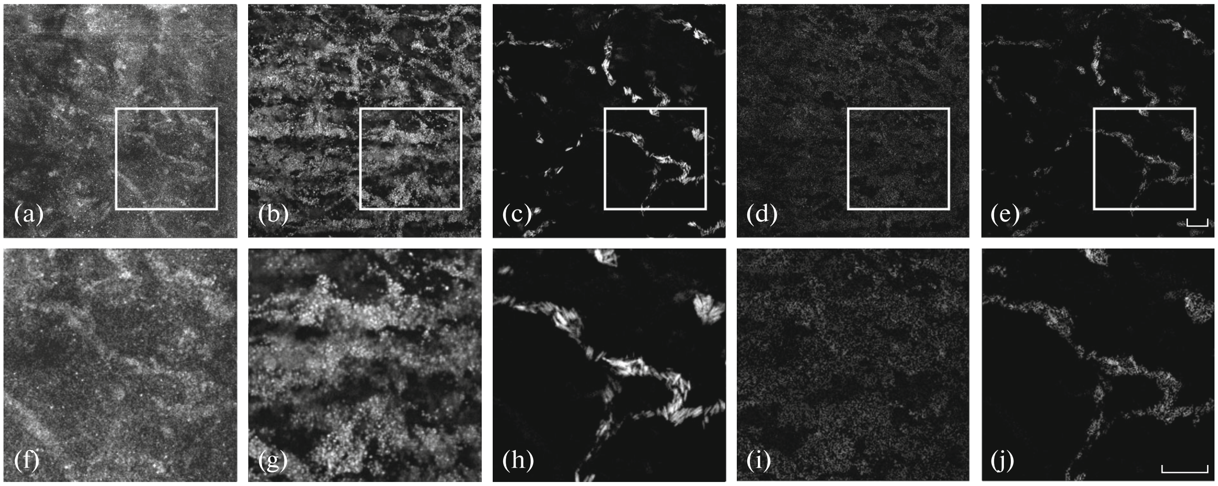

Fig. 7. Triple fluorescence staining of somatic muscle fibers of the earthworm with (a, f) antibodies to the α1G subunit of the voltage-gated Ca^{2+}-channel of $Ca_V3.1$ type, (b, g) antibodies to the presynaptic protein synaptophysin, and (c, h) staining of ACh-receptor with TMR-B.

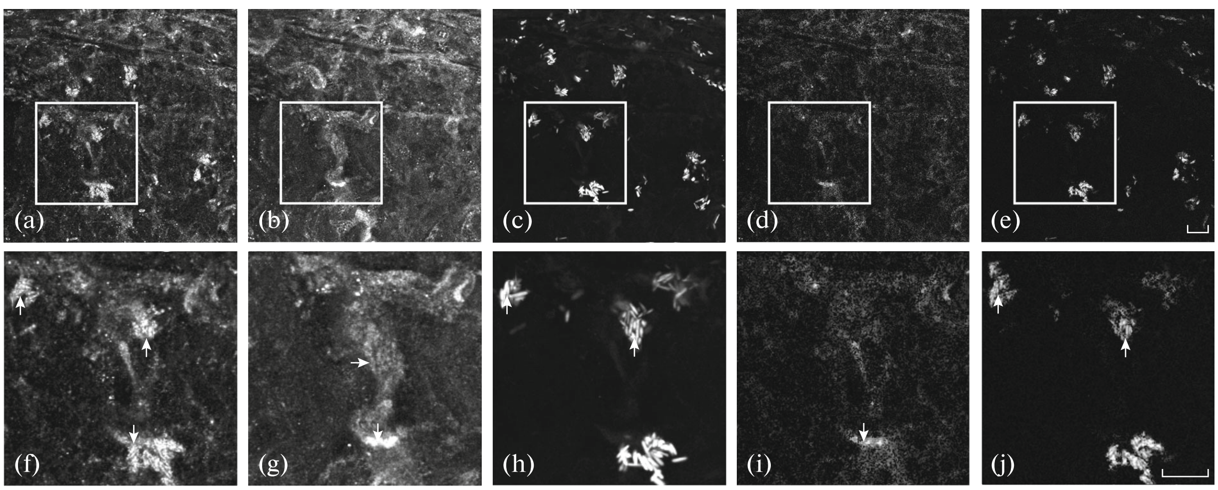

Fig. 8. Triple fluorescence staining of somatic muscle fibers of the earthworm with (a, f) antibodies to the α1H subunit of the voltage-gated Ca^{2+}-channel of $Ca_V3.2$ type, (b, g) antibodies to the presynaptic protein synaptophysin, and (c, h) staining of ACh-receptor with TMR-B.

