Activation of neuronal nicotinic receptors inhibits acetylcholine release in the neuromuscular junction by increasing Ca2+ flux through Cav1 channels

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Abstract

Background and Purpose: Cholinergic neurotransmission is a key signal pathway in the peripheral nervous system (PNS) and in several branches of the central nervous system (CNS). Despite the fact that it has been studied extensively for a long period of time, some aspects of its regulation still have not yet been established. One is relationship between nicotine-induced autoregulation of acetylcholine (ACh) release with changes in the concentration of presynaptic calcium levels. Experimental Approach: The mouse neuromuscular junction of m. Levator Auris Longus was chosen as the model of the cholinergic synapse. ACh release was assessed by electrophysiological methods. Changes in the calcium transients were recorded using a calcium-sensitive dye. Functional interaction between nicotinic ACh receptors and calcium channels was investigated pharmacologically using specific agonists and antagonists. Key Results: Nicotine hydrogen tartrate salt (considered as a stable form for potential therapeutic delivery of nicotine) effects on the parameters of ACh release from the nerve ending were analyzed. Nicotine application (10 μM) decrease the amount of evoked ACh release, while calcium transient increase in the motor nerve terminal. Both of these effects of nicotine were abolished by the neuronal ACh receptor antagonist dihydro-beta-erythroidine and Cav1 blockers, verapamil and nitrendipine. Conclusion and Implications: Neuronal nicotinic ACh receptors activation decreases the number of ACh quanta released by boosting calcium influx through Cav1 channels. Understanding of mechanisms of autoregulation of ACh release is important for the searching new approaches treat diseases associated with cholinergic dysfunction.

Activation of neuronal nicotinic receptors inhibits acetylcholine release in the neuromuscular junction by increasing Ca^{2+} flux through Ca_v1 channels

Running title: Neuronal nAChRs and Cav1 channels in regulation of ACh release

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Author contributions

N.Z. and D.S. conceived and designed research; N.Z. and A. Yu. performed experiments; N.Z., A. Yu, A.M. and D.S. did the data analysis and interpretation. N.Z., D.S and A.M. drafted the manuscript. All authors approved final version of the manuscript.

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Experimental Approach:

The mouse neuromuscular junction of m. Levator Auris Longus was chosen as the model of the cholinergic synapse. ACh release was assessed by electrophysiological methods. Changes in the calcium transients were recorded using a calcium-sensitive dye. Functional interaction between nicotinic ACh receptors and calcium channels was investigated pharmacologically using specific agonists and antagonists.

Key Results:

Nicotine hydrogen tartrate salt (considered as a stable form for potential therapeutic delivery of nicotine) effects on the parameters of ACh release from the nerve ending were analyzed. Nicotine application (10 μM) decrease the amount of evoked ACh release, while calcium transient increase in the motor nerve terminal. Both of these effects of nicotine were abolished by the neuronal ACh receptor antagonist dihydro-beta-erythroidine and Ca_v1 blockers, verapamil and nitrendipine.

Conclusion and Implications:

Neuronal nicotinic ACh receptors activation decreases the number of ACh quanta released by boosting calcium influx through Ca_v1 channels. Understanding of mechanisms of autoregulation of ACh release is important for the searching new approaches treat diseases associated with cholinergic dysfunction.

Keywords: neuromuscular junction, neurotransmitter release, acetylcholine, nicotinic receptor, calcium channel, calcium transient.

Abbreviations: ACh, acetylcholine; CaM, Calmodulin; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; CNS, central nervous system; DHβE, dihydro-β-erythroidine hydrobromide; DMSO, dimethylsulfoxide; EPP, evoked endplate potentials; LAL, levator auris longus; mEPP, miniature endplate potentials;

NMJ, neuromuscular junction; nNAChRs, neuronal nicotinic acetylcholine receptors; PNS, peripheral nervous system; QC, quantal content; VGCCs, voltage-gated calcium channels

What is already known

Cholinergic neurotransmission is essential for the nervous system functioning.

One of the mechanisms of autoregulation of ACh release is triggered by activation of nNAChRs.

What this study adds

Activation of nNAChRs is accompanied by an increase in the entry of Ca^{2+}.

Autoregulation of ACh release is mediated by modulating of function of VGCCs of Ca_v1 type

What is the clinical significance

Investigation of cholinergic neurotransmission is important for understanding of principles of the nervous system activity.

Our results may be useful for developing approaches to treat diseases associated with cholinergic dysfunction.

Introduction

Acetylcholine (ACh) is the main neurotransmitter in the peripheral nervous system of vertebrates and humans. In particular, it is responsible for the transmission of signals from the motor nerve to skeletal muscle (Del Castillo & Katz, 1957; Ciani & Edwards, 1963). Since neuromuscular junction (NMJ) is a key linker in the initiation of any motor act (from voluntary movement of the limbs to breathing and contraction of the vocal cords), investigation of the regulation of neuromuscular transmission is of great importance for both fundamental neurobiology and applied medicine.

Since the midst of the 20^{th} century, the data began to accumulate indicating that ACh, released in the synaptic cleft from the nerve endings, activates presynaptic cholinergic receptors, thus exerting a modulatory effect on the neurotransmission process by changing the amount and/or dynamics of subsequent portions of neurotransmitter release (Ciani & Edwards, 1963; Starke et al., 1989; Bowman et al., 1990; Prior et al., 1995; Miller, 1998; Nikolsky et al., 2004). Initially pharmacologically, and later by other methods it has been shown that both ionotropic nicotinic and metabotropic muscarinic cholinergic receptors are present in the motor nerve terminal, and activation of these receptors can lead to autoregulation of ACh release (Bowman et al., 1990; Miller, 1990; Santafé et al., 2004).

