Effect of NO-Generating Compound $NaNO_2$ on Ultrastructure of Synaptic Vesicles of Glutamatergic Synapses

N. V. Samosudova, V. P. Reutov*, and N. P. Larionova

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Ultrastructure of synaptic vesicles in axon terminals of granule cells from isolated cerebellum of *Rana temporaria* frogs under the influence of NO-generating compound $NaNO_2$ in various concentrations and electrical stimulation was evaluated by the method of electron microscopy. NO-generating compound in low concentration induced translocation of synaptic vesicles and formation of small clusters. The size and structure of synaptic vesicles remained unchanged under these conditions. Increasing the concentration of $NaNO_2$ led to swelling of synaptic vesicles, formation of arranged heaps from individual vesicles or fusion of their content. Electrical stimulation of the cerebellum in the presence of $NaNO_2$ increased damage to synaptic vesicles. These experimental data model some stages observed in stroke. The formation of clusters from synaptic vesicles is a compensatory and adaptive response maintaining the structure of synaptic vesicles and protecting neurons from high concentrations of glutamate. Glutamate produces a toxic effect on nerve cells and glial cells of the cerebellum under pathological conditions, which is accompanied by impairment of signal transduction from presynaptic to postsynaptic neurons.

Key Words: *synaptic vesicles; nitric oxide; active zone; cerebellum*

Information transmission in the brain is associated with activity of synapses and release of neurotransmitters from synaptic vesicles (SV). Structural diversity of synapses is manifested in variations in the shape of synaptic elements (boutons and spinules), which depends on physiological and pathological conditions. We hypothesized that changes in the shape of synapses induced by the influence of NO-generating compound on the cerebellum probably result from the interaction of NO and free radical products with unsaturated fatty acids (lipids) and individual amino acids (synaptic membrane proteins) [1-3].

Activation of NO synthesis under pathological conditions, including aging and cardiovascular disorders (infarction, stroke, and brain injury), is usually followed by structural changes in membranes of nerve and glial cells. They play a key role in dysfunction of cells and synaptic contacts [1,3,4]. It cannot be excluded that NO modulates function of SV, which serve as the major component in synaptic signal transduction. Much attention was paid to the structure and function of SV, mechanisms of neurotransmitter release (*e.g.*, fusion of the presynaptic and postsynaptic membrane), and recovery of vesicles [5-11]. However, the role of NO in ultrastructural changes and redistribution of SV in neurons remains unclear.

Laboratory No. 12, A. A. Kharkevich Institute for Information Transmission Problems, Russian Academy of Sciences; *Laboratory for Ultrastructural Bases of a Conditioned Response, Institute of Higher Nervous Activity and Neurophysiology, Russian Academy of Sciences, Moscow, Russia

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Here we studied the effect of NO-generating compound on variations in the size and shape of SV and redistribution of these vesicles in boutons of cerebellar granule cells.

MATERIALS AND METHODS

Experiments were performed on cerebellums from 20 adult frogs (*Rana temporaria*). The cerebellums were fixed under various conditions: immediately after isolation (normal, 4 cerebellums); after incubation in Ringer's solution with $NaNO_2$ (10 μM, 2 h, 3 cerebellums); after stimulation with $NaNO_2$ (10 μM, 1 h) and incubation (1 h) in the same solution (4 cerebellums); after incubation of the cerebellums in Ringer's solution with $NaNO_2$ at concentrations of 1 mM (2 h, 3 cerebellums) and 5 mM (2 h, 3 cerebellums); and after stimulation of the cerebellums with $NaNO_2$ (1 mM, 1 h, 3 cerebellums) and incubation in the same solution (1 h).