($Ca_V1.3$) and α1F ($Ca_V1.4$) are distributed over all membranes of muscle cells. Ca^{2+}-channels of $Ca_V2.1$ type with the α1A subunit are concentrated in the region of neuromuscular synapses. Ca^{2+}-channels of the $Ca_V2.3$ type, containing the α1E subunit, are also, although to a lesser extent, concentrated in the area of cholinergic synapses. Finally, Ca^{2+}-channels of Ca_V3 type with α1H ($Ca_V3.2$) and α1I ($Ca_V3.3$) subunits are also concentrated almost exclusively in the area of motor nerve terminals, while Ca^{2+}-channels of $Ca_V3.1$ type with the α1G subunit are localized on muscle cell membranes.

We are the first to report here that the synaptophysin protein is involved in the molecular machine of the vesicular cycle of nerve terminals. It complements the family of analogous proteins, such as synaptotagmin 1 and syntaxin 1, previously identified in the neuromuscular synapses of earthworms (Volkov et al., 2012).

Taken together, our findings suggest that the presynaptic membrane of the cholinergic synapses of the somatic muscle in the earthworm contains the following types of voltage-gated Ca^{2+}-channels (in accordance with the identified isoforms of the α1 subunit): $Ca_v1.1$ and $Ca_v1.2$ (with the α1S and α1C subunits),

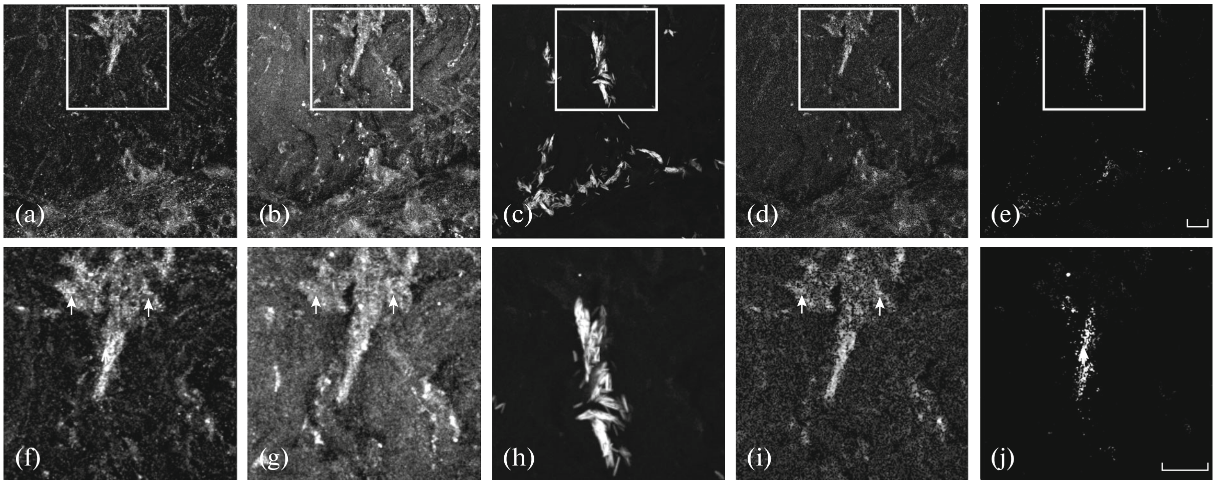

Fig. 9. Triple fluorescence staining of somatic muscle fibers of the earthworm with (a, f) antibodies to the α1I subunit of the voltage-gated Ca^{2+}-channel of $Ca_V3.3$ type, (b, g) antibodies to the presynaptic protein synaptophysin, and (c, h) staining of ACh-receptor with TMR-B.

$Ca_V2.1$ (α1A), $Ca_V2.3$ (α1E), and $Ca_V3.2$ and $Ca_V3.3$ (α1H, α1I), as well as the Ca^{2+}-channel $Ca_V2.2$ (α1B) (Volkov et al., 2012). Ca^{2+}-channels $Ca_V1.3$ and $Ca_V1.4$ (with subunits α1D and α1F) and $Ca_V3.1$ channels (α1G) are predominantly located on muscle membranes.

In conclusion, annelid neuromuscular synapses and cells of the somatic muscle have almost all known types of voltage-gated Ca^{2+}-channels common for both vertebrates (Catterall, 2000) and invertebrates (Jeziorski et al., 2000). However, they exhibit a specific distribution over the excitable membranes of nerve and muscle cells.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest. The authors declare that they have no conflict of interest

Statement on the welfare of animals. The authors declare that all work with animals was done in accordance with Russian law and the *Guide for the Care and Use of Laboratory Animals* (http://www.nap.edu/openbook.php?isbn=0309053773).

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