When studying autoregulation mediated by muscarinic cholinergic receptors, it was found that activation of the M_1-subtype receptors led to facilitation of the release. In contrast, activation of the M_2-subtype caused inhibition of the ACh quanta release (Oliveira et al., 2002; Santafé et al., 2003). Both M_1- and M_2-mediated mechanisms depend on calcium influx (Santafe et al., 2003; Slutsky et al., 2003; Khaziev et al., 2016; Zhilyakov et al., 2019).

Studies of the mechanisms of autoregulation of ACh release mediated by nicotinic cholinergic receptors are complicated by the fact that the predominant population of these proteins is located in the postsynaptic membrane. Their activation is accompanied by depolarization of sarcolemma and subsequent generation of action potential, which ultimately leads to muscle contraction. The data collected by to date indicate that activation of presynaptic nicotinic cholinergic receptors leads to inhibition of the process of ACh release (Van der Kloot, 1993; Prior & Singh, 2000; Balezina et al., 2006).

Also, experimental evidence was obtained indicating possible involvement of voltage-gated calcium channels (VGCCs) of L-type (Ca_v1) in modulation of neurotransmission (Prior & Singh, 2000). Meanwhile, the results of a number of studies demonstrate that neither the Cav1 type nor the N-type (Cav2.2) VGCCs participates in the evoked release of ACh in mammalians mature neuromuscular contacts (Penner & Dreyer, 1986; Atchison, 1989; Protti et al., 1991; Bowersox et al., 1995).

Thus, the question on the role of calcium channels in the mechanisms of regulation of ACh release, mediated by nicotinic cholinergic receptors, remains open as of now.

In the present study, using a pharmacological approach, electrophysiological techniques and the method of optical registration of changes in the calcium level in the motor nerve ending, we made the following observations. An agonist of nicotinic receptors (at a concentration not significantly affecting the state of the postsynaptic membrane) leads to a decrease in the amount of released ACh quanta. This effect is accompanied not by a decrease, but by an increase of calcium ions entry into the motor nerve terminal. Our data suggest that nicotinic cholinergic receptors responsible for the mechanism of ACh release autoregulation are the receptors of neuronal type. Activation of these receptors leads to upregulation of Ca_v1 type of VGCCs, resulting the enhancement of Ca^{2+} entry into the nerve ending.

Methods

Animals

Mice BALB/C (20-23g, 2-3 months old) of either sex were used in this study. All animal care and experimental protocol met the requirements of the European Communities Council Directive 86/609/EEC and was approved by the Ethical Committee of Kazan Medical University. Animals were housed in group of 10 animals divided by gender inside plastic cages with plenty of food (standard mice chow) and water *ad libitum* . The temperature (22degC) of the room was kept constant and 12-h light/dark cycle was imposed. Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the British Journal of Pharmacology (Lilley et al., 2020).

Tissue preparations and solutions

Animals were euthanised by cervical dislocation in accordance with the approved project protocol. Muscle *levator auris longus* (m. LAL) was quickly removed (Angaut-Petit et al., 1987), then put down onto a Sylgard(r) chamber with bubbled (95% O_2 and 5% CO_2) Ringer solution (pH 7.4) containing (in mM): NaCl 135, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, NaH_2PO_4 1, $NaHCO_3$ 11.9, glucose 11. Bath solution temperature was controlled by Peltier semiconductor device. Experiments were performed at 20.0 +- 0.3 degC. Muscle contractions were prevented by using μ-conotoxin GIIIB in 2 μM concentration (Hill et al., 1996).

Electrophysiology

We used standard intracellular recording technique (Thesleff, 1958; Santafé et al., 2003). Microelectrodes were prepared from borosilicate glass (World Precision Instruments, Sarasota, FL, USA) using a P97 micropipette puller (Sutter Instrument, Novato, CA, USA). Recording electrodes were 20-30 MΩ and filled with 3 M KCl. To record evoked and spontaneous (miniature) endplate potentials (EPPs and mEPPs, respectively) amplifier Axoclamp 900A and digitizer DigiData 1440A (Axon Instruments, San Jose, CA, USA) were used. Membrane potential was at -60 to -80 mV and recorded by miniDigi 1B (Axon Instruments, San Jose, CA, USA). The experiments with membrane potential deviations over 7 mV were declined. The nerve was stimulated with rectangular suprathreshold stimuli (0.2 ms duration, 0.5 Hz frequency) *via* a suction electrode connected to an isolated pulse stimulator, model 2100 (A-M Systems, Sequim, WA, USA). All electronic devices were driven by software pClamp v.10.4. Bandwidth was from 1 Hz to 10 kHz. After collecting 35 EPPs, mEPPs during 2 min were recorded. Quantal content estimated as ratio of averaged EPPs to mEPPs amplitude.

