EJN fens EUROPEAN JOURNAL OF NEUROSCIENCE

European Journal of Neuroscience, pp. 1–9, 2012 doi:10.1111/ejn.12029

Regulation of acetylcholinesterase activity by nitric oxide in rat neuromuscular junction via *N*-methyl-D-aspartate receptor activation

Konstantin A. Petrov,[1,2] Artem I. Malomouzh,[1] Irina V. Kovyazina,[1] Eric Krejci,[3] Alexandra D. Nikitashina,[1,2,4] Svetlana E. Proskurina,[4] Vladimir V. Zobov[2,4] and Evgeny E. Nikolsky[1,5]

[1]Kazan Institute of Biochemistry and Biophysics, Russian Academy of Sciences, PO Box 30, Kazan, 420111, Russia
[2]A.E. Arbuzov Institute of Organic and Physical Chemistry, Russian Academy of Sciences, Kazan, Russia
[3]Centre d'Etude de la Sensori-Motricité (CESeM), Université Paris Descartes, CNRS, UMR8194, Paris, France
[4]Kazan State University, Kazan, Russia
[5]Kazan State Medical University, Kazan, Russia

Keywords: acetylcholine, glutamate, mammalian cholinergic synapse, nitric oxide synthase

Abstract

Acetylcholinesterase (AChE) is an enzyme that hydrolyses the neurotransmitter acetylcholine, thereby limiting spillover and duration of action. This study demonstrates the existence of an endogenous mechanism for the regulation of synaptic AChE activity. At the rat *extensor digitorum longus* neuromuscular junction, activation of *N*-methyl-D-aspartate (NMDA) receptors by combined application of glutamate and glycine led to enhancement of nitric oxide (NO) production, resulting in partial AChE inhibition. Partial AChE inhibition was measured using increases in miniature endplate current amplitude. AChE inhibition by paraoxon, inactivation of NO synthase by N^{ω}-nitro-L-arginine methyl ester, and NMDA receptor blockade by DL-2-amino-5-phosphopentanoic acid prevented the increase in miniature endplate current amplitude caused by amino acids. High-frequency (10 Hz) motor nerve stimulation in a glycine-containing bathing solution also resulted in an increase in the amplitude of miniature endplate currents recorded during the interstimulus intervals. Pretreatment with an NO synthase inhibitor and NMDA receptor blockade fully eliminated this effect. This suggests that endogenous glutamate, released into the synaptic cleft as a co-mediator of acetylcholine, is capable of triggering the NMDA receptor/NO synthase-mediated pathway that modulates synaptic AChE activity. Therefore, in addition to well-established modes of synaptic plasticity (e.g. changes in the effectiveness of neurotransmitter release and/or the sensitivity of the postsynaptic membrane), another mechanism exists based on the prompt regulation of AChE activity.

Introduction

In contrast to glutamatergic, dopaminergic, noradrenergic, and other synapses where the neurotransmitter lifespan in the synaptic cleft is limited by diffusion or uptake, to terminate acetylcholine action, cholinergic synapses employ the enzyme acetylcholinesterase (AChE) (Katz & Miledi, 1973). To a large extent, AChE activity determines the lifespan of acetylcholine in the synaptic cleft and, thereby, the amplitude and duration of postsynaptic potentials. Therefore, AChE inhibition by different exogenous compounds is widely used in medical practice when the safety factor for synaptic transmission is reduced (myasthenia gravis, Alzheimer's disease, etc.).

It is known that the density of AChE in the synaptic cleft is not constant and can adapt to changes in synaptic activity (Rotundo, 2003; Rotundo *et al.*, 2008). The rate of AChE synthesis and therefore the amount of the enzyme in the cleft may depend on the pattern of neuronal firing (Pregelj *et al.*, 2007). However, all established mechanisms of AChE regulation require dozens of hours, as synthesis, secretion, and anchoring of the enzyme are required for activity. Ambient changes and modulation of physiological AChE activity patterns can occur rapidly. Therefore, it is likely that faster regulatory pathways for AChE activity (either activation or inhibition) exist.