Electrical stimulation of the surface of the molecular layer (primarily parallel fibers, PF) was delivered through bipolar electrodes (0.1 Hz, 10^{-4}-10^{-5} A). Oxygenated Ringer's solution consisted of 115 mM Na^+, 2.5 mM K^+, 1.2 mM Ca^{2+}, 6.0 mM $NaHCO_3$, and 2 g/liter glucose (pH 7.2-7.4). $NaNO_2$ is capable of generating NO due to the reduction of NO_2^- into NO [2]. Fixation was performed with 2.5% solution of glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2), which contained 0.5% tannin acid and 3% sucrose. The sample was postfixed in 1% OsO_4 (pH 7.2) in the same buffer at 4°C for 1 h, dehydrated in ethyl alcohol at increasing concentrations, absolute alcohol, and acetone, and embedded into a mixture of Epon and araldite. The sections were stained with uranyl acetate and lead citrate and examined under a JEM-100SX electron microscope (accelerating voltage 90 kV).

RESULTS

Under normal conditions, SV have a diameter of 60 nm and are regularly distributed over axon terminals (boutons) of cerebellar granule cells. They also contain mitochondria, microtubules, and electron-dense active zone (Fig. 1, *a*). Bouton vesicles are bound to each other, actin threads, and microtubules by the protein synapsin 1 [7-9].

In the presence of NO-generating compound ($NaNO_2$, 10 μM), SV were displaced from the presynaptic zone and formed small clusters. The shape and size of SV, as well as the structure of cytoplasmic and mitochondrial membranes remained practically unchanged.

During electrical stimulation of PF in the presence of NO-generating compound ($NaNO_2$, 10 μM), displaced vesicles were arranged in dense clusters. The structure of these vesicles changed, they became closer to each other, and their diameter decreased to 40 nm (Fig. 1, *b*). Dense clusters looked like a crystal grid. The cluster included several subgroups, which were arranged at an angle to each other (Fig. 1, *b*).

