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REVIEWS

Modern Concepts of Cholinergic Neurotransmission at the Motor Synapse

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Abstract—Cholinergic synaptic contact between motor neuron and skeletal muscle fiber is perhaps one of the core objects for investigations of molecular mechanisms underlying the communication between neurons and innervated cells. In the studies conducted on this object in the past few decades, a large amount of experimental data was obtained that substantially complemented a traditional view on synaptic transmission. In particular, it was established that (i) acetylcholine is released from the nerve ending in both quantal and non-quantal ways; (ii) molecular mechanisms of the processes of the quantal acetylcholine release—spontaneous and evoked by electrical stimuli—have unique features and can be regulated independently; (iii) acetylcholine release from the nerve ending is accompanied by a release of a number of synaptically active molecules modulating the processes of secretion or reception of the main mediator; (iv) signal molecules affecting the process of cholinergic neurotransmission can be released not only from the nerve ending but also from glial cells and muscle fiber; (v) molecular mechanisms of the regulation of synaptic transmission are highly diverse and go beyond the alteration of the number of the released acetylcholine quanta. Thus, the neuromuscular junction shall be deemed currently as complicated and adaptive synapse characterized by a wide range of multiloop intercellular signaling pathways between presynaptic motor neuron ending, muscle fiber, and glial cells ensuring a high safety factor of synaptic transmission and the possibility of its fine tuning.

Keywords: cholinergic synapse, quantal and non-quantal release of mediator, cotransmission, neuromodulators, synaptic plasticity

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INTRODUCTION

Regulation, integration and hierarchy of cells within a multicellular organism, including a human one, are realized due to humoral and nervous systems. The nervous system is the key link in perceiving of information from external and internal environments, analysis of this information, and initiation of a response (including a motor one) of the whole body to an incoming signal. The nervous system functions because neurons can generate and conduct electrical signals that are transmitted to adjacent innervated cells in the regions of specialized cell junctions (*synapses*). The vast majority of these junctions are so-called chemical synapses, that is, junctions, in which an electric signal causes a release of a chemical mediator (*neurotransmitter*) from the neuron ending. The neurotransmitter diffuses through a synaptic cleft and interacts with special receptor proteins at the postsynaptic membrane of the innervated cell. Further events at the postsynaptic membrane depend on the receptor nature: either an electric signal is generated or/and the signal cascade of reactions that changes metabolism of the innervated cell is triggered.

A process of a signal transmission from a neuron to another cell is often referred to as *neurotransmission* and characterized further by an "ergicity"; that is, we specify a chemical structure of a mediator responsible for the transmission of an electric signal. For example, in the case of synapses, whose neurotransmitter is acetylcholine, we speak of a *cholinergic* nature of the transmission.

A wide range of factors makes the study of cholinergic signal transmission a challenging issue. First, the cholinergic neurons are presented in a number of brain structures (including brainstem, mesencephalon, and cerebral cortex), and their axons innervate various zones of cortex and a number of subcortical zones. The cholinergic system plays a key role in the mechanisms of attention [1], memory, and learning [2]. Second, the cholinergic transmission is the main system

† Deceased.

of a signal transmission in the parasympathetic division of the autonomous nervous system innervating smooth muscles and glands of gastro-intestinal tract, secretory and reproductive organs, lungs, atria, salivary and lacrimal glands, and eye muscles. Third, the cholinergic neurotransmission underlies the signal transmission from a motor neuron to a skeletal muscle and thus serves as the key element in the initiation of any motion—an arbitrary motion of a body or a limb, breathing, contraction of vocal chordae, etc.

For historical reasons, a cholinergic neuromuscular junction—intercellular junction between the motor nerve ending and a skeletal muscle fiber—has became one of the key model object for investigations not only of cholinergic signal transmission but also of the synaptic transmission in general. It is the studies on neuromuscular preparations of a frog, lizard, mouse, and rat that enabled revealing and examination of numerous aspects of a synapse formation, development, morphology, and molecular structure, as well as demonstration of the basic principles of functioning of the pre- and postsynaptic regions. We can say therefore that this type of the intercellular contact is the best investigated synaptic junction. Until recently, we believed that we know practically everything about cholinergic transmission. However, novel electrophysiological, genetic, pharmacological, biochemical, and immunohistochemical technologies have revealed a wide range of phenomena and processes that necessitate updating, if not a complete revision, of our concepts of chemical neurotransmission in general and of the cholinergic one, in particular. This review suggests to examine both underexplored and recently disclosed aspects of the cholinergic synapse functioning as exemplified by a neuromuscular junction, the structure and main functioning details of which will be briefly surveyed.

NEUROMUSCULAR SYNAPSE: A BRIEF DESCRIPTION OF STRUCTURE AND FUNCTIONING

A detailed description of a neuromuscular junction anatomy can be found in [3–5].

Somata of motor neurons innervating the locomotor muscles are localized in frontal horns, and the axons come out to the periphery through their ventral roots (Fig. 1). As a rule, each muscle fiber of a mammal, is innervated by one motor neuron and has only one synaptic junction. From the other hand, one motor neuron usually innervates several fibers, and thus forms a functional *motor unit*. Immediately before the contact, an axon loses its myelin sheath composed by the myelinating Schwann cells and forms branches of nerve endings. Some non-myelinating, so called perisynaptic, Schwann cells are localized above these terminals and compactly cover them with their processes. The terminal Schwann cells, motor nerve ending, and postsynaptic site of sarcolemma (also termed *end plate*) form altogether a neuromuscular junction.

A motor nerve ending contains a big amount of synaptic vesicles filled with acetylcholine synthesized in the nerve terminal from choline and acetyl coenzyme A with the participation of acetylcholine transferase [2.3.1.6] and transferred into synaptic vesicles with a vesicular acetylcholine transporter at the expense of gradient of protons pumped into the vesicles by the ATPase. As a result, one synaptic vesicle of a vertebrate nerve terminal contains about 5000–10000 molecules of acetylcholine, and during exocytosis this portion of the neurotransmitter (named a *quantum* due to its relative stability) is released into a synaptic cleft.

