Chapter 7

Non-Cholinergic Signaling Pathways at Vertebrate Neuromuscular Junctions

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Additional information is available at the end of the chapter

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1. Introduction

Neuromuscular junction (NMJ) is the functional contact (synapse) between an axon of motor neuron and muscle fiber. It is generally accepted to consider this contact only as a specialized morpho-functional structure, where chemical transmission (via release of the acetylcholine (ACh)) of electrical signal from motor neuron to muscle fiber occurs, ultimately causing the muscle to contract. This synaptic contact is probably one of the most studied synapses since it has relatively large size and easy accessibility for various experimental manipulations. A great body of data is received on the development, molecular organization, morphology, and physiology of both pre- and postsynaptic regions of the NMJ. It's not so long ago that it seemed that practically all was known about the NMJ. However, due to the significant progress in the improvement and application of electrophysiological, genetic, pharmacological, biochemical and immunohistochemical methods a number of previously unknown aspects of neuron and muscle interaction were revealed. So, according to numerous studies, not only ACh (which by the way does not always lead to a contraction of the muscle fiber) is released in the vertebrate neuromuscular synapse, but also a number of other synaptically active molecules. And these molecules can be released from both nerve terminal (anterograde signal), and from muscle fiber (retrograde signal).

Before starting the consideration of the facts relating to the yet poorly studied non-cholinergic signaling, it should be recalled main points of the structure and functioning of the NMJ.

2. Neuromuscular junction organization: Brief overview

Detailed descriptions of the NMJ anatomy can be found in [1-7].

Motor neurons in the ventral region of the spinal cord send axons out toward the periphery (Fig. 1). In mammals and many higher vertebrates, each muscle fiber typically has a single

synaptic site innervated by a single motor axon branch. In front of the contact, the motor axon loses its myelin sheath and forms nerve terminal branches. Several non-myelinating Schwann cells are located over these nerve terminal branches and make processes that are closely covered to them. Terminal Schwann cells, motor nerve terminal branches and the postsynaptic specializations of sarcolemma (also known as a motor end plate) together form the neuromuscular junction (or myoneural junction).

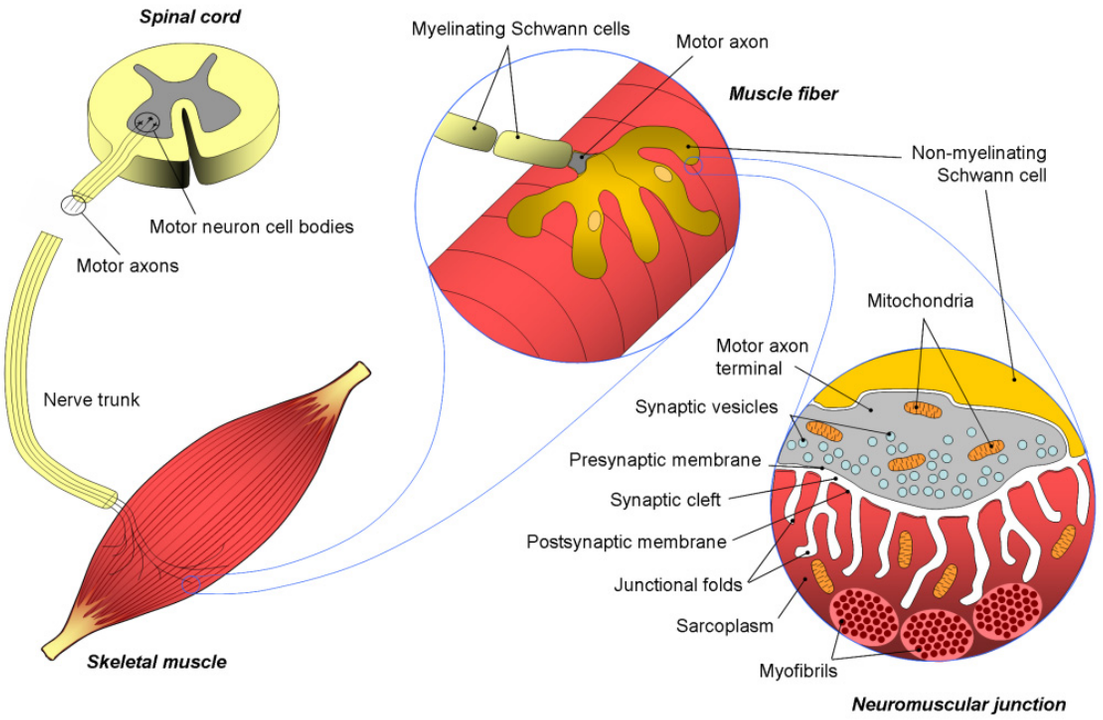

Figure 1. The anatomy of vertebrate neuromuscular junction

The motor nerve ending contains a large number of small synaptic vesicles which store molecules of the neurotransmitter ACh. The latter is synthesized in nerve terminals from choline and acetyl coenzyme A by the cytoplasmic enzyme choline acetyltransferase (ChAT) and transferred by a vesicular ACh transporter (VAChT) into synaptic vesicles. The transmitter contained in a single vesicle (in vertebrate NMJ it is about 5000 – 10000 molecules of ACh) is often referred to as a 'quantum', because during vesicle exocytosis relatively stable portion of chemical substance is released.