Recently, spermine NONOate, an equimolar donor of nitric oxide (NO), was shown to inhibit the activity of cortical AChE (Udayabanu *et al.*, 2008), suggesting that NO could be an endogenous AChE modulator. At the neuromuscular junction, NO participates in modulation of acetylcholine release from motor nerve endings (Mukhtarov *et al.*, 2000; Malomouzh *et al.*, 2003; Pinard & Robitaille, 2008). Specifically, some studies (Grozdanovic & Gossrau, 1998; Luck *et al.*, 2000) have shown that NO synthase is coupled to glutamate *N*-methyl-D-aspartate (NMDA) receptors at the neuromuscular junction. Activation of NMDA receptors results in calcium ion influx, which is necessary for the enzymatic synthesis of NO. One previous study demonstrated the precise postsynaptic localization of NMDA receptors in the *extensor digitorum longus*, *soleus*

Correspondence: Konstantin A. Petrov, [1]Kazan Institute of Biochemistry and Biophysics, as above.
E-mail: kpetrov2005@mail.ru

Received 31 May 2012, revised 14 September 2012, accepted 20 September 2012

muscle, and the diaphragm (Malomouzh *et al.*, 2011). Due to this coupling with NMDA receptors, enhanced synaptic activity (e.g. rhythmic high-frequency stimulation) may result in the accumulation of glutamate, a co-mediator of acetylcholine, in the synaptic cleft. In addition to modulation of quantal (Pinard *et al.*, 2003) and non-quantal (Malomouzh *et al.*, 2003) acetylcholine secretions, glutamate can act as a depressant of synaptic AChE activity.

The purpose of this study was to evaluate the putative role of NO in the regulation of AChE activity at the mammalian neuromuscular junction. As NO-mediated processes are likely more evident in muscle with greater NO synthase activity (Kobzik *et al.*, 1994; Hussain *et al.*, 1997; Kapur *et al.*, 1997), all experiments were performed using fast-twitch *extensor digitorum longus* (EDL) muscle fibres. The results demonstrated that the NO donor, *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP), depresses total AChE activity in EDL homogenates. In EDL motor endplates, SNAP application resulted in alterations of miniature endplate currents (mEPCs) (i.e. changes in amplitude and temporal parameters) typical for AChE inhibition. Furthermore, enhancement of endogenous NO production, via NMDA receptor activation, causes partial AChE inhibition in the rat neuromuscular junction.

Materials and methods

Preparations and solutions

Male Wistar rats (200–250 g body weight) were used for all experiments. The animals were kept in sawdust-lined plastic cages in a well-ventilated room. A standard diet and water were available at all times. The experiments were carried out in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC) and the experimental protocol was approved by the Animal Care and Use Committee of Kazan State Medical University. Experiments were performed on isolated nerve–muscle preparations of the EDL excised from ether-anaesthetized rats. Isolated muscles with a nerve stump (length 10–15 mm) were placed into a chamber and superfused (at a rate of 2–3 mL/min) with oxygenated Ringer–Krebs rat solution with the following composition (in mM): 120.0 NaCl, 5.0 KCl, 2.0 $CaCl_2$, 1.0 $MgCl_2$, 23.0 $NaHCO_3$, 1.0 NaH_2PO_4, and 11.0 glucose. The pH was maintained at 7.2–7.4.

Biochemical estimation of acetylcholinesterase activity

The EDL homogenates were prepared in a Potter homogenizer with 0.05 M Tris-HCl, 1% Tween 20, 1 M NaCl, and 2 mM EDTA at pH 7.0, in a 1 : 4 ratio at 4 °C. The activity of AChE was measured according to the modified Ellman's method (Ellman *et al.*, 1961). Tetra-isopropyl pyrophosphoramide (50 μM) (Koelle *et al.*, 1974) was added to inhibit butyrylcholinesterase. Gergel' & Cederbaum (1997) showed the ability of NO to interfere with Ellman's reaction, causing bleaching (in the absence of AChE). The following protocol was used to evaluate the contribution of bleaching to the estimate of AChE activity obtained using Ellman's method. Muscle homogenates were incubated with substrate (acetylthiocholine) for 5, 10, 15, and 20 min, followed by the addition of 10 μM neostigmine (Sigma), an AChE inhibitor, to stop hydrolysis by AChE. As a blank, 10 μM neostigmine was added at the beginning of the reaction. For all conditions, 0.5 mM 5,5′-dithiobis (2-nitrobenzoic acid) was then added and the absorbance at 412 nm was measured. AChE activity was calculated as the variation in optical density over time.