Calcium transient recording

Nerve motor endings were loaded with high-affinity calcium-sensitive dye Oregon Green 488 BAPTA-1 Hexapotassium Salt 1 mM (Molecular Probes, Eugene, OR, USA) through the nerve stump, as described previously (Samigullin, Khaziev, Zhilyakov, Sudakov, et al., 2017). The fluorescence signal was recorded using an imaging setup based on an Olympus BX-51 microscope with a x40 water-immersion objective (Tokyo, Japan). Calcium transient registration performed via high-sensitivity Red Shirt Imaging NeuroCCD-smq camera (RedShirtImaging, Decatur, GA, USA), 500 fps (exposure time 2 ms) at 80x80 pixels, what was sufficient for calcium transient registration with good temporal resolution. As the source of light Polychrome V

(Till Photonics, Munich, Germany) was used, with set light wavelength 488 nm. The following filter set was used to isolate the fluorescent signal: 505DCXT dichroic mirror, E520LP emission (Chroma, Bellows Falls, VT, USA). We used Turbo-SM software (RedShirtImaging, Decatur, GA, USA) for data recording. In each experiment, 8 fluorescence responses were recorded then averaged. This was an optimal amount to obtain the data of sufficient quality and to reduce excitotoxicity and photobleaching of fluorophore.

To analyze recorded images ImageJ software (NIH, Bethesda, MD, USA) was used. We picked regions of interest in motor nerve ending image and background manually. Subsequent data processing was performed in Excel (Microsoft, Redmond, WA, USA). Background values were averaged and subtracted from signal ones. Data were represented as a ratio: $(\Delta F/F0 - 1) \times 100$ %, where ΔF is the fluorescence intensity during stimulation, F0 is the fluorescence intensity at rest (Samigullin, Khaziev, Zhilyakov, Bukharaeva, et al., 2017).

Materials

Nicotine hydrogen tartrate salt (nicotine),*nitrendipine*,*verapamil*,*ω-αγατοξιν ΙΆ*, cadmium chloride, dimethylsulfoxide (DMSO) were obtained from Sigma Aldrich (St. Louis, MO, USA). Dihydro-β-erythroidine hydrobromide (*ΔΗβΕ*) (TOCRIS, Bristol, UK), μ-conotoxinGIIIB (Peptide institute Inc., Osaka, Japan). Drugs were dissolved in distilled water with the exceptions of nitrendipine and verapamil which were dissolved in DMSO. Further dilutions for all drugs were done in Ringer solution. In experiments with drugs which were dissolved in DMSO, the same concentration of DMSO was added to the control solution as was present in the solution with the agent. Finally, the DMSO concentration in the solution did not exceed 0.01%.

Data and statistical analysis

Data collection and statistical analysis comply with the recommendations of the British Journal of Pharmacology on experimental design and analysis in pharmacology (Curtis et al., 2018). The number of experiments in each experimental group was selected on the basis of observing a statistically significant effect while using the minimum number of animals (3R principles) and on the experience from the previous studies. Animals were randomly assigned in the different experimental groups with each group having the same number of animals by design. Blinding of the operator was not feasible, but data analysis was performed semi-blinded by an independent analyst.

Statistical analysis was performed using Statistica 6.1 Base (Tulsa, OK, USA). Shapiro–Wilk normality test was used to analyze the data distribution. Null-hypothesis testing was performed by ANOVA. One-way ANOVA followed by Dunnett's or Tukey's test for multiple comparison post hoc was used. For related groups one-way repeated ANOVA test was performed. Data are presented as mean ± SEM. Values of $P < 0.05$ were considered significant.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY (http://www.guidetopharmacology.org) and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander et al., 2019).

Results

Effects of nicotine on the electrophysiological parameters of the neuromuscular junction

Using the intracellular microelectrode technique, we recorded the resting membrane potential (RMP) of the muscle fiber, amplitude of miniature endplate potentials (mEPPs), frequency of occurrence of mEPPs and the amplitude of the evoked potentials of the end plate (EPPs).

Alterations in RMP and MEPP amplitude indicate the postsynaptic action of the pharmacological agent. While changes in the frequency of occurrence of mEPPs suggest presynaptic action of the drug. The EPP amplitude, in turn, can vary due to changes at both pre- and post-synaptic levels. Therefore, it was necessary

to assess the effect of nicotine on every parameter mentioned above to determine the optimal effective concentration of nicotine to study the autoregulation.

Control RMP value of muscle fibers was -71.48 ± 0.77 mV (n = 5, 30 NMJs). Application of nicotine at concentrations of 0.1 μM, 1 and 5 μM did not affect the RMP significantly, providing the value of -69.72 ± 0.90 mV (n = 5, 30 NMJs), -70.91 ± 0.78 mV (n = 5, 30 NMJs), and -70.85 ± 0.87 mV (n = 5, 30 NMJs), respectively (Figure 1A). A slight significant depolarization was observed when nicotine concentration was increased to 10 μM (-67.04 ± 0.79 mV; n = 5, 30 NMJs); at a concentration of 50 μM a more pronounced depolarization was observed and the mean RMP value decreased to -56.94 ± 1.29 mV (n = 5, 30 NMJs; Figure 1A).

Another sign of postsynaptic action of nicotine was the change in the amplitude of mEPP. The mean value of the amplitude of the spontaneous signal in control was 0.88 ± 0.05 mV (n = 5, 30 NMJs). Application of nicotine in concentration up to 10 μM did not affect mEPP amplitude significantly; the value was: 0.83 ± 0.04 mV (n = 5, 30 NMJs) for 0.1 μM, 0.84 ±0.05 mV (n = 5, 30 NMJs) for 1 μM, 0.96 ± 0.06 mV (n = 5, 30 NMJs) for 5 μM, and 0.83 ± 0.05 mV (n = 5, 30 NMJs) for 10 μM (Figure 1B). Significant decrease in the mEPPs amplitude to 0.55 ±. 0.04 mV (n = 5, 30 NMJs) was observed only with 50 μM nicotine (Figure 1B).