Acetylcholine diffuses through a synaptic cleft (50–100 nm) and activates acetylcholine receptors of a postsynaptic membrane possessing prominent invaginations of sarcolemma that form so-called *junctional folds*. The main population of the acetylcholine receptors is localized at the crests of these folds, while their bottom is occupied by voltage-sensitive sodium channels. Interaction between acetylcholine and its receptor causes opening of a sodium-selective channel and influx of positively charged ions into a muscle fiber. A soon as depolarization of a postsynaptic membrane reaches a certain level, voltage-sensitive sodium channels open and generate an action potential that spreads along a muscle fiber membrane and finally causes its contraction. Duration of the acetylcholine action is limited by activity of the localized in a synaptic cleft acetylcholinesterase [3.1.1.7] that hydrolyzes acetylcholine into choline and acetate. Choline, in turn, with the help of a transport protein returns into the nerve terminal (*high-affinity choline uptake*) and is used for the synthesis of new acetylcholine molecules.

It is worth mentioning that the release of acetylcholine from a motor nerve terminal does not always cause contraction of a muscle fiber, and a motor neuron by itself not only induces contractile activity of a muscle but realizes control of a wide range of morphological and functional properties of a muscle fiber. Such an effect is termed *neurotrophic* and is often realized through alterations of gene expression in a muscle fiber [6–8]. By now, it is recognized that acetylcholine is released both in portions (*quantal release*) and in the form of single molecules (*non-quantal release*).

QUANTAL RELEASE OF ACETYLCHOLINE

Since early studies of B. Katz [9, 10] and up to recent days, the vast majority of the experimental data can be put into frames of the so-called *quantum-vesicle theory* of the mediator release, according to which one quantum of a mediator is the contents of one synaptic vesicle. A quantum release is realized through exocytosis, in course of which a membrane of a synaptic vesicle fuses with a membrane of a nerve ending followed by the discharge of the vesicle content into the synaptic cleft. A quantum of acetylcholine, interacting with a population of postsynaptic ionotropic cholinorecep-

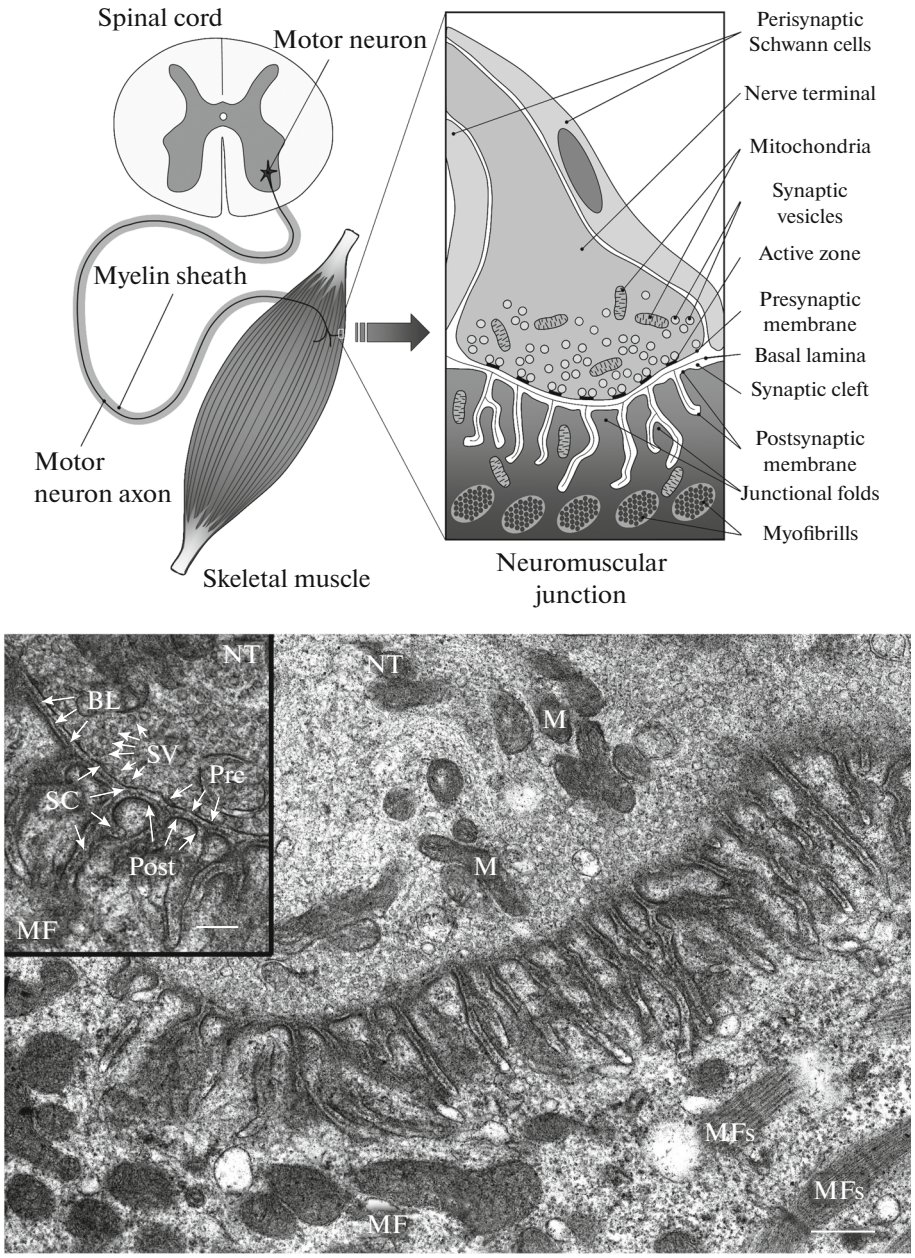

Fig. 1. Structure of a mammal neuromuscular junction. *Upper panel*: a neuromuscular junction anatomy. *Lower panel*: electron microphotograph of a neuromuscular synapse of a rat diaphragm. NT, nerve terminal; MF, muscle fiber; M, mitochondria; MFs, myofibrils; SV, synaptic vesicles; Pre, presynaptic membrane; SC, synaptic cleft; BL, basal lamina; Post, postsynaptic membrane. Scale bar, 1 μm; scale bar in the box, 0.5 μm.

tors located alongside of a discharge point, causes a slight alteration in the synaptic zone potential, designated as a *miniature end plate potential* (MEPP). In homoiothermals, in the absence of nerve firing, frequency of the MEPP occurrence varies about 1 signal per second; such a neurotransmission process is classified as a *spontaneous quantum release* (Fig. 2a).