ACh diffuses across the synaptic cleft (50 – 100 nm) to be received by ACh receptors on the postsynaptic sarcolemma. One of the most striking structural features of this region is the deep infolding of sarcolemma. The crests of the folds contain a high density of ACh receptors whereas in the depths of the folds a density of voltage-gated sodium channels are presents. The binding of ACh to receptors causes the opening of cation-selective ion channels and allows a net flux of positive charge into the skeletal muscle. When rising depolarization is adequate to open voltage-gated sodium channels, the threshold for action potential generation is reached. Then action potential sweeps across the muscle fiber membrane and the muscle fiber contracts. The neurotransmitter action is terminated by

localized in synaptic cleft enzyme acetylcholinesterase (AChE) hydrolyzing ACh to choline and acetate. Choline is recycled into the motor nerve terminal by a high-affinity uptake system, making it available for the resynthesis of ACh.

3. Neurotransmission in neuromuscular junction

First of all it must be recalled, that ACh release from the motor nerve ending does not always leads to muscle fiber contraction, and motor neuron not only induce a contractile activity in the muscle, but also control of a number of morphological and functional properties of muscle fibers. This latter influence is usually referred to as neurotrophic and it often involve the control of gene expressions in the muscle [8-13]. At the NMJ have revealed the presence of several distinct types of ACh release: spontaneous quantal, nerve impulse evoked quantal and non-quantal release. Molecular mechanisms, features and functional significance of these secretion types are described in detail and systematized in reviews [6,14-17]. Here we will briefly consider these processes.

Spontaneous quantal release. Low amplitude (0.5 – 1 mV) potentials, called as miniature endplate potentials (mEPPs; Fig. 2A) appear in the synaptic area of sarcolemma as the result of release of a single synaptic vesicle content ('quantum'). Average mEPPs frequency in vertebrates is about 1 per second in the absence of nerve stimulation.

Nerve impulse evoked quantal release. The nerve action potential leads to opening of voltage-gated Ca^{2+} channels, resulting in the local influx of Ca^{2+} into motor nerve ending. This leads to the relatively synchronous release of 20-400 ACh quanta, what in its turn causes depolarization of the postsynaptic membrane by several – several tens of mV. This potential is recorded as an endplate potential (EPP; Fig. 2B). The number of quanta released by a single nerve impulse is known as the 'quantal content' of the EPP.

Non-quantal release. In the absence of nerve stimulation the amount of ACh released by non-vesicular manner is a hundred times greater than ACh, released by spontaneous quantal release. Electrophysiologically, the intensity of the non-quantal ACh secretion process can be evaluated only in terms of AChE inhibition [17; Fig. 2C]. Currently there is still no clear answer to the question of which protein is mediated by this type of neurotransmitter release. Nevertheless it is established that this process is not a passive leakage, and it is regulated by various synaptic active molecules, regardless of quantal release processes [17] and is blocked by increasing of Mg^{2+} concentrations [18], as well as inhibitors of both vesicular ACh transport (vesamicol) [19,20] and choline uptake (hemicholinium-3) [21].

Although molecular mechanisms of action of spontaneously released mediator are not yet fully established, the majority of data indicate that tonic neurotransmitter release is one of the neurotrophic control factors whereas the physiological role of the evoked quantal ACh release is to ensure clear transmission of the electric impulse from the motor nerve to the muscle fiber [15,17]. At the same time obtained experimental results suggest a possible trophic role of ACh released by quantal manner in response to the nerve action potential [8,22]. However, until recent time, the fact that other signaling molecules can be released

together with the ACh from motor nerve endings and participate in the neuromuscular transmission was ignored.