The NO donor, SNAP (100–500 μM), was also added to the same volume. At 412 nm, the addition of SNAP caused a 3–5% attenuation of absorbance (bleaching) in a concentration-dependent manner. These results show that the bleaching caused by NO was similar in magnitude to the measurement error. Therefore, in this study, the contribution of bleaching caused by NO could be considered insignificant.

From the dose–response curve for SNAP, the SNAP concentration required for half-maximal inhibition (IC_{50}) was estimated.

Electrophysiological recordings

The mEPCs were recorded at the endplate region of the muscle fibres, using the standard two-electrode voltage-clamp technique at 20–22 °C. Glass microelectrodes (resistance 10–15 MΩ) were filled with 3 M KCl. The membrane potential was held at −60 mV (except when otherwise stated). At least 200 mEPCs were recorded for each muscle fibre. The recorded responses were filtered (band-pass 0.03–10 kHz) and digitized at 10–20 μs intervals. The mEPCs were analysed using an original computer program designed to determine the amplitude and e-fold decay time constant (Petrov *et al.*, 2011). The decay time constant (τ) was estimated as the time interval between 0.8 and 0.367 of the mEPC amplitude (e-fold decrease). Recorded mEPC parameters from individual endplates were averaged. Each experimental group consisted of mean values measured in approximately 20–30 endplates.

Voltage-gated Na^+ channels were inhibited by adding 0.1 μM tetrodotoxin to the superfusing medium to increase the threshold for muscle action potential and prevent contractions after anti-AChE treatment (10 μM paraoxon). In experiments with nerve stimulation, μ-conotoxin GIIIB (2 μM) was applied for 20 min to prevent muscle contractions. In order to minimize stimulus artefact, nerve stimulation (10 Hz) was elicited by the application of supra-maximal stimuli (duration 0.1 ms) to the motor nerve through a pair of platinum electrodes located in a small, adjacent moist compartment.

Data analysis

In all cases, data were reported as mean ± SEM and n indicates the number of endplates measured in each group (except as otherwise stated). Two-group comparisons were performed using either a paired or unpaired two-tailed Student's t-test. Differences between groups were considered statistically significant at $P < 0.05$. Statistical comparisons were performed with ORIGINPRO 8.0 (OriginLab Corporation, Northampton, MA, USA).

Chemicals

Paraoxon, tetrodotoxin, glutamate, glycine, DL-2-amino-5-phosphopentanoic acid (AP-5), N^{ω}-nitro-L-arginine methyl ester (L-NAME), SNAP, cycloheximide, okadaic acid (OA), tetraisopropyl pyrophosphoramide, acetylthiocholine, 5,5′-dithiobis (2-nitrobenzoic acid), and Ellman's reagent were all obtained from Sigma-Aldrich (St Louis, MO USA). μ-Conotoxin was obtained from Alamone Labs (Israel). All drugs were applied via the superfusate.

Results

Nitric oxide donor, S-nitroso-N-acetyl-DL-penicillamine, inhibits acetylcholinesterase activity in muscle homogenates

To determine whether NO molecules can inhibit AChE, biochemical assays for AChE activity were performed in EDL homogenates using different concentrations of the NO donor, SNAP. In the presence of SNAP, a dose-dependent decrease in AChE activity was

observed, culminating in complete AChE inhibition (Fig. 1). The estimated IC_{50} for SNAP was 230 μM. SNAP is not an equimolar NO donor. For example, using the NO-selective electrode, Hermann & Erxleben (2001) showed that 1 mM of SNAP produced approximately 8 μM of NO. Therefore, an IC_{50} of 230 μM corresponds to 1.8 μM of NO in solution.

Exogenous nitric oxide increases the amplitude and duration of miniature endplate currents

The amplitude and duration of spontaneous endplate currents change rapidly after AChE inhibition (Katz & Miledi, 1973). Notably, the current amplitude increases almost linearly in proportion to AChE inhibition, whereas the decay time constant increases only when 80–90% of AChE activity is inhibited (Anglister *et al.*, 1994). Therefore, alterations in current amplitude and decay time constant can be used to follow AChE inhibition in the synaptic cleft