In response to action potential, a transmitter, in the quantities from several units to several hundred units, is released from a nerve terminal. A number of a transmitter quanta causing occurrence of a certain generated postsynaptic response is termed a *quantal content* of a postsynaptic signal, and a process of transmission generated by an electric stimulus as an *evoked quantal release* (Fig. 2b).

A quantal-vesicle theory of transmission implies availability of a mechanism for synaptic vesicles recycling. Indeed, a membrane of a synaptic vesicle, being fused with a presynaptic one, is included into the composition of the last one, and then, in course of the

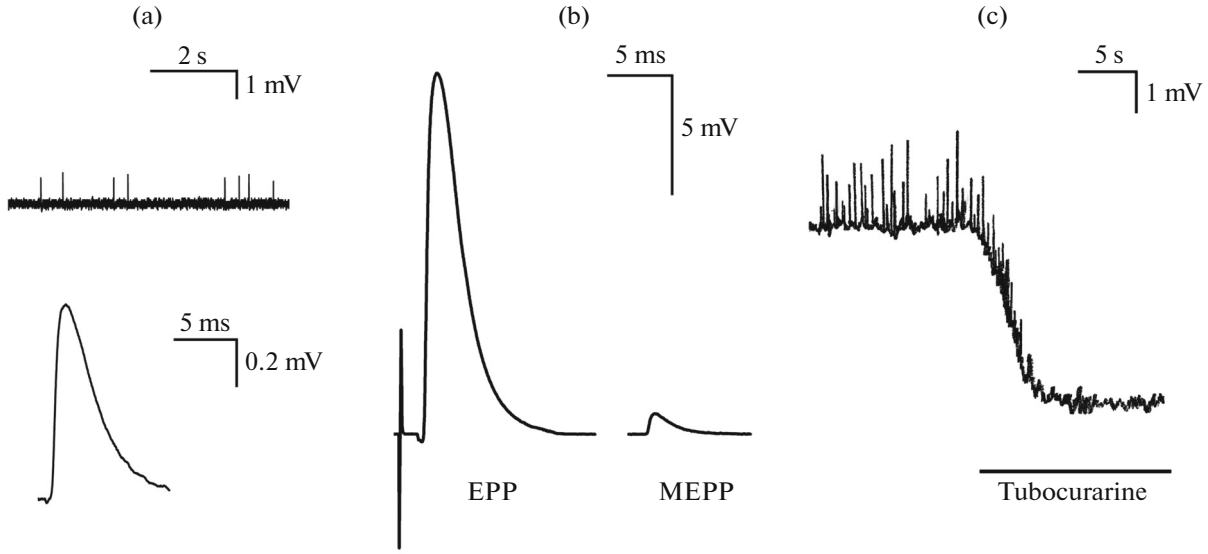

Fig. 2. Electrophysiological recordings of acetylcholine release processes in the neuromuscular junction of a rat diaphragm with the use of intracellular microelectrodes introduced into the postsynaptic region of a muscle fiber (by Malomouzh et al. [11, 12] modified). (a) Miniature end plate potentials (MEPPs) as a result of spontaneously released acetylcholine quanta effect on postsynaptic membrane (spontaneous quantal release). *Below* is an extended view of one MEPP. (b) End plate potential (EPP) elicited by acetylcholine on the postsynaptic membrane; the transmitter is released in several tens of quanta in response to an electrical stimulus (evoked quantal secretion). *Right*: MEPP recorded in the same synapse. (c) Hyperpolarizing effect (H-effect) of tubocurarine on the muscle fiber membrane potential at inhibited acetylcholinesterase; this indirectly reflects the intensity of the non-quantal acetylcholine release.

endocytosis, is involved into formation of new synaptic vesicles, which after "pinching-off" and filling with transmitter molecules are ready to exocytosis again. Such a cyclic process was referred to as a *vesicle cycle*, and in our days a remarkable progress has been made in its investigations [13, 14].

As was already mentioned, the vast majority of the experimental data on neurotransmission fits a classical quantum-vesicle theory. However, some recently found factors, on first reading, cannot be explained in terms of this theory. One of them is multimodal distributions of amplitudes of miniature signals and so-called "subminiature" postsynaptic responses observed in some cases. Just these observations founded a *multivesicular* hypothesis of a quantal formation [15], according to which a miniature postsynaptic response is a response of a postsynaptic membrane to the simultaneous release of contents of several vesicles located within closely adjacent active zones, while a subminiature response is a response of a postsynaptic membrane to the release of contents of only one synaptic vesicle. However, this hypothesis is open to criticism, as the polymodality of amplitudes of spontaneous postsynaptic responses can be a result of irregular filling of a vesicle with a transmitter or of different receptor density in various regions of a postsynaptic membrane.

Another possible interpretation of amplitudes variability in the spontaneous postsynaptic responses deserves some more attention. This concept fits to a postulate "one vesicle = one quantum of the transmitter". This is a mechanism, referred as "*kiss-and-run*", in which a synaptic vesicle immediately after making a pore in the membrane of a nerve ending loses its contact with the axolemma and returns into the cytoplasm. In such a case no full fusion of the membranes takes place and amount of the released transmitter depends both on diameter and "lifetime" of a newly formed pore. This provides a possibility to alter the amplitude of a post-synaptic signal. Whether such a mechanism of neurotransmission is possible and what is its physiological sense, if any, is actively debated [16], however, the process is investigated up to now, and the models for its examination include cholinergic neuromuscular synapse [17]. A functional meaning of the "incomplete fusion" between a synaptic vesicle and a presynaptic membrane may grow in course of the high frequency firing and its sense may be in a faster and more effective repeat use of synaptic vesicles in a vesicular cycle (relative to the mechanism of the "complete fusion") [18].

Analysis of the fractional transmitter release in a neuromuscular junction will be incomplete without discussion of such a phenomenon as giant MEPPs. These signals can be characterized not only by essentially higher and greatly varying amplitude (of several mV) but by a much slower rise time (5–10 ms against 0.1–0.6 ms for "typical" MEPPs), and independence from concentrations of external calcium, magnesium, and potassium [19]. Such agents as ethanol and ouabain increasing frequency of "standard" MEPPs never

affect an expected level of spontaneous giant postsynaptic responses. Moreover, the occurrence of the last ones is never blocked by botulin toxin A, removing occurrence of the "typical" MEPPs [19, 20]. So, the giant spontaneous postsynaptic responses could be either a result of the delayed simultaneous release of the acetylcholine from a synaptic vesicles cluster or a release of transmitter from the large synaptic vesicles or endosome-like structures with a time-delayed interval. Now, the discussions of possible participation of endosomes in the neurotransmission become more and more frequent. As the investigations suggest, not only a "classical" endocytosis of isolated vesicles but formation of large invaginations, which later form endosome-like structures, take place in a nerve ending in the period of the intensive nerve stimulation (at a higher exocytosis level). Formation of such structures enables internalization of large regions of a membrane and, in such a way, an essential correction of a nerve ending area in the period of higher activity. This process is named "*bulk endocytosis*" [21].