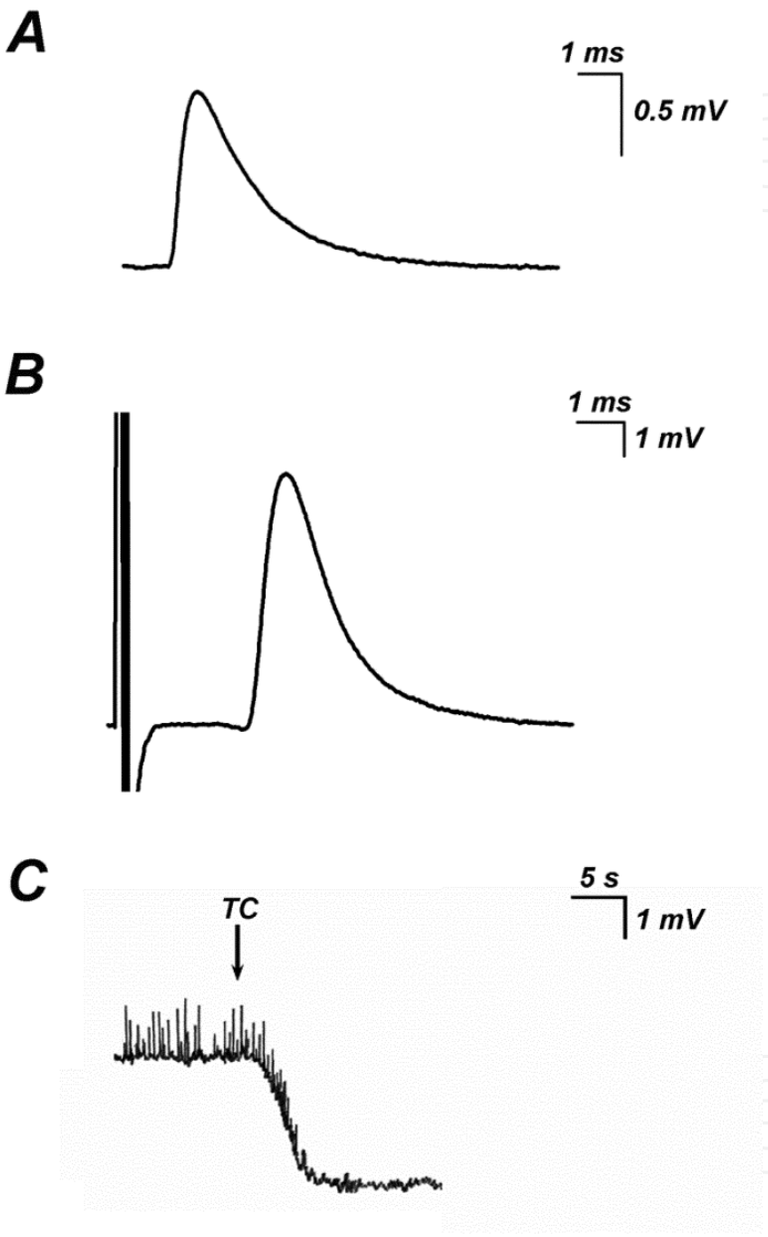

Figure 2. Electrophysiological registration of different types of ACh release at the rat neuromuscular junction. **A** – Spontaneous miniature endplate potential (averaging of about 100 signals in a single fiber) as a result of the action of individual quantum of ACh molecules. **B** – Evoked endplate potential (averaging of about 100 signals in a single fiber) as a result of synchronous release of a number of quanta of neurotransmitter in response to a nerve impulse. In this case, the phasic ACh release was reduced by high Mg^{2+} in the Ringer's solution to prevent muscle contraction. **C** – Endplate hyperpolarization (H-effect) following blockade of skeletal muscle postsynaptic nicotinic receptors by (+)-tubocurarine (TC) and cholinesterase as a result of the action of ACh, released predominantly in a non-quantal manner.

4. Cotransmission and neuromodulation

In neurobiology for decades the 'Dale's principle' dominated, according to which, one neuron synthesizes, stores, and releases a single transmitter liberated from all own's axon terminals. In this regard, vertebrate motoneuron for a long time considered as a cell capable to release ACh only. However by the early 90's a large amount of experimental data was obtained, the analysis of which led to the formation of the modern theory of 'cotransmission' [23-28]. According to this theory, one or several types of synaptically active molecules – cotransmitters (coexisting transmitters) are released from the neuron together with basic mediator. These cotransmitters are capable of exerting its own effects in the target cell, regulating the release of primary neurotransmitter (presynaptic modulation) or modulating the physiological response in the postsynaptic cell (postsynaptic modulation). At present, it can be stated that the phenomenon of corelease of several neurotransmitters from the nerve endings is the rule rather than the exception for the entire nervous system, including peripheral part [24,25,27,28].

Some signaling molecules that do not meet the definition of 'cotransmitters' are involved in the functioning of the synaptic apparatus too. They are released from either neuron, but independently of the primary neurotransmitter, or have a glial origin or they are released from the postsynaptic cell and, along with cotransmitters, exert their modulating and/or neurotrophic effects.