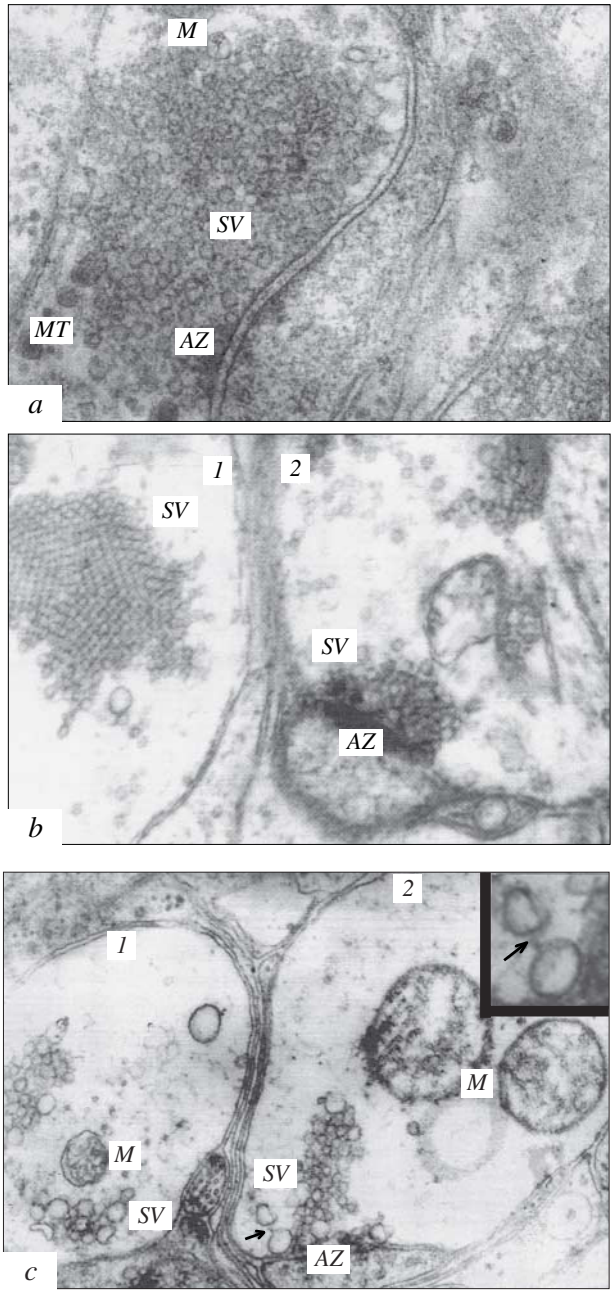

Fig. 1. Structure of presynaptic terminal in the granule cell axon. Normal conditions (×48,000, *a*); after electrical stimulation of the cerebellum in the presence of 10 μM $NaNO_2$ (×36,000, *b*); and after treatment with 5 mM $NaNO_2$ (×35,000, *c*). Insert: preserved bonds between individual SV (×83,000). Here and in Fig. 2: *M*, mitochondria; *MT*, microtubules.