In contrast to the amplitude of mEPPs, the effect of nicotine on the frequency of occurrence of spontaneous signals was detected at significantly lower concentrations. That is, the average value of the frequency of mEPPs upon application of 0.1 and 1 μM nicotine was 1.64 ± 0.15 Hz (n = 5, 30 NMJs) and 1.22 ± 0.12 Hz (n = 5, 30 NMJs), respectively, and did not differ from the control value of 1.57 ± 0.14 Hz; n = 5, 30 NMJs (Figure 1C). After application of 5 μM nicotine the frequency significantly decreased to 1.07 ± 0.08 Hz (n = 5, 30 NMJs) and inhibition was further enhanced to 0.98 ± 0.07 Hz, n = 5, for 10 μM and 0.57 ± 0.06 Hz, n = 5, for 50 μM (Figure 1C).

The amplitude of the EPP, which reflects the level of evoked ACh release and depends on changes in the sensitivity of the postsynaptic membrane in the area of the neuromuscular contact, was 32.80 ± 1.02 mV (n = 5, 30 NMJs) in control. Application of nicotine at concentrations of 0.1 μM, 1 and 5 μM did not alter the average amplitude of EPP, which was equal to 31.93 ± 1.21 mV (n = 5, 30 NMJs), 32.43 ± 1.11 mV (n = 5, 30 NMJs), and 32.33 ± 1.18 mV (n = 5, 30 NMJs), respectively (Figure 1D). However, nicotine produced a decrease in EPPs amplitude, starting at the concentration of 10 μM (28.36 ± 1.27 mV; n = 5, 30 NMJs), while at 50 μM the amplitude decreased almost twofold to 16.89 ± 1.09 mV (n = 5, 30 NMJs; Figure 1D).

Thus, for further investigations of the ACh release autoregulation mechanisms, concentration of 10 μM nicotine was chosen. When using nicotine at this concentration, a decrease in the EPP amplitude was observable, while there were no changes in the mEPP amplitude (with only a slight depolarization of the sarcolemma).

Activation of neuronal nicotinic receptors leads to downregulation of the EPP quantal content

Under control conditions, the quantal content (QC) was 46.8 ± 4.5. The bath application of nicotine (10 μM) decreased the QC of EPP significantly by 12.0 ± 4.4% (n = 5, 7 NMJs; Figure 2).

The nicotine-induced decrease in the number of ACh quanta released in response to stimulation of the motor nerve suggests the involvement of presynaptic cholinergic receptors. Using the antagonist of neuronal nicotinic ACh receptors (nNAChRs) DHβE (Moroni et al., 2006; Wonnacott, 2014), we obtained the data supporting this suggestion. Application of DHβE alone at a concentration of 1 μM did not change the QC of EPP (101.5 ± 1.3%; n = 6, 9 NMJs, Figure 2), however, after the pretreatment with DHβE, the inhibitory effect of nicotine on the quantal release of ACh was completely abolished (105.5 ± 6.6%; n = 6, 9 NMJs; Figure 2).

Activation of neuronal nicotinic receptors induces an increase of calcium level in motor nerve terminal

Since the process of evoked release of a neurotransmitter is triggered by the entry of calcium ions into the nerve ending (Katz B., 1969; Crawford, 1974), it was suggested that the inhibitory effect of nicotine on the ACh release could be related to a decrease in Ca^{2+} influx.

The amplitude changes of the optical signal ($\Delta F/F0$) from the calcium dye loaded into the nerve terminal in response to a single stimulus (with the same characteristics as during EPP registration) averaged about 30% (Figure 3). Nicotine application did not lead to a decrease, as expected, but caused a significant increase in the amplitude of the calcium transient by 13.7 ± 4.3% (n = 5, 8 NMJs; Figure 4). Thus, in the presence of a nicotinic receptor agonist, the presynaptic calcium level in response to nerve stimulation increases more strongly than in its absence. Is this increase indeed triggered by nNAChRs, which activation of leads to a decrease in subsequent ACh release? The answer to this question was obtained in the experiments with an antagonist of nicotinic receptors, DHβE.

Application of the antagonist alone led to a decrease in the calcium transient significantly by 12.9 ± 1.5% (n = 5, 15 NMJs; Figure 4), however, after pretreatment with DHβE, the calcium signal-enhancing effect of nicotine was completely abolished (100.0 ± 0.8%; n = 5; 15 NMJs; Figure 4).

Neuronal nicotinic receptors alter calcium level in presynaptic terminal by gating L-type (Ca_v1) calcium channels

To identify the source of the increase in the calcium signal upon activation of presynaptic nNAChRs, a nonselective blocker of calcium-permeable channels, cadmium chloride at a concentration of 10 μM, was used. After application of cadmium chloride, a decrease in the amplitude of the calcium transient was observed by 54.5 ± 2.7% (n = 5, 9 NMJs). In the presence of cadmium the effect of nicotine on the alterations in calcium levels was completely abolished (101.4 ± 3.4%; n = 5, 9 NMJs; Figure 5). Therefore, the observed increase in the presynaptic calcium level upon activation of nNAChRs is mediated by proteins (channels) which are permeable for Ca^{2+}. Further experiments were carried out to establish which type of VGCCs is involved in nicotine-induced increases in calcium transients.