5. Purinergic signaling

Purines in NMJ. Adenosine 5′-triphosphate (ATP) one of the purine compounds, which plays a crucial role in energy exchange and metabolism in all living cells. It is formed from adenosine 5′-diphosphate (ADP) and inorganic phosphate by the enzyme ATP synthase, localized in mitochondria. These organelles are abundantly represented in all synapses, including NMJ, where they concentrated both presynaptic and postsynaptic regions. The ATP concentration is about 2-5 mM in the cytoplasm of most neurons, whereas directly in the synaptic vesicles it is higher for at least by 2 orders, including vesicles of cholinergic terminals [29,30], where it is pumped by ADP/ATP translocase [31,32]. It is necessary to note that in ACh contained vesicles besides ATP, small amounts of ADP and traces of adenosine 5′-monophosphate (AMP) were observed [29,30].

Release and metabolism of purines in the synaptic NMJ cleft. In experiments on rat and frog neuromuscular preparations clear evidence of Ca^{2+}-dependent corelease of ATP and ACh from motor nerve terminals was obtained [33-35]. At the same time, there are data suggested that ATP is released from skeletal muscle cells in response to muscle contraction [35-37]. And if in the first case, mechanism of a signaling molecule release is exocytosis of synaptic vesicle, in the case of the muscle fiber molecular mechanism of ATP discharge is not yet fully established. At the present time pannexin (Pnx) hemichannels have also been proposed as relevant ATP conduits [38]. In any case, nowadays the fact of a significant increase of the ATP concentration and its derivatives in the synaptic cleft of the NMJ after motor nerve stimulation is well established [29,39].

Like many signaling molecules, ATP released from the cell is metabolized in the extracellular space. ATP is broken down to ADP and AMP by extracellular ATPases [29]. Further, as shown directly in the rat NMJ, AMP was either dephosphorylated into adenosine by ecto-5′-nucleotidase or deaminated into inosine monophosphate by ecto-AMP deaminase [39]. Inosine is an inactive metabolite [40], but adenosine is a signaling molecule that activates its own receptors [41]. Formed adenosine is removed from the synaptic cleft of the NMJ by dipyridamole-sensitive adenosine uptake system, and there are reasons to believe that adenosine uptake is more important than adenosine deamination in the regulation of extracellular adenosine concentrations [40].

Purine receptors in NMJ. Purine receptors are divided into 2 large groups: adenosine or P1 receptors and P2 receptors, which activated by nucleotides ATP, ADP, uridine 5′-diphosphate and uridine 5′-triphosphate [29,41-43]. All P1 receptors (A_1, A_{2A}, A_{2B} and A_3) are G protein-coupled receptors, while P2 receptors are consist of two distinct families: P2X receptors, which are ligand-gated ion channels for cations, and P2Y receptors, which are G protein-coupled receptors. Seven mammalian P2X receptor subtypes ($P2X_{1-7}$) and eight mammalian P2Y receptor subtypes have yet been cloned and functionally defined as P2 receptors ($P2Y_{1,2,4,6,11,12,13,14}$) [29,41].

Pharmacological evidence of the presynaptic localization of adenosine (P1) receptors were obtained on preparations of NMJ, both in amphibians [44] and mammals [45,46]. In the latter case the presence of A_1 and A_{2A} receptor subtypes on the nerve ending was defined. Subsequently, confirmation of exclusively presynaptic localization of A_{2A} receptors in the the NMJ of mouse was obtained by the means of immunohistochemistry [47]. At the same time, on the plasma membrane of human skeletal fiber adenosine A_{2A} and A_{2B} receptors were revealed by means of immunohistochemistry [48].

As for P2 receptors, the following is known at present time. $P2X_7$ receptor subunits were found on presynaptic motor nerve terminals of mouse, but there is no evidence for $P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$ or $P2X_6$ receptor subunits [49]. According to a number of electrophysiological studies metabotropic P2Y receptors are also localized on the motor nerve endings of both amphibian and mammals NMJ [44,50-52]. However, P2Y receptors were found on the postsynaptic membrane of skeletal muscle fiber. Moreover, the presence of $P2Y_1$ and $P2Y_2$ receptors on the plasma membrane was precisely established [53,54]. Developing mammalian skeletal muscle fibers are able to express 4 subtypes of metabotropic purine receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_{11}$) and, what is interesting, all types of P2X receptors which, apparently, are absent on the mature innervated muscle fibers [29,55-57].

The physiological significance of purinergic signaling in NMJ. Quite a lot of evidence indicating the significant role of purinergic signaling in the processes of establishment, development and maintenance of NMJ is accumulated. In developing of the *Xenopus* neuromuscular synapses ATP increased the intensity of the ACh release from the nerve ending and enhanced the responses of the muscle membrane to ACh [58,59]. Moreover, ATP modulates agrin-induced ACh receptor aggregation via activation of $P2Y_1$ receptors [60] and regulates

the expression of AChE and ACh receptor genes via activation of $P2Y_2$ receptors [53]. Activation of ionotropic ($P2X_4$, $P2X_5$ and $P2X_7$) and metabotropic $P2Y_1$ and $P2Y_4$ purinoreceptors participates in forming the calcium transients of multinucleated myotubes [56]. The significance of purinergic signaling in the development of neuromuscular synapses is perfectly demonstrated in the work [57], where authors investigated the NMJ in knockout mice ($P2X_2$). It should be recalled that this type of receptor is absent on mature muscle fiber, where it is expressed only in the early stages of development. It was found that the neuromuscular contacts in these knockout animals have significant structural abnormalities, followed by muscle fiber atrophy.