Thus, the mechanism of synaptic vesicles endocytosis underlies a quantal transmitter release. As soon as the fraction mechanism of a neurotransmitter release had been discovered it was postulated that spontaneous and evoked quantal secretions are two versions of one the same process. The only difference between these versions is a different probability of a quantal release, whose value at rest is rather low and grows essentially at calcium ions entry through potential-dependent calcium channels that open, when a nerve ending is depolarized with an action potential. However, some new data tell on differences in the mechanisms of the spontaneous and evoked quantal secretion. For example, it was recently demonstrated that the evoked quantal release of acetylcholine can be selectively (skipping a process of the spontaneous quantal release) controlled with choline [22] or activation of vanilloid (TRPV1) receptors [23] and receptors to γ-aminobutyric acid [12]. Other models of synaptic contacts, by contrast, prove the selective regulation of only spontaneous process of quantal release of the transmitter [24, 25]. Moreover, some authors revealed cases of the reverse regulation of these two neurotransmission forms [26, 27]. What could be the nature of differences in mechanisms of the spontaneous and evoked quantal release of a neurotransmitter? By far, the experiments have proved the following hypotheses: (i) mechanisms of the spontaneous and evoked neurotransmitter releases have different calcium-dependence; (ii) processes of the exocytosis mediating the spontaneous and evoked quantal releases of the transmitter have different "molecular machineries"; (iii) spontaneous and evoked neurotransmission involve different populations of vesicles. More detailed analysis of the current data showing differences in the processes of the spontaneous and evoked quantal release of a neurotransmitter and arguing that these are the independent signaling pathways are provided in [28, 29].

So, summing all the above mentioned, we may say that in spite of the numerous available studies of the quantal acetylcholine release, a lot of aspects of the quantal neurotransmitter release still require further investigations. And, while a physiological role of the evoked quantal release of the transmitter is obvious (this is enabling of transmission of an electrical impulse from a neuron to an innervated cell through a synaptic cleft), the place of the spontaneous quantal release is not found yet, though we can suppose that a transmitter released in such a way participates in the neurotrophic control of a postsynaptic cell properties. However, according to the current concepts, it is acetylcholine released in a non-quantal way that plays the main part in the neurotrophic control.

NON-QUANTAL RELEASE OF ACETYLCHOLINE

An idea that a transmitter can be released not only in a quantal form was first proposed by J. Mitchell and A. Silver [30], who demonstrated the lack of correlation between amount of acetylcholine released into the incubation medium from a resting neuromuscular preparation and MEPP frequency at alterations of temperature and potassium concentration. Further experiments and estimations showed that in the absence of the nerve firing, only a small portion of acetylcholine (several percent) is released in the form of quanta, while the rest portion of the transmitter is either of non-synaptic origin (muscular) or released form a nerve permanently through a cytoplasm "leakage" [31, 32]. Possible acetylcholine synthesis and release by a muscle tissue became a rather unexpected phenomenon [33]. It was found that about a half transmitter released from a neuromuscular preparation at rest is of a "muscle" origin. Nevertheless, though a considerable amount of acetylcholine is released just in the synapse region, it is much greater than the amount that can be released through the spontaneous quantal secretion.

In 1977, a technique for electrophysiological registration of the non-quantal acetylcholine release in a neuromuscular junction was developed [34, 35] (Fig. 2c). The method is based on a concept that, while in the absence of nerve firing the major part of acetylcholine is released in a non-quantal mode, in the condition when the acetylcholinesterase is inhibited (making the enzymatic hydrolysis of acetylcholine impossible) the transmitter will be accumulated in the synaptic cleft in an amount sufficient to cause depolarization detectable by the electrophysiological techniques. At first approximation, depolarization should be proportional to the transmitter concentration in the synaptic cleft. However, B. Katz suggested [34] that a more objective and convenient estimation of the intensity of the non-quantal release would be not a degree of depolarization but its removal by the cholinergic receptor block-

ade (H-effect, from *hyperpolarization*). Later, it was shown that the H-effect can be registered only in a synaptic region and disappears completely in several hours after the nerve crush, while the spontaneous quantal release remains unchanged to that moment [36–38]. These data, first, show that the H-effect is a parameter of acetylcholine release from a nerve and, second, correspond to the biochemically registered decrease in the acetylcholine release from a denervated neuromuscular preparation in the same time frames [30, 39]. Hence, the H-effect indeed is an indicator of the non-quantal acetylcholine release intensity on the condition that the sensitivity of a postsynaptic membrane to the transmitter remains constant.

It is noteworthy that the H-effect can be registered without acetylcholinesterase inhibitor as well, though only in the junctions, where activity of the enzyme is either absent or initially low. For example, Sun and M.-M. Poo [40], using a *Xenopus* "motor neuron—myocyte" co-culture, registered a significant hyperpolarization (5–8 mV) of a muscle cell membrane, following a local application of d-tubocurarine and α-bungarotoxin (in the absence of any acetylcholinesterase inhibitors), and the value of this hyperpolarization did not depend on the MEPP frequency.Vyskocil and Vrbova [41] registered the H-effect (1.8 mV) not using cholinesterase inhibitors as well, but at later stages of synaptogenesis, namely, in the neuromuscular synapses in diaphragms of 7–9-day-young rats that already have the acetylcholinesterase in their synaptic clefts but with lower density than in the synapses of mature animals. Minic et al. [42], in turn, recorded the H-effect without additional use of cholinesterase inhibitors, but, in this case, in the synapses of mature mice knocked out by a certain type of collagen, which provides anchoring of acetylcholinesterase in the synaptic cleft.

What is the mechanism underlying the process of the non-quantal acetylcholine release? Immediately after this phenomenon was first described, a hypothesis on their mechanism was proposed. It suggested that non-quantal acetylcholine release was a simple diffusion of acetylcholine molecules through the lipid bilayer along a concentration gradient. For that matter, a term "leakage" became the most used name for the non-quantal release [34, 35]. It is expected that the molecules of acetylcholine cannot overcome the lipid barrier but such a possibility is not denied completely [43]. On the other hand, the vast majority of experimental data tell that the non-quantal release is an active and fine-tuned process accomplished with the help of a special carrier protein, that is, one of two transport systems: vesicular transporter of acetylcholine and a transporter realizing the reuptake of a transmitter from a synaptic cleft (high-affinity choline uptake system).

Hypothesis of vesicular acetylcholine transporters suggests that acetylcholine can be pumped into a synaptic cleft with the help of vesicular transporters that are inbuilt into a presynaptic membrane in course of exocytosis (at the quantal neurotransmitter release) [44]. Indeed, an inhibitor of acetylcholine transport into vesicles (vesamicol) decreases the H-effect essentially. Strictly speaking, this is the only valid argument in favor of the vesicular transporters hypothesis. Recently, we verified this hypothesis using another methodical approach: we evaluated intensity of a non-quantal acetylcholine release in the conditions of the endocytosis blockade [45]. It was found that an essential inhibition of the process by dynasore (an inhibitor of dynamine, one of the key proteins of the "machinery" of the synaptic vesicle endocytosis) did not change a value of the H-effect. Moreover, the increased concentrations of dynasore began to exert an essential potentiating effect on the spontaneous quantal release, which should result in additional increase in amount of vesicular transporter built-in into a membrane. However, such a situation did not cause any increase in the non-quantal acetylcholine release either. It should be noted that the absence of whatever correlation between the processes of spontaneous quantal and non-quantal release was noticed much earlier and can find their endorsements up to now. For example, intensities of these two forms of the neurotransmitter release change independently of each other at denervation and reinnervation, temperature changes, and at various concentrations of magnesium, potassium, and calcium ions. Moreover, these processes of the acetylcholine release have different oxygen sensitivities and may have different endogenous regulatory mechanisms. More details of these processes are presented in reviews [46, 47]. So, we can suppose that the activity of a vesicular acetylcholine transporter could hardly underlay a mechanism of the non-quantal acetylcholine release from nerve.

Here is an alternative *hypothesis* explaining a mechanism of the non-quantal acetylcholine release: it is transported by the *high-affinity system of choline uptake*; in other words, this system acts as a transporter. For example, experiments on a mouse neuromuscular synapse showed that substitution of Na^+ with Li^+, as well as application of hemicholinium-3 (a selective inhibitor of the choline uptake system), very rapidly abolishes the H-effect [38]. On the other hand, enhancement of performance efficiency of this transporter activity by the addition of choline or by a low-frequency nerve stimulation delays considerably a fall of the post-denervation H-effect. Nevertheless, the hypothesis of high affinity system of a transmitter reuptake has some bottleneck: we have no direct and incontestable evidences that choline transporters can pump the acetylcholine outward from a nerve ending cytoplasm. At the same time, abundant evidences obtained on non-cholinergic synapses indicate that transport proteins participating in the transmitter uptake can release the same transmitter out of the cell cytoplasm into its environment [47, 48]. This can be considered as a valid argument in favor of participation

of these proteins in the non-quantal acetylcholine release.

A number of evidences indicating that non-quantal secretion is not just a passive leakage of a transmitter and not a laboratory effect but an active process very sensitive to a nerve integrity may imply a participation of this form of the acetylcholine release in the realization of the neurotrophic control of a muscle fiber properties. This idea gets more and more evidences. First, after a motor nerve is transected, the non-quantal acetylcholine release stops much earlier than the quantal one. And, vice versa, it is just non-quantal release to be the first restored in course of reinnervation, while process of the spontaneous quantal release appears only several days later [36]. Second, the acetylcholine released in a non-quantal manner was shown to be a necessary factor responsible for the switch from a polyneuronal character of innervation to a mononeuronal one at the initial stages of a skeletal muscle innervation [41]. Third, a wide range of data attests to the fact that just a transmitter fraction released in the non-quantal mode at rest controls the maintenance of the resting membrane potential at the level typical for the innervated muscle condition [49–51]. Fall of a muscle fiber membrane potential (primarily, in a synaptic region) is one of the first postdenervation changes and its development meets the same time frames as decrease in intensity of non-quantal release. Note that it occurs at the background of an unchanged level of spontaneous quantal transmitter release. Administration of acetylcholinesterase inhibitors and cholinomimetics in concentrations simulating an effect of the "non-quantal" acetylcholine delays this fall of the muscle fiber membrane potential. Ability of acetylcholine in nanomolar concentrations to activate a Na^+,K^+-ATPase of a muscle fiber is one of the possible mechanisms underlying such a cholinomimetic activity [52].

Though examination of the CNS synaptic contacts, instead of the peripheral regions, is now the main focus of neurophysiological studies, the interest to process of the non-quantal acetylcholine release is growing. This is partially due to the evidences of the non-quantal acetylcholine release from parasympathetical neurons innervating both smooth [53] and cardiac muscles [54, 55].

Thus, we may conclude that a process of non-quantal acetylcholine release (along with the spontaneous and evoked quantal releases) should be considered as an independent form of a neurotransmitter release playing a quite certain physiological role related to, at least, realization of a neurotrophic control of an innervated cell.

Until recently, the fact that other signal molecules capable of participation in a neuromuscular transmission can be released from a nerve ending along with the acetylcholine practically never was considered.

COTRANSMISSION AND NEUROMODULATION

For decades, a Dale's principle, according to which one neuron can synthesize, store and release one neurotransmitter, was the main principle of neurobiology. As a result, a vertebrate motor neuron for a long time was considered as a cell capable of release of only acetylcholine. However, in the early 1990s, the analysis of a big amount of laboratory data led to the establishment of a current *cotransmission* concept [56–58]. According to this theory, a neuron can release one or more species of synaptically active molecules, *cotransmitters*, able to exert an effect of their own on target cells, to control release of the main transmitter (*presynaptic modulation*) or modulate a transmitter physiological response in a receiving cell (*postsynaptic modulation*). It could be postulated that a phenomenon of co-release of several transmitters from a nerve ending is rather a rule than an exclusion for the entire nervous system, including its peripheral part [56–58].

In addition, a synaptic apparatus involves in its functioning some types of signal molecules that do not meet the determination "cotransmitters". These can be released either from a neuron, but independently from a main transmitter, or from a postsynaptic cell, or be of the glial origin but they exert a modulating or neurotrophic effect cell along with cotransmitters. Let us review some most examined cotransmitters and neuromodulators that can play a certain signal role in functioning of the cholinergic neurotransmission.

Purines. It is well known that adenosine-5'-triphosphate (ATP) plays a key role in substance and energy metabolisms of any living cell, and its cytoplasmic concentration in most neurons is 2–5 mmol/L. It was found that ATP concentrations inside synaptic vesicles of cholinergic terminals are higher by, at least, two orders. In addition, small amounts of adenosine-5'-diphosphate (ADP) and adenosine-5'-monophosphate (AMP) were found in the acetylcholine-containing vesicles. One of the first facts telling for a combined calcium-dependent release of ATP and acetylcholine from the motor nerve terminals became known in 1975 [59]. For now, the fact of essential increase in the concentrations of ATP and its derivatives (including ADP, AMP and adenosine by itself) in a synaptic cleft of a neuromuscular synapse after the motor nerve stimulation is well recognized [60]. It is worth mentioning that purines can be released not from a nerve ending only (as acetylcholine cotransmitters) but from skeletal muscle fibers [61] and perisynaptic Schwann cells [62] as well. In such a case, ATP can release in a vesicular mode through a membrane channel formed by protein pannexin 1 [63, 64]. More recently it was demonstrated that purinergic regulation of cholinergic neurotransmission is impaired in mice with knock-out of pannexin-1 gene [65].

Careful attention to a signal role of ATP and improved methods of examination resulted in reveal of

a wide spectrum of both adenosine (P1) and purine (P2) receptors in the region of a neuromuscular junction. The most data tell in favor of the presynaptic localization of these receptors, though some subtypes of P2 receptors were found at the membranes of the skeletal muscle fibers as well. Moreover, some studies show alterations of purine receptors expression in course of ontogenesis, pointing to a certain role of purinergic signaling in the processes of formation, development and regulation of a neuromuscular junction. It was found that ATP modulated expression of genes coding acetylcholinesterase and acetylcholine receptors, as well as regulated aggregation of the last in the synapse region [66, 67]. It was found that in mice with knock-out of receptor $P2X_2$, a neuromuscular synapse had considerable structural anomalies followed by muscle atrophy [68]. In a mature neuromuscular synapse, ATP and adenosine considerably decrease intensities of both evoked and spontaneous quantal forms of acetylcholine release, activating the presynaptic purine P2Y and adenosine P1 receptors [60]. Non-quantal acetylcholine release is reduced at activation of P2Y receptors, too [69].

Glutamic acid (glutamate). Amounts of glutamate and its derivatives dominate all other amino acids of the nerve tissue. This amino acid not only plays the key metabolic role [70], but acts as a main excitatory neurotransmitter in the synaptic contacts of CNS. It was found that glutamate concentration in motor nerve endings of mammals was comparable with its concentration in a hippocampus (a brain structure rich in glutamatergic contacts). Glutamate molecules are immediately associated with synaptic vesicles, and this may enable the cooperative release of acetylcholine and glutamate from the motor nerve endings [71]. It is worth mentioning that the processes of labeled glutamate capture by frog motor neurons, of the amino acid transport along the nerve, and its release from the motor nerve ending were found as far as in 1967 [72]. In the late twentieth century and in the early twenty first, the fact of glutamate and acetylcholine cooperative release was repeatedly proved in various models of cholinergic synapses.

Ionotropic kainate receptors, as well as receptors to the α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) and N-methyl-*D*-aspartate (NMDA), whose activation potentiates the quantal acetylcholine release, were found at developing neuromuscular synapses of amphibians [73]. The same time, in the mature amphibians motor synapses only metabotropic glutamate receptors are found, whose activation, on the contrary, leads to the inhibition of the main transmitter release [74]. Only glutamate ionotropic NMDA and AMPA receptors are found in the mammal neuromuscular junction; all experimental data point to the entirely postsynaptic localization of these proteins [75, 76], and their activation does not exert immediate effects on the processes of quantal acetylcholine release. An NMDA receptor mediated mechanism of the non-quantal acetylcholine release inhibition by glutamate was found [77]. Activation of glutamate receptors both in amphibian [74], and in mammals [77] can be followed with enhancement in synthesis of molecular nitric oxide (NO), so we can suppose that the amino acid can participate in a wide spectrum of physiological functions, because an impact of NO-mediated signaling was found in the processes of metabolism and contraction of a muscle fiber [78].

N-acetylaspartylglutamate (NAAG). NAAG is the most abundant neuropeptide of a mammal nervous system that can exert a signaling function in synapses, activating some glutamate receptors or acting as a glutamate precursor generated immediately in the extracellular space as a result of glutamate carboxypeptidase II hydrolysis [79]. NAAG was found in motor nerve endings, while the glutamate carboxypeptidase II was found at membranes of perisynaptic Schwann cells [80]. Experiments with a rat neuromuscular synapse showed that NAAG can inhibit the non-quantal acetylcholine release [81]. Mechanism of the neuropeptide action is realized through its enzymatic hydrolysis followed by generation of glutamate molecules that, as was shown earlier [77], activate the postsynaptic glutamate receptors of NMDA and, in such a way, activate the NO-mediated mechanism of inhibition of the non-quantal acetylcholine release intensity. Recently, biochemical and electrophysiological evidences obtained in a lizard neuromuscular junction, showed that NAAG is an acetylcholine cotransmitter and can modulate its release, activating the glutamate receptors [82].

Substance P. This peptide was found in the neurons of both central and peripheral nervous systems, where it functions as a neurotransmitter and as a neuromodulator [83]. In the frog neuromuscular synapses, substance P was found in the motor nerve endings [84], while in the rodents it was found in the muscle fibers [85].

Substance P affects all compartments of an amphibian neuromuscular synapse: a motor nerve terminal, a postsynaptic membrane and Schwann cells. Specifically, the following effects were demonstrated: (i) a potentiating effect of the neuropeptide on the spontaneous and evoked quantal acetylcholine release [86]; (ii) decrease in postsynaptic membrane acetylcholine sensitivity in the presence of substance P [87], and (iii) induction of Ca^{2+} exit from depot in the Schwann cells [88]. Besides, in a mammal neuromuscular synapse a potentiating presynaptic action of substance P is registered [89].