Application of the specific P/Q-type ($Ca_v2.1$) VGCCs blocker ω-agatoxin IVA at a concentration of 40 nM that blocks only a certain proportion of channels (Protti & Uchitel, 1993) led to a significant decrease in the calcium transient by 67 ± 4.4 % (n = 5, 5 NMJs; Figure 5). In case of partial blockade of the main type of VGCCs $Ca_v2.1$, nicotine application (10 μM) led to an increase in the amplitude of the calcium transient significantly by 29.9 ± 3.8% (n = 5, 10 NMJs, Figure 5). Therefore, the effect of activation of nNAChRs on the intracellular calcium level is not mediated by $Ca_v2.1$ channels.

Ca_v1 calcium channel blockers such as verapamil (50 μM) and nitrendipine (25 μM), produced significant calcium transient decrease by 25 ± 4.4% (n = 5, 9 NMJs) and 18.8 ± 1.1% (n = 5, 17 NMJs), respectively (Figure 6). Application of nicotine after pretreatment by these blockers did not cause any changes in the calcium transient: the amplitudes were 101.8 ± 1.5% (n = 5, 9 NMJs) and 100.7 ± 1.2% (n = 5, 17 NMJs), respectively (Figure 6). These data allow us to conclude that observed increase in the calcium level in the nerve ending upon activation of nNAChRs by an exogenous agonist is due to mediation by Ca_v1 type VGCCs. Therefore, the phenomenon of endogenous activation of presynaptic cholinergic receptors discovered by us should also be mediated by calcium channels of this type. Indeed, the calcium transient-reducing effect of DHβE, when Ca_v1 channels were blocked by nitrendipine, was completely abolished (100.0 ± 0.8%; n = 5, 7 NMJs; Figure 6).

Thus, the results obtained allow us to conclude that activation of nNAChRs leads to an additional increase in the entry of Ca^{2+} into the nerve ending through VGCCs of the Ca_v1 type. Therefore, if this is the mechanism underlying the decrease in the quantal content upon activation of this type of cholinergic receptors, then it should be expected that the blockade of Ca_v1 type of calcium channels will eliminate the nicotine-induced decrease in the amount of released ACh quanta. Examining this assumption became the scope of the next step of the study.

Nicotine-induced decrease in acetylcholine release mediated by L-type (Ca_v1) calcium channels

To assess the possible role of Ca_v1 type of calcium channels in the nicotine-induced mechanism of ACh release autoinhibition, verapamil and nitrendipine were used at the same concentrations as in experiments with calcium transients.

Verapamil and nitrendipine application resulted in a significant decrease in the QC by 14.2 ± 3.2% (n = 5, 6 NMJs) and 11.2 ± 2.9% (n = 5, 6 NMJs), respectively (Figure 7). Nicotine application after pre-treatment with Ca_v1 channel blockers did not cause any changes in the evoked ACh release, and the QC was 101.6 ± 1.9% (n = 5, 6 NMJs) and 103.8 ± 2.4% (n = 5, 6 NMJs), respectively (Figure 7).

Discussion

Effects of nicotine on ACh release

The results of our study demonstrate that in the mouse neuromuscular preparation of m.LAL, nicotine at concentrations up to 1 μM has no effect either on the processes of ACh release from the nerve terminal, or on the processes of its interaction with the postsynaptic membrane. At the concentration of 5 μM, the presynaptic effect of nicotine appears to become detectable (inhibition of the spontaneous release of ACh due to the activation of presynaptic cholinergic receptors). An increase in concentration to 10 μM enhances the presynaptic effect of the alkaloid (suppression of not only spontaneous, but also of the evoked ACh release) and leads to a weak postsynaptic effect (decrease in RMP due to activation of postsynaptic cholinergic receptors). With an increase in nicotine concentration to 50 μM, dramatic changes become evident in all recorded parameters of neurotransmission.

Thus, nicotine at a concentration of 10 μM exerts both postsynaptic and presynaptic inhibitory effects on the neuromuscular synapse, in particular, causing a decrease in the number of ACh quanta released in response to action potential. Similar decrease in the QC during activation of cholinergic receptors has been noted earlier (Tian et al., 1994; Prior & Singh, 2000; Balezina et al., 2006; Khaziev et al., 2016), however, these results were obtained on other preparations and in conditions of initially reduced QC, or in cut fiber preparations.

Presynaptic cholinergic receptors and the role of calcium influx in the mechanism of ACh release autoinhibition

Since the inhibitory effect of nicotine on the QC was completely abolished by application of the antagonist of neuronal cholinergic receptors DHβE, it was concluded that these receptors are involved in the cholinergic mechanism of regulation of ACh release. DHβE binds to β2 subunits of neuronal receptors and is a selective antagonist for non-α7 nACh receptors (Stauderman et al., 2000). In the heteromeric receptors of ganglionic neurons the primary α subunit is α3, whereas in the rodent central nervous system the primary α subunit is α4 (Papke et al., 2010). The α4β2 nNAChR is the most abundant subtype expressed in the brain, and studies have demonstrated that this receptor subtype is located presynaptically (Karadsheh et al., 2004). Therefore, we can assume that in the neuromuscular synapse neuronal cholinergic receptors have the α4β2 subunit composition. This assumption is supported by the data that at 1 μM DHβE, that we used in this study, the mouse α4β2 receptors are almost completely blocked, while the α3β4 subunit receptors remain essentially active (Papke et al., 2010).

In the next step of the mechanism of autoinhibition triggered by nNAChRs, it was necessary to answer the following key question: how is the activation of the presynaptic nicotinic receptors coupled to changes in the intracellular calcium level? Previous data (Tian et al., 1994; Prior & Singh, 2000; Wang et al., 2018) were indicating such a coupling, but there was no direct evidence found for this prior to our study. Using of standard electrophysiological methods, combined with the fluorescent method for registration of calcium transients, which reflect changes in the calcium level within the presynaptic terminal upon action potential arrival, enabled us to obtain the data on changes in the ACh. Our results demonstrate that activation of nNAChRs (sensitive to DHβE), leading to a decrease in ACh release, is accompanied by an increase in the level of calcium in the nerve terminal. Another important observation made was a significant effect of DHβE on the amplitude of the calcium transient when applied alone. This may indicate that there exists a background tonic activation of nNAChRs which results in a tonic increase in calcium entry into the nerve

terminal.

The complete absence of the effect of nicotine on the calcium signal after pre-treatment with cadmium (10 μM), which is a nonselective blocker of all types of calcium Ca_v channels (Hess et al., 1984) allows two conclusions to be put forward: (i) the increase in calcium level in the nerve terminal is mediated by transmembrane proteins permeable for Ca^{2+} from the environment; (ii) the observed entry of Ca^{2+} is mediated by channels other than those of nNAChRs. The last suggestion is very important, because it has been shown earlier that nNAChRs are more permeable to Ca^{2+} as compared to permeability of their muscle-type counterparts (Radford Deckera & Dani, 1990; Gotti & Clementi, 2004). At the same time, it was shown that cadmium up to a concentration of 200 μM does not block currents through nNAChRs (Wheeler et al., 2006), but significantly blocks currents through VGCCs (Lansman et al., 1986; Samigullin, Khaziev, Zhilyakov, Sudakov, et al., 2017).

After inactivation of VGCCs of $Ca_v2.1$ type, which are key to triggering the process of evoked ACh release (Nachshen & Blaustein, 1979; Protti & Uchitel, 1993; Katz et al., 1996) the effect of nicotine on calcium entry into the terminal was preserved, while after blockade of Ca_v1 type channels, it was completely abolished. It should be noted that the possibility of the involvement of these channels in the evoked release of ACh quanta remained under debate until recent times: (Prior & Singh, 2000; Urbano et al., 2002; Pagani et al., 2004; Perissinotti et al., 2008) *versus* (Penner & Dreyer, 1986; Atchison, 1989; Protti et al., 1991; Bowersox et al., 1995). We have obtained a clear evidence of the involvement of the Ca_v1 type of calcium channels in regulation of bulk calcium level in the terminal and of process of neurotransmission in the mammalian neuromuscular junction.

Critical issues in establishing the coupling between nNAChRs and L-type (Ca_v1) calcium channels while using a pharmacological approach

The phenylalkylamine (verapamil), dihydropyridine (nitrendipine) and benzothiazepine classes of Ca_v1 type calcium channel blockers are capable of blocking nNAChRs (Houlihan et al., 2000; Wheeler et al., 2006). The absence of the effects of nicotine (both on the calcium transient and on the QC) after the application of verapamil and nitrendipine may well be related to simple direct blockade of nNAChRs (sensitive to DHβE and permeable to Ca^{2+}). Indeed, DHβE, verapamil and nitrendipine all lead to a decrease in the transients. At the same time, all three pharmacological agents abolish the effect of nicotine.

If even direct block of nNAChRs by verapamil and nitrendipine does take place, a number of additional facts still point to the involvement of the Ca_v1 calcium channels in the mechanism of modulation of calcium entry and the process of ACh release in the nerve terminal. So, after application of all three agents, the calcium entry decreases, however, the effect of verapamil and nitrendipine is almost two times more pronounced than that of DHβE. Further on, the QC does not change after the addition of DHβE, whereas in the presence of verapamil and nitrendipine it is decreased by more than 10%. In addition, activation of cholinergic receptors (by nicotine) and presumed blockade of cholinergic receptors (by verapamil and nitrendipine) have not an opposite, but a unidirectional effect - a decrease in the QC. And the last but not the least, the absence of the effect of nicotine on the calcium transient after application of cadmium, which does not affect the functioning of nNAChRs (including α4β2 nNAChRs) or even potentiates them (Wheeler et al., 2006; Garduño et al., 2012), indicates that in our case a pharmacological effect on two different targets takes place.

How activation of nNAChRs modulates the L-type (Ca_v1) channel functioning

nNAChRs have a high Ca^{2+} permeability (Gotti & Clementi, 2004), which does not have direct effect on the calcium transient amplitude. However, the calcium entry through these receptors can lead to two potential outcomes and one can observe an increase in the amplitude of the calcium transient as a result of two mechanisms: i) the CDF process (calcium dependent facilitation) of the Ca_v1 type channel is triggered (Kim et al., 2008) (Figure 8a) ; ii) the CDI (calcium dependent inactivation) process of Ca_v1 type calcium channels, mediated by the interaction between CaM and Ca^{2+} channel, is disrupted by increasing CaMKII activity (Abiria & Colbran, 2010) (Figure 8b) . However, according to (Prior & Singh, 2000), decrease in ACh release caused by activation of nNAChRs is not associated with CaM.

The existence of a functional interaction between nNAChRs and the channels of the Ca_v1 type was established in primary culture of neurons in the mouse cerebral cortex (Katsura et al., 2002). It should be noted that this work revealed the interaction of calcium channels with ∝4β2 nNAChRs (Potentially, a similar interaction takes place in the muscle-nerve junction). The authors believe that activation of presynaptic receptors leads to depolarization sufficient for opening of Ca_v1 calcium channels and entry of calcium into the neuron (Katsura et al., 2002) (Figure 8c) .

The results obtained on the neuromuscular preparation suggest that the Ca_v1 type of calcium channels is located far from the active zone (Polo-Parada et al., 2001), therefore, Ca^{2+} entering through them cannot directly interact with the exocytosis machine. It has been shown that there is a functional interaction between the Ca_v1 type and Ca^{2+}-activated $K^+(K_{Ca})$ channels (Flink et al., 2003).

We hypothesize that nNAChRs, Ca_v1 type calcium channels, and K_{Ca} channels can form a cluster on the presynaptic membrane. Activation of ones starts the process of autoinhibition of ACh release. In this case, entering Ca^{2+} through Ca_v1 type channels can activate K_{Ca}channels, which leads to membrane hyperpolarization and, as a consequence, a decrease in the amount of released neurotransmitter quanta. However, this hypothesis will need to be proven in further research.

Conclusion

In the present study we found that activation of presynaptic nNAChRs leads to a decrease in the quantal ACh release from the nerve ending. This negative feedback mechanism is mediated by modulating of function of VGCCs of Ca_v1 type, which leads to an increase in the entry of Ca^{2+} into the nerve terminal (Figure 9). Understanding of peculiarities of action of ACh (nicotine) on the nNAChR-containing nerve endings (not only in cholinergic synapses (Seth et al., 2002; Garduño et al., 2012)) has of broad scientific and clinical significance, since cholinergic nicotinic signaling (in addition to neuromuscular transmission and synaptic transmission in ganglia) is involved in the setting of a variety of processes, including anxiety, depression, arousal, memory, and attention (Hogg et al., 2003; Picciotto, 2003).

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Figure legends

Figure 1. Effects of nicotine on the electrophysiological parameters registered at the mouse neuromuscular junction. Changes in absolute values are shown (a) resting membrane potential (RMP) of muscle fibers, (b) amplitudes of miniature endplate potentials (mEPP), (c) the frequency of the mEPPs and (d) amplitudes of evoked endplate potentials (EPP) in control and 15 min after nicotine application (the range from 0.1 to 50 μM). Results are expressed as mean ± SEM of five independent experiments. Asterisk (*) indicates significant effect ($P< 0.05$, one-way ANOVA test with Dunnet's post-hoc comparison).

Figure 2. Nicotine inhibits evoked release of ACh quanta (quantal content, QC) by activating of nNAChRs. Panels on the top are representative traces of EPP and mEPP (50 signals averaged) in separate experiments with nicotine application (Nic, 10 μM; a) and nicotine application after pretreatment with the neuronal cholinergic receptor antagonist DHβE (1 μM; b). (c) Results are expressed as mean ± SEM and SD of QC, as percentage with nicotine (n = 5, 7 NMJs), DHβE (n = 6, 9 NMJs) and DHβE + nicotine (n = 6, 9 NMJs) applications versus control. Asterisk (*) indicates significant effect ($P < 0.05$, one-way repeated ANOVA test with Tukey's post-hoc comparison).

Figure 3. Pseudo-color calcium images of a motor nerve terminal loaded with Oregon Green 488 BAPTA-1 Hexapotassium Salt. The axon is imaged before and during single electrical stimulus (0.2 ms duration). Bar is 20 μm.

Figure 4. Nicotine increases the calcium transient in the motor nerve ending by activation of neuronal ACh receptors; blockade of the receptors leads to a decrease in the amplitude of the calcium signal. Panels on the top are representative traces of calcium transient from separate experiments with nicotine application (Nic, 10 μM; a) and nicotine application after pretreatment with nNAChRs antagonist DHβE (1 μM; b). (c) mean ± SEM and SD of the amplitude of the calcium signal, expressed as a percentage of control when applying nicotine (n = 5, 8 NMJs), DHβE (n = 5, 15 NMJs) and DHβE + nicotine (n = 5, 15 NMJs). Asterisk (*) indicates significant effect ($P < 0.05$, one-way repeated ANOVA test with Tukey's post-hoc comparison).

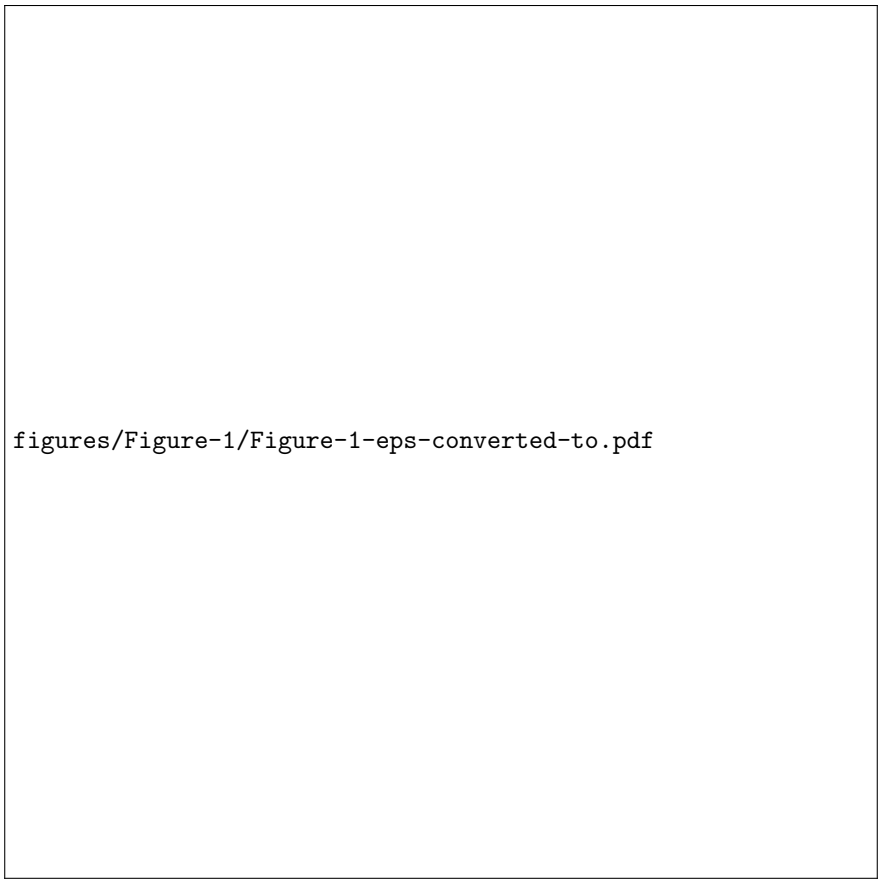
Figure 5. The calcium transient-enhancing effect of nicotine is abolished after nonspecific calcium channel blockade, but not after inhibition of $Ca_v2.1$ type calcium channels. Panels on the top are representative traces of calcium transient from individual experiments: (a) effect of the nonspecific calcium channel blocker $CdCl_2$ (Cd, 10 μM); (b) no effect of nicotine (Nic, 10 μM) after pretreatment with $CdCl_2$; (c) effect of $Ca_v2.1$ VGCCs blocker ω-agatoxin IVA (Aga, 40 nM); and (d) effect of nicotine on the calcium transient after pre-incubation with ω-agatoxin IVA. (e) mean ± SEM and SD of calcium signal amplitudes obtained in the above series and expressed as a percentage of control or value after $CdCl_2$ (n = 5, 9 NMJs), $CdCl_2$+ Nic (n = 5, 9 NMJs), Aga (n = 5, 5 NMJs;) and Aga + Nic (n = 5, 7 NMJs) application. Asterisk (*) indicates significant effect ($P< 0.05$, one-way repeated ANOVA test).

Figure 6. Lack of the effect of nicotine (an increase in the amplitude of the calcium transient) and DHβE (a decrease in the amplitude of the calcium transient) after blockade of the Ca_v1 channels. Panels on the top are representative traces of calcium transient from individual experiments: (a) effect of Ca_v1 calcium channel blocker nitrendipine (Nitre, 25 μM); (b) lack of nicotine (Nic, 10 μM) effect after pre-application of nitrendipine; (c) lack of DHβE (1 μM) effect after nitrendipine pre-treatment; (d) effect of Ca_v1 type VGCCs blocker verapamil (50 μM); (e) no effect of nicotine after pre-application of verapamil. (f) mean ± SEM and SD of calcium signal amplitudes obtained in the above series and expressed as a percentage of control or value after Nitre (n = 5, 17 NMJs), Nitre + Nic (n = 5, 17 NMJs), Nitre + DHβE (n = 5, 7 NMJs), verapamil (n = 5, 9 NMJs) and verapamil +Nic (n = 5, 9 NMJs) application (five independent experiments). Asterisk (*) indicates significant effect ($P< 0.05$, one-way repeated ANOVA test).

Figure 7. Nicotine-induced decrease in the ACh release (quantal content, QC) involves L-type Ca_v1 channels. Panels on the top are representative traces of EPP and mEPP (50 signals averaged) from individual experiments: (a and b) lack of nicotine (Nic, 10 μM) effect after pre-application with Ca_v1 VGCCs blockers nitrendipine (Nitre, 25 μM) and verapamil (50 μM); (c) mean ± SEM and SD of QC, expressed as a percentage of control or value after Nitre (n = 5, 6 NMJs), Nitre +Nic (n = 5, 6 NMJs), Verapamil (n = 5, 6 NMJs) and Verapamil + Nic (n = 5, 6 NMJs) application (five independent experiments). Asterisk (*) indicates significant effect ($P< 0.05$, one-way repeated ANOVA test with Tukey's post-hoc comparison).

Figure 8 - Schematic drawing showing possible mechanisms of the coupling between nAChRs and L-type (Ca_v1) calcium channels in the motor nerve terminal. (a) Calcium dependent facilitation of the Ca_v1 type channel, mediated by CaM and triggered by calcium (Kim et al., 2008), which enters through nNAChR; (b) calcium dependent inactivation process of Ca_v1 type calcium channels, mediated by interaction between CaM and Ca^{2+}channel, is disrupted by an increase in CaMKII activity (Abiria & Colbran, 2010); (c) opening of Ca_v1 calcium channels and entry of calcium into terminal caused by depolarization due to activation of neuronal AChRs (Katsura et al., 2002).

Figure 9. - Working model of the mechanism of autoregulation of ACh release in the peripheral cholinergic synapse via nNAChRs. Activation of nNAChRs is accompanied by an increase in the entry of calcium ions into the motor nerve terminal through the L-type (Cav1) calcium channels. Latter are involved in both the process of evoked ACh release and its modulation.



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