In addition to the role of ATP in the process of synaptogenesis a lot of data is obtained about the modulator effects of purines on the processes of ACh release in the mature vertebrate NMJ. So, it was found that ATP and adenosine significantly reduced the intensity of both evoked and spontaneous quantal release of ACh, activating presynaptic purine receptors [44,50,51,61,62]. However adenosine can also facilitate the quantal release of ACh what, apparently, depends on the pattern of motor nerve stimulation [46]. Extracellular ATP induces presynaptic inhibition of ACh release via its own P2Y receptors, which modulate voltage-gated Ca^{2+} channels [50,51]. Adenosine also inhibits quantal release of ACh, acting through P1 receptors and its mechanism of action does not affect the operation of calcium channels [50,51]. As for the influence of purines on the non-quantal release of ACh it is established that its intensity remains unchanged in the presence of adenosine, but it decreases via activation of P2Y receptors by the ATP molecules and this mechanism is not coupled to presynaptic voltage-dependent Ca^{2+} channels [52,63].

Postsynaptic modulator effects of purines in the mature neuromuscular synapse were also established. So it was found that ATP can increase ACh receptor activity [64-66] and inhibit chloride channels in mammalian skeletal muscle [54].

6. Glutamatergic signaling

The origin and localization of glutamate in NMJ. Glutamate and its derivatives are dominant in terms of numbers among all amino acids in nervous tissue. This amino acid plays not only a central metabolic role [67,68], but also acts as the primary excitatory neurotransmitter in the central nervous system [69-71].

In experiments on the culture of spinal neurons and skeletal muscle fibers of *Xenopus* embryos it was shown that glutamate is present in the growth cone of developing motor neurons and in the nerve endings forming synaptic contact with muscle fiber [72]. Significant immunoreactivity to glutamate has been identified directly in the nerve endings of mammals [73,74], and the level of immunoreactivity in terminals that innervate the extensor digitorum longus was higher than in the nerve endings of soleus muscle. Concentration of glutamate in the motor nerve endings of the extensor digitorum longus was estimated in the range 10-20 mM [74]. Furthermore, authors were able to demonstrate

direct association of glutamate with synaptic vesicles what supposes the joint release of ACh and glutamate in the synaptic cleft [74].

Glutamate release from the motor neuron. The uptake of labeled glutamate by frog motor neurons and its release from the motor nerve terminals were demonstrated in one of the first studies indicating the possible involvement of glutamate in the functioning of the vertebrate NMJ [75]. The detection of the vesicular glutamate transporters (VGLUT1 and VGLUT2) in the motor neurons of the spinal cord testifies for vesicular release of amino acid from cholinergic terminals [76]. The VGLUT3 transporter was found directly in the motor nerve terminals [77]. Clear evidence for co-operative glutamate release with ACh was obtained in the study of synaptosomes from nerve terminals of the *Torpedo* electric organ [78]. Previously this object considered as 'purely cholinergic system' and used as a classical model for studying the general aspects of the cholinergic neurotransmission. The authors have shown the corelease of these two mediators in Ca^{2+}-dependent manner under the action of depolarizing agents [78]. This fact of a simultaneous release ACh and amino acid was confirmed later by other authors [79]. Electrophysiological data showing action potential induced corelease of glutamate and ACh from of mammals motor neurons was obtained recently [80,81].

Glutamate receptors in the NMJ. Nowadays this is probably the most studied aspect of glutamatergic signaling in the neuromuscular synapse. A wide range of ionotropic glutamate receptors (kainate, AMPA and NMDA) has been found in synaptic contact in experiments on the culture of neurons and myocytes of *Xenopus* [72,82,83], and data indicate about predominantly presynaptic localization of these receptor structures. In the later stages of amphibians development, namely in tadpoles and adult frogs metabotropic glutamate receptors were found [80,84-86], which, apparently, are localized postsynaptically [80,85]. In contrast to the amphibian NMJ, in the endplate of mammals to date were found only ionotropic glutamate NMDA and AMPA receptors, and all the experimental data show exclusively postsynaptic localization of these proteins [87-92].

Removal of glutamate from the synaptic cleft. Any extracellular enzyme which inactivates glutamate in extracellular space is not found until now [67]. It is interesting to note that the uptake of glutamate molecules from the solution, where muscle was incubated, and the transport of amino acid on sciatic nerve to the spinal cord was demonstrated even in 1967 [75]. Currently, five membrane transporters of this amino acid are identified and only two of them are widespread: GLAST (EAAT1) and GLT (EAAT2). The presence of glutamate transporter GLAST, which is localized mainly on the presynaptic membrane of Schwann cell, was revealed in experiments on the frog nerve-muscle preparation [80]. GLAST and GLT transporters were found in mammals in the area of endplate and they are fairly well represented in the synapses of both fast and slow muscles [93]. And it was established that most of them are localized deep in postsynaptic folds [93].

The physiological significance of glutamatergic signaling in the NMJ. It is established that glutamate affects the processes of ACh release from motor nerve endings, and the mechanism of modulator action, based on available data, in mammals and amphibians is different. Moreover, in amphibian it, apparently, changes during ontogenesis. Thus, it is

shown that glutamate facilitates the quantal release of ACh at early stages of establishment and maturation of the NMJ in frog [72,82,94], whereas in adult animals, on the contrary, the amino acid inhibits the quantal release of ACh [80,84-86]. At the same time any effect of glutamate on the quantal release of ACh in the NMJ of mammals was not established [89], however, the inhibition of non-quantal ACh release was revealed [89,90]. And since this type of the mediator is able to perform trophic function [17], in this case, glutamate may be considered as a regulator of neurotrophic control of the properties of the postsynaptic membrane. Due to the fact that the activation of glutamate receptors both in amphibians [85], and mammals [88-90] may be accompanied with increased synthesis of nitric oxide molecules (NO), then it should be assumed that the amino acid is able to participate in a wide range of physiological functions, since the contribution of NO-mediated signaling was revealed in metabolism and contraction of muscle fibers [95,96].

7. Peptidergic signaling

N-Acetylaspartylglutamate (NAAG) is the most abundant and widely distributed neuropeptide in the mammalian central nervous system, able to perform signaling function in the interneuronal synapses [97].

High concentrations of NAAG have been found in spinal cord motoneurons and motor components of cranial nerve nuclei [98-100]. Moreover, this dipeptide was found in sciatic nerve [98,101] and phrenic nerve terminals [87]. NAAG can be involved in neurotransmission as: (i) direct agonist of glutamate ionotropic NMDA receptors and metabotropic GluR3 receptors and (ii) as a glutamate precursor, which is formed directly in the extracellular space during hydrolysis by the enzyme glutamate carboxypeptidase II (GCP II), also known as N-acetylated α-linked acidic dipeptidase (NAALADase) [102].This peptidase is a membrane-bound protein which was detected in non-myelinating presynaptic Schwann cells surrounding motor nerve terminals [87,103].

Experiments on rat NMJ showed that NAAG is able to depress non-quantal ACh release [90]. The mechanism of neuropeptide action is realized through its extracellular hydrolysis by the GCP II with the formation of glutamate molecules, which, as was shown earlier [89], activate glutamate postsynaptic NMDA receptors and thereby trigger the NO-mediated mechanism of reducing the intensity of the non-quantal ACh release [104].

Substance P. This peptide belongs to the tachykinin neuropeptide family, found in neurons of both central and peripheral nervous system, where it performs neurotransmitter and neuromodulator functions [105]. The main receptor for substance P is G protein-coupled neurokinin 1 (NK-1) receptor.

The presence of substance P in frog motor nerve endings was shown by immunohistochemistry [106]. Later, data demonstrating the neuropeptide release during the stimulation of the motor nerve was obtained [107]. NK-1 receptors, localized in perisynaptic Schwann cells NMJ were found by the same authors. Substance P was not found by

immunohistochemistry in the motor nerve endings of rodents [108,109], however, it was found in the muscle fibers. Soleus muscle had a significantly higher content (0.61 ng/g) than the extensor digitorum longus (0.22 ng/g) [109].

In studying the signaling function of substance P in the frog NMJ its influence on all compartments NMJ was revealed: on motor nerve terminal, on postsynaptic membrane and on Schwann cell. So, following effects were shown: (i) facilitating effect of neuropeptide (at a concentration till 1 μM) on spontaneous and evoked quantal release of ACh [110]; (ii) reduction of the sensitivity of the postsynaptic membrane to ACh at the concentration peptide above than 1 μM [111,112]; and (iii) induction of Ca^{2+} release from internal stores in Schwann cells [107]. In the mammalian NMJ also was noted presynaptic facilitatory action of substance P. Neuropeptide facilitated the indirect twitch responses of the rat diaphragm and increased amount of ACh released into the bathing medium in response to tetanic stimulation of the phrenic nerve [113].

Calcitonin gene-related peptide (CGRP). This peptide is distributed throughout the central and peripheral nervous systems and exhibits a range of biological effects [114]. CGRP mediates its effects via G protein-coupled receptor called calcitonin receptor-like receptor (CALCRL).

Frog motor neurons express CGRP-like immunoreactivity and this immunoreactivity in motor nerve terminals is confined within so called 'large dense-core vesicles' [115]. CGRP-like immunoreactivity was found in the mouse and rat motor nerve terminals [116,117]. In rat hind limb CGRP-like immunoreactivity is heterogeneously present in the endplates and, apparently, correlates with the muscle fibers phenotype [118]. Motoneurons of small and slow-twitch motor units in general have lower levels than motoneurons of large and fast-twitch motor units [119]. It is established that the CGRP is released by nerve impulse activity [120]. Calcrl mRNA and CALCRL protein were found directly in postsynaptic region of rats muscle fibers [121]. The CGRP receptor, and its two associated components (RAMP1 and RCP), are highly concentrated at the adult avian NMJ where they co-localize with AChE and ACh receptors [122].

Physiological role of CGRP was revealed not only at establishment and development NMJ, but also in the process of its functioning. Thus, on cultured chick myotubes it was shown that the CGRP stimulates the turnover of phosphoinositides and the accumulation of inositol phosphates [123] and also increases the number of surface ACh receptors [124]. In 1-day-old *Xenopus* nerve-muscle cultures CGRP enhances the postsynaptic response at developing NMJs by increasing the burst duration of embryonic ACh channels [125]. Moreover, neuropeptide plays a key role in the trophic regulation of AChE at the NMJ not only during synaptogenesis, but lifelong [122,126].

In experiments on mature rodent neuromuscular synapse it was shown that CGRP enhances muscle contraction during stimulation of the nerve fibers or direct stimulation of the muscle [117]. The ability of neuropeptide to enhance the intensity of spontaneous quantal ACh release was revealed [127]. The effect of the CGRP facilitating the secretion of ACh was also described in the frog neuromuscular synapse [128].

8. Nitric oxide signaling

Biosynthesis of NO in NMJ. NO is a free radical short-living (half-life 4-6 s) gas, which is formed from L-arginine in the body by the enzyme NO-synthase. Three isoforms of this enzyme were identified: neuronal (type I), inducible (type II) and endothelial (type III) [96,129]. Health skeletal muscular tissue expresses both endothelial and neuronal isoforms of the NO-synthase [96]. Endothelial isoform is co-localized with mitochondria of skeletal muscle fibers [130], while the neuronal NO-synthase is concentrated in the NMJ [88,131,132]. 'Anchoring' of the enzyme in the postsynaptic membrane is provided by interaction with the dystrophin-associated protein α1-syntrophin [131]. Moreover, experiments on skeletal myotubes showed that neuronal NO-synthase is able to interact directly with the NMDA receptor via the PSD-95 protein [133]. In mature muscle of health rodents and human the expression of inducible NO-synthase is absent or represented very poorly [134,135], however, it can significantly increased under certain pathological conditions [135,136].

Neuronal and endothelial NO-synthases are activated by calcium and calmodulin, whereas the inducible isoform binds irreversibly to the calmodulin right after the translation, so this enzyme produces NO independently of changes in intracellular calcium concentration [96]. It is established that during muscle contraction the activity of NO-synthases increases by several times [137,138]. It is well explained by the increase of cytosolic calcium concentration, which facilitates the interaction of the enzyme with calmodulin. According to several authors skeletal muscle produces from 2 to 25 (average ~ 10) pmol $\cdot$ min^{-1} $\cdot$ mg^{-1} of nitric oxide [137,139,140].

It is interesting to note that, apparently, in amphibians the localization of NO-synthases is differ from mammals. So, in frog NMJ NO-synthase immunostaining was found at the membrane and occasionally in the cytoplasm of perisynaptic Schwann cells and was not detected in the nerve terminal or muscle [141].

Physiological effects of NO in NMJ. The mechanism of NO signaling function is based on its interaction with thiol groups and/or transition metals in proteins. Most of the NO physiological responses are mediated by S-nitrosylation of redox centers and interactions with heme or nonheme iron and copper. Thus, the binding of NO with heme-containing protein leads to changes in the activity of the latter: in the case of cytochrome-c oxidase - inhibition and in case of guanylate cyclase - activation [86].

NO-mediated signaling plays a certain role in the formation of the NMJ. In particular, the role of NO both in presynaptic and postsynaptic differentiation of NMJ was shown [142,143]. In mature neuromuscular synapse physiological significance of NO-mediated signaling was revealed in processes metabolism and contraction of muscle fiber, as well as in modulation of ACh release from the motor nerve ending.

It is shown that the NO-synthase activity can modulate mitochondrial respiration in skeletal muscle. So, inhibitory effect of NO on oxygen consumption of muscle tissue was revealed [144,130]. Modulatory influence of NO was demonstrated with respect to carbohydrate metabolism. It was shown that NO-synthases blocking inhibits the reuptake of 2-

deoxyglucose, whereas exogenous NO molecules donor leads to its increase [138,139]. On the other hand, the possibility of NO to inhibit the activity of glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase was revealed [145]. Finally, data on the NO-mediated inhibition of the creatine kinase activity in skeletal muscle was obtained [146]. It can lead to decrease in the synthesis of ATP from creatine phosphate.

The action of NO on contractile function of muscle fibers is complex. *L. Kobzik* and co-workers [137] showed that NO-synthase inhibition, inactivation of extracellular NO and inhibition of guanylate cyclase increase the amplitude of muscle contractions, which is reduced in the presence of NO donor and with an increase the cGMP concentration. At the same time *R. Morrison* and co-workers [147] demonstrated reduction of the maximum rate of contraction of muscle fibers on rat diaphragm during NO-synthase inhibition. This did not happen, when the donor of NO was added together with the blocker of enzyme. Similar results were obtained also on mouse extensor digitorum longus [148]. Interesting data is presented in [149], where it is shown that L-arginine (the substrate for the NO synthesis) increases the amplitude of muscle contraction in response to nerve stimulation of isolated rat diaphragm, but it leads to a reduction at direct muscles stimulation. Both effects removed by NO-synthase inhibition and were not appeared after D-arginine application. The authors suggest that NO enhances contractile function by acting on presynaptic level, and reduces it when acting on postsynaptic. One of the major potential targets for NO on postsynaptic compartment is the ryanodine receptors of sarcoplasmic reticulum. NO can both facilitate and inhibit the activity of the ryanodine receptors [150,151] what, apparently, explained by the presence of several areas in this protein, interacting with the NO molecules [96].

Namely the fact of finding the post-synaptic localization of NO-synthase and modulating effect of NO molecules on the process of ACh release from motor nerve terminals allow us to declare that this signaling molecule acts as a retrograde synaptic mediator in the NMJ. NO reduces the intensity of both spontaneous and evoked quantal ACh release in the neuromuscular synapse of the frog [152,153]. The inhibitory action of nitric oxide on spontaneous and induced synaptic currents was shown also in the developing neuromuscular contacts *Xenopus laevis* [154]. In contrast with endplate of amphibian, NO has no effect on spontaneous and evoked forms of quantal ACh release, but significantly reduces the intensity of non-quantal release of ACh in mammalian NMJ [104]. However, as was shown later, NO can modulate the quantal release in mammals, enhancing transmitter release from motor nerve via a cGMP pathway, but it occurs only when adenosine A_1 receptors were blocked [155]. It is also necessary to note the fact that in the synapse of amphibians and mammals, the fact endogenous tonic effect of NO on ACh release processes was confirmed repeatedly [86,89,104,153].

9. Conclusion

Until now, many people share the opinion that intercellular contact between motor neuron and skeletal muscle fiber is very well studied morpho-functional structure, which provide the one-way transmission of electrical impulse from the motor neuron to the muscle for the

initiation of the contractile act. However, this opinion is totally wrong and one of the proofs for that is this review which describes a number of most studied signaling pathways mediated by molecules that previously were not considered in the aspect of the functioning of the NMJ. Experimental facts proving; (i) the formation of these molecules in the neuromuscular synapse; (ii) their release in the synaptic cleft; (iii) the interaction with specific receptor proteins; and (iv) the existence of a specific physiological effect for each of these signaling molecules are presented and analyzed here. It is necessary to emphasize that the author intentionally considered those signaling molecules (ATP, glutamate, NAAG, substance P and NO), which act as an individual neurotransmitter in the mature organism, but in synapses of other parts of the nervous system [27,29,69,105,156,157]. CGRP, in its turn, also plays its role in mature intercellular contact, acting as a cotransmitter in sensory-motor neurons [27].

A number of signaling molecules which are also participate in the signaling between motor neuron, Schwann cell and skeletal muscle fiber remained beyond the review. At least nerve growth factor (NGF), glial-cell-line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4 (NT-4) and transforming growth factor-beta 2 (TGF-β2) are among them. The main role in regulating of neuronal survival, plasticity, growth, and death is ascribed to them. However, it turned out that these proteins act also as regulators of the maintenance, function, and regeneration of skeletal muscle fibers [158]. So, it was shown that BDNF, NT-3, NT-4 are expressed both in motor neurons and in muscle fibers. GDNF, in its turn, is expressed in Schwann cell and in muscle fiber. Activity-dependent synthesis and release of these factors in extracellular space have been reported. Receptors for all these factors were revealed in mature NMJ, their participation in the regulation of neuromuscular transmission was shown also at the expense of influence on the processes of ACh release [158-160].

Thus, NMJ is a rather complicated and flexible compartment for multicircuit intercellular communication between a motor neuron and muscle fiber, what provides the synaptic plasticity and reliability of synaptic transmission.

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