Calcitonin gene-related peptide (CGRP). This peptide, the same as the above mentioned, is rather typical both for central and peripheral divisions of nervous system, playing roles of neurotransmitter and neuromodulator through activation of own metabotropic receptor (CALCRL). CGRP was found in the motor nerve terminals of amphibians and mammals, and its release at stimulation of a motor nerve is proven [90].

CGRP participates both in processes of formation and development of a neuromuscular junction and in the process of a mature synapse functioning. For example, it was demonstrated that CGRP stimulates phosphoinositide turnover in chicken skeletal muscle cells in culture [91] and increases numbers of membrane acetylcholine receptors [92]. Moreover, it was found that the neuropeptide plays a key role in the trophic regulation of acetylcholinesterase in the neuromuscular junction during the whole life and not only in the course of synaptogenesis [93]. In a mature rodent neuromuscular junction, it was shown that CGRP enhances muscle contractions [94]. This may be due to ability of the neuropeptide to potentiate the processes of the quantal acetylcholine release, and increase in a transmitter quantal size is one of the most examined mechanisms of a CGRP-mediated facilitation [95, 96].

Nitric oxide (NO). Formation of NO from *L*-arginine is catalyzed by NO synthase. Three isoforms of this enzyme are identified: neuronal (type I), inducible (type II) and endothelial (type III). A healthy skeletal muscle expresses both endothelial and neuronal isoforms of NO synthase. The endothelial isoform is localized near mitochondria of skeletal muscle fibers, while the neuronal NO synthase is concentrated in the neuromuscular synapse region [78]. Fixation of the enzyme in a postsynaptic membrane enables its binding with a protein α_1-syntrophin, associated with dystrophin. Besides, it was demonstrated that in a muscle fiber the neuronal synthase can interact immediately with the NMDA receptor through a protein PSD-95 [97]. It was found that at a muscle contraction, activity of NO synthase increases several times, this can be explained by increase in cytosol calcium, required for the enzymatic synthesis of NO molecules. According to several authors, a skeletal muscle can produce 2–25 (in average ~10) pmole mg^{-1} min^{-1} of nitric oxide [78].

Mechanism of NO signal function is based on its interaction with thiol groups and/or transition metals within the proteins. For the most part, NO physiological responses are mediated by S-nitrosylation of redox centers and interactions with heme or nonheme iron and copper. Thus, NO binding to a heme-containing protein leads to the conformational changes of the last and this, in turn, affects its activity: inhibition at interaction with cytochrome *c* oxidase, and activation at binding with guanylate cyclase [78].

In a neuromuscular junction, the NO-mediated signaling participates in processes of metabolism and contraction of a muscle fiber, as well as in the regulation of acetylcholine release from a motor nerve ending. For example, NO inhibits oxygen consumption by muscle tissue [98], as well as modulates carbohydrate metabolism. It was found that blockade of NO synthase inhibits the 2-desoxyglucose uptake, while an exogenous donor of NO molecules leads to its increase [99]. NO-mediated inhibition of creatine kinase in the skeletal muscles was shown [100], this inhibition can reduce the ATP synthesis from creatine phosphate.

NO effects on a contractile function of the skeletal muscles are not unambiguous. Blockade of NO synthase, inactivation of extracellular NO, and inhibition of guanylate cyclase increase amplitude of muscle contractions which is decreased in the presence of NO donor and upon elevation of the cGMP concentration [101]. The same time, decrease in maximal speed of muscle fibers contraction of a rat diaphragm was registered at blockade of NO synthase, while addition of an NO molecules donor together with the enzyme prevented this phenomenon [102]. Ambiel and Alves-Do-Prado [103] presented interesting data demonstrating that *L*-arginine, a substrate for NO synthesis, increases amplitude of muscle contractions in response to stimulation of nerve of an isolated rat diaphragm, but leads to its decrease at direct stimulation of the muscle. Both effects were removed by the blockade of NO synthase and were not caused by application of *D*-arginine. It is likely that NO enhances a muscle contraction acting at the presynaptic level and inhibits it acting at the postsynaptic level.

The postsynaptic localization of NO synthase and modulating effects of NO molecules on the processes of acetylcholine release from a nerve terminal imply that this signal molecule plays a role of a retrograde synaptic transmitter in a neuromuscular synapse. It was found that NO decreases intensities of both spontaneous, and evoked quantal release of acetylcholine in a frog neuromuscular synapse [104]. In contrast to an endplate of poikilothermic animals, in mammalian endplates NO does not affect either spontaneous or evoked forms of acetylcholine quantal release but essentially decreases intensity of the non-quantal acetylcholine release [105] and, as was recently demonstrated, inhibits activity of acetylcholinesterase [106].

Neurotrophins. Properly speaking, this is a common name of a group of secreted proteins supporting survivability of neurons, and stimulating their development and activity. It was found that a wide range of neurotrophins, namely, *nerve growth factor (NGF)*, *brain-derived neurotrophic factor (BDNF)*, *glial-cell-line-derived neurotrophic factor (GDNF)*, *neurotrophin-3 (NT-3)* and *neurotrophin-4 (NT-4)* play an important role in regulation of properties of neuromuscular junction and muscular fiber not only in early ontogenesis [107–109]. For example, BDNF, NT-3 and NT-4 are expressed both in motor neurons and in muscle fibers, while GDNF in Schwann cells and in muscle fibers. All the listed proteins are released into a synaptic cleft. Receptors to all these factors are found in a mature neuromuscular junction. They were shown to participate in the regulation of neuromuscular transmission, for example, due to affecting the processes of acetylcholine release [107, 109].

Thus, a neuromuscular junction is a complex morpho-functional structure with numerous intercellular

signaling pathways between a motor neuron, glial cell, and muscle fiber that promotes a signal transmission safety and possibility of its fine tuning.

REGULATION MECHANISMS OF CHOLINERGIC TRANSMISSION

Studies of cholinergic neurotransmission are actual first of all in the context of fundamental aspects of the regulation process. Numerous data were obtained on the neuromuscular junction model. These data base the current concepts of so called *synaptic plasticity*. The term implies the processes leading to alterations of responses in postsynaptic cells during impulse activity. In accordance with the presynaptic activity, as well as activation of one or another signaling pathway, the postsynaptic responses strengthen (*enhancement*) or weaken (*depression*). *Short-term* and *long-term* types of plasticity are distinguished. The first one is based mainly on alterations in kinetics of biochemical and metabolic processes (for a time period from milliseconds to tens minutes), the second one is involves alterations in gene expression and synthesis of new proteins (from several minutes and more, sometimes up to several days). This specification according to a process duration was established after the main attention of neurophysiologists had switched from peripheral synapses to the CNS synapses. Several phenomena were found, including *long-term potentiation (LTP*) and *long-term depression (LTD*) [110]. Neuromuscular synapse is still one of the most convenient subjects for examination of the short-term plasticity and below we review mechanisms of its processes.

Change of a quantal content of synaptic signals. Increase or decrease in amount of the transmitter quanta released in response to an electric impulse is, maybe, one of the most obvious and most examined mechanisms underlying synaptic processes of enhancement or depression, respectively [111, 112]. Increase in a quantal content can be a result of increase in plasma calcium due to: (i) so-called residual calcium, that is, calcium remaining in nerve terminals after the previous (conditioning) stimuli; (ii) modulation of calcium channels activity in a plasma membrane (increase in time of open channel or involvement of previously "silent" channels); (iii) inhibition of calcium-sequencing systems activity and vice versa; (iv) stimulation of calcium release from the intracellular stores. In addition, enhancement of transmitter quanta release can be a result of both involvement of previously "silent" active zones and increase in pool of vesicles ready for release. A decrease in the number of vesicles just from this pool is considered as the main cause of a quantal content decrease. Since an evoked quantal release is calcium dependent, any effect (signaling pathway) decreasing intracellular calcium or preventing its interaction with the calcium-activated proteins of exocytosis will lead to a decrease in the quantal content of an evoked response.

Change of a quantum size. A term "quantum" implies nondivisibility of a portion, nevertheless, an essential body of data telling that a transmitter amount in a quantum can vary both downwards and upwards is accumulated by now [13, 43]. The degree of vesicle occupation by transmitter can depend on: (i) concentration of the transmitter in the cytoplasm; (ii) activity of ATPase creating a proton gradient at a vesicle membrane; (iii) amount and activity of vesicular transporters pumping a transmitter.

Change of synchrony in transmitter quanta release. One more thesis of a quantal-vesicular theory underlying a concept of the evoked quantal release is revised now: synchrony of a transmitter portion release in response to an electrical stimulus. Thorough analysis of the evoked release showed that independent quanta forming a postsynaptic potential in response to a single stimulus are released not simultaneously. This can be proved by a considerable variability in the real synaptic delay of separate quanta released after nerve stimulation. It was proven that variability of time period, after which quanta of transmitter are released (degree of asynchronism), can be altered at activation of certain signaling pathways affecting, this way or another, calcium metabolism in a nerve ending. As a result, at the same quantal content and increase in release asynchrony, amplitude of postsynaptic response decreases and, vice versa, increase in degree of synchrony of transmitter quanta release leads to increase in a signal amplitude in an innervated cell. Today, variation of kinetics of a transmitter release is considered as a separate mechanism of the neurotransmission regulation [113, 114].

Variation of acetylcholinesterase activity. Inhibition of activity of an enzyme, which hydrolyzes acetylcholine, leads to increase in a number of repeated bindings of transmitter molecules with receptor, followed by an essential increase in amplitude and duration of postsynaptic responses. Until quite recently, this mechanism of neurotransmission regulation was considered only in view of pharmacotherapy of myasthenia, an autoimmune disease, associated with a syndrome of pathological muscle fatigability. However, we found an endogenous mechanism of acetylcholinesterase activity inhibition by NO molecules [106], and it let us to consider the process as an independent mechanism enabling the synaptic plasticity.

Change in the of postsynaptical membrane sensitivity to the transmitter. Pathological conditions of a wide spectrum vary density and distribution of acetylcholine receptors [111]. For example, development of *myasthenia gravis* dramatically changes amount of acetylcholine receptors; this is expressed in decrease in postsynaptic responses amplitude. From the other hand, a number of diseases are caused by delay of kinetics of ion channels activity (so called, "*slow channel syndrome*"). These diseases follow mutations in genes coding the subunits of acetylcholine receptors. An essential delay of the decay phase of the postsynap-

tic signal occurs due to a longer open state of the acetylcholine receptor channel. As for the properties of a native receptor, we should mention such process as *desensitization* (essential sensitivity decrease of a receptor exposed to a prolonged effect of a rather high concentration of ligand). Development of this process is followed by the fall of the postsynaptic response amplitude. Phosphorylation of the receptor by certain isoforms of the protein kinase C, in turn, removes it from the desensitized state and its amplitude returns to the norm [115].

In summary, cholinergic neurotransmission has a wide range of mechanisms regulating the signal transmission process by itself. Everything suggests that multiple images and stages of modulation mechanisms enable, from one hand, fine tuning of the synaptic machinery at whole, and, from the other hand, duplication of certain regulating mechanisms thus ensuring a high safety factor of a signal transmission.

CONCLUSIONS

Contacts between a motor nerve and a skeletal muscle fiber are under thorough investigations since 1950s. According to PubMed, about 400–500 articles on physiology of this cholinergic contact are published yearly, since the mid-1970s. The available data suggest a complete revision of an existing simplified concept telling that a neuromuscular junction is an intercellular contact, in which a chemical transmission of an electrical signal from a motor neuron to a muscle fiber initiates a contraction process. At the moment, a neuromuscular junction (and indeed any chemical synapse) should be considered as a quite complex and rather flexible morphofunctional structure, constructed from a wide range of multiloop intercellular signal pathways between a presynaptic neuron ending, innervated cell, and adjacent glial cells. A chemical signal transmission is not limited to the release of only one species of signal molecules in the response to an electrical impulse, and not always completed by an occurrence of a potential in the innervated cell.

New aspects were recently found in functioning of cholinergic system at signal transmission: (i) interaction between the processes of spontaneous quantal, evoked quantal and non-quantal secretion of a transmitter, (ii) determination of a physiological meaning of the spontaneous mediator release, (iii) mechanisms of release and role of the wide range of synaptically active molecules (cotransmitters, modulators, and retrograde transmitters), (iv) mechanisms of synaptic transmission regulation and correction at development of certain diseases, involving disturbances in cholinergic neurotransmission, and some other. Investigation of these problems will be important for understanding of fundamental principles of the brain activity and a search for new approaches to pathogenic therapy of a wide range of diseases.

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