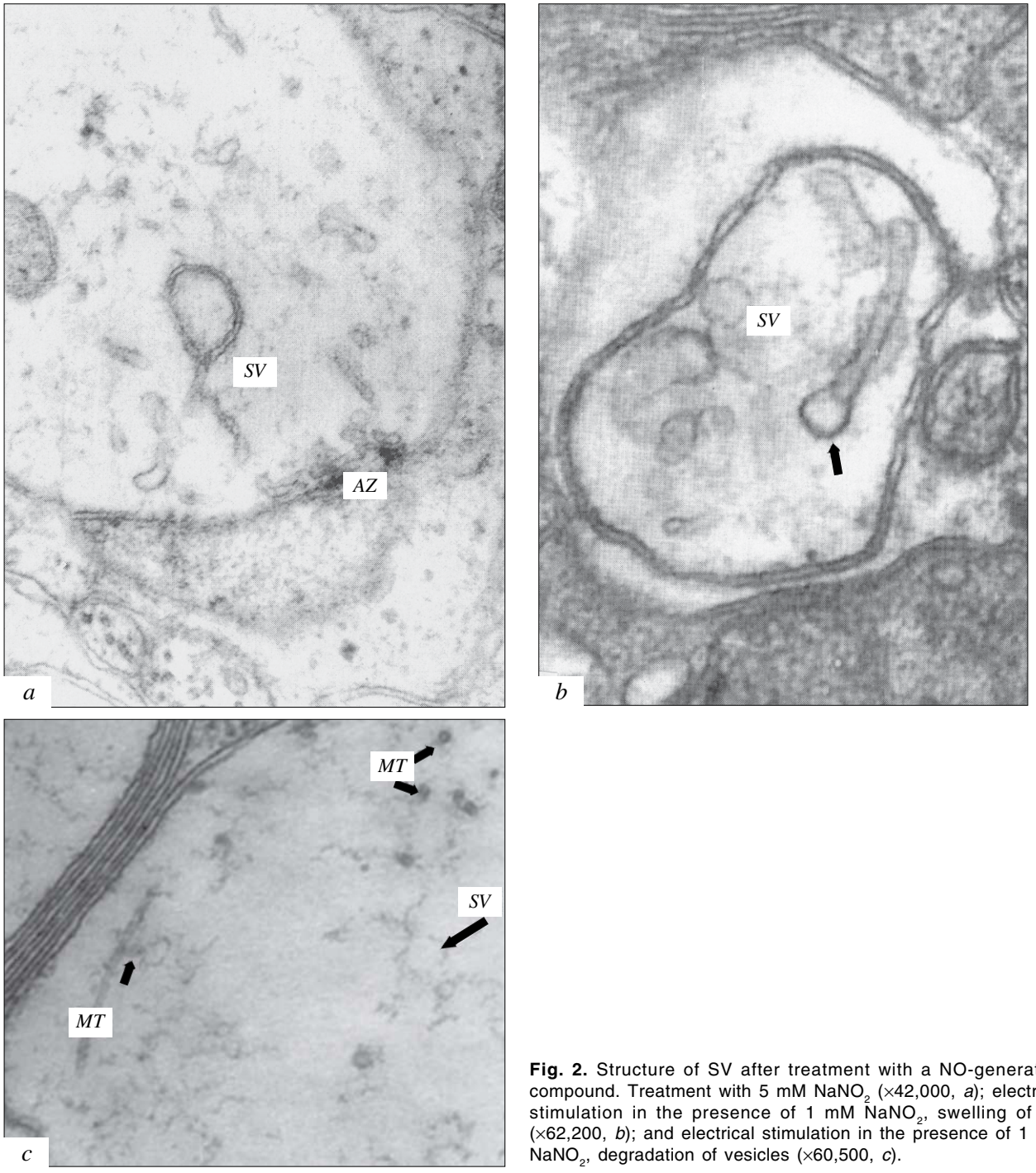

Fig. 2. Structure of SV after treatment with a NO-generating compound. Treatment with 5 mM $NaNO_2$ (×42,000, *a*); electrical stimulation in the presence of 1 mM $NaNO_2$, swelling of SV (×62,200, *b*); and electrical stimulation in the presence of 1 mM $NaNO_2$, degradation of vesicles (×60,500, *c*).

Incubation of the cerebellum with 1 mM $NaNO_2$ was followed by translocation and arrangement of SV in active zone (AZ). The shape and size of SV remained practically unchanged under these conditions. Electrical stimulation of PF in the presence of 1 mM $NaNO_2$ was also accompanied by significant structural changes in SV and other organelles in boutons of cerebellar granule cells. Severe swelling of SV could be followed by fusion of vesicles and formation of elongated sacs (Fig. 2, *b*). Otherwise, SV membranes became loosened into individual fragments. These fragments were sometimes bound to each other. Mitochondria practically disappeared from these boutons (Fig. 2, *c*).

Increasing the concentration of $NaNO_2$ to 5 mM (without electrical stimulation) resulted in swelling of boutons and SV. The arranged heaps were formed from swollen SV. The shape of SV was altered under these conditions. SV became

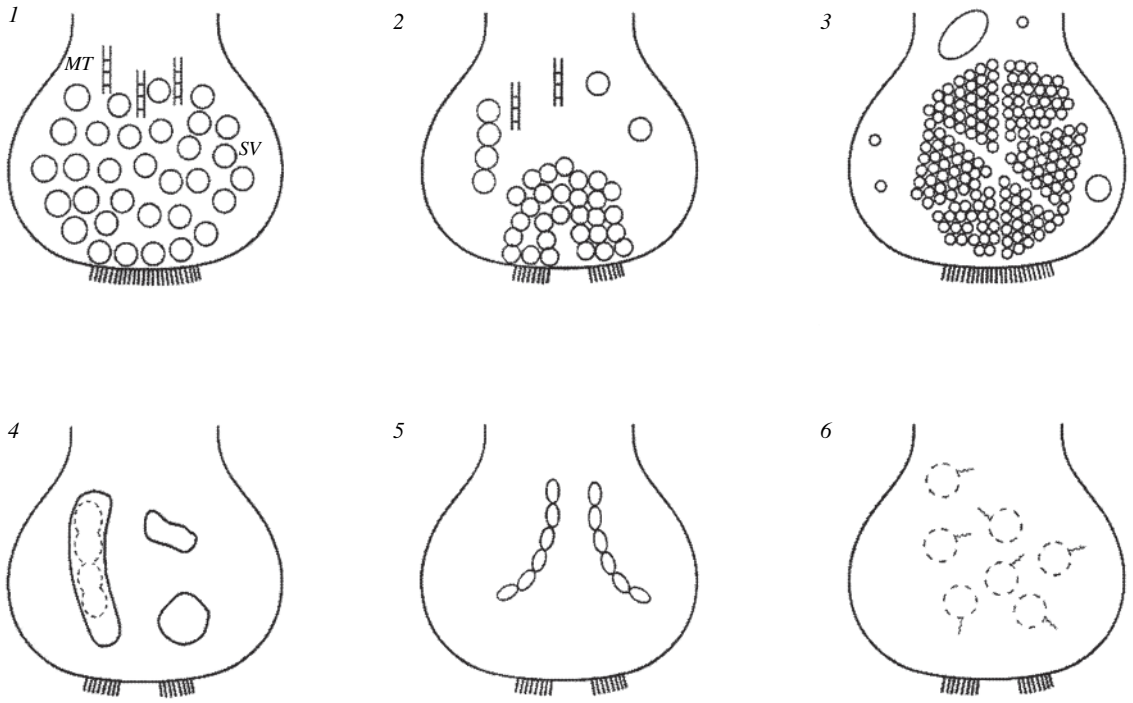

Fig. 3. Scheme of SV distribution in the bouton. Normal conditions (SV of 60 nm, *1*); treatment with 10 μM $NaNO_2$ (*2*); 10 μM $NaNO_2$ and electrical stimulation (SV of 40-45 nm, *3*); 5 mM $NaNO_2$ (SV of 120-140 nm, *4*); 5 mM $NaNO_2$ (SV of 25-27 nm, *5*); and 1 mM $NaNO_2$ and electrical stimulation, fragments of SV (*6*).

flattened (Fig. 1, *c*). The diameter of SV usually increased to 120-130 nm. Mitochondria were swollen and had no cristae. They were transformed into mitochondrial ghosts (Fig. 1, *c*). Individual vesicles were bound to each other (Fig. 1, *c*). Apart from swollen vesicles, several boutons included chains of strongly compressed SV with a size of 25-27 nm. They were not bound to AZ (Fig. 2, *a*). Figure 3 illustrates the scheme of sequential changes in the ultrastructure of SV, which are induced by $NaNO_2$ at increasing concentrations.

3D-reconstruction of AZ revealed the presence of special proteins (beams and ribs) that fasten SV [11]. Moreover, AZ contains proteins for fusion of the presynaptic membrane and SV membrane (*e.g.*, syntaxin). Each SV is surrounded by the membrane, which consists of the lipid bilayer and embedded transport proteins. These proteins are specific for each type of neurotransmitters (in this instance, for glutamate). Vesicular membranes include proteins and phospholipids (1:3 ratio). Phospholipids are presented by phosphatidylcholine, cholesterol, and inositol triphosphate (40, 10, and 5%, respectively).

Our results indicate that NO-generating compounds in low doses (*e.g.*, 10 μM $NaNO_2$) impair the relationship between SV and fastening proteins. These changes result from nitration or nitrosation/nitrosylation of protein amino acids. Moreover, phosphorylation of synapsin 1 with calmodulin-dependent protein kinase is followed by its degradation [8]. Electrical stimulation of the cerebellum in the presence of NO-generating compound (10 μM) was followed by changes in the structure of SV clusters. Their diameter decreased from 60 to 40 nm (by 1.5 times). These clusters looked like a crystal grid. Probably, the displacement and formation of clusters are affected by neurotrophic factor (neurotrophin). The formation of this factor during stimulation is associated with dissociation of the adhesion complex (cadherin and catenin) under β-catenin phosphorylation [5]. Published data show that neurotrophin is present in synapses of granule cells and Purkinje cells [6]. During activation of synapses, neurotrophin causes the displacement of SV due to dissociation of the cadherin-β-catenin adhesion complex.

NO and NO_2 can interact with individual amino acids (proteins) and unsaturated fatty acids (lipids), which leads to the formation of paramagnetic sites in proteins and unsaturated fatty acids of SV membranes. The process is followed by protein-protein and protein-lipid interactions, which results in polymerization. It cannon be excluded that protein-protein and protein-lipid interactions contribute to the formation of arranged heaps from SV. Otherwise, fusion of the SV content is accompanied by the formation of a common elongated sac.

Our findings suggest that the formation of clusters from SV is a compensatory and adaptive response, which maintains the structure of SV and protects neurons from glutamate in high concentration. This experimental approach serves as a model for several stages of structural changes in SV, which occur during stroke and traumas. The data can be used in the study of information transmission in the brain under pathological conditions (strokes and craniocerebral traumas) and exposure to strong chemical or physical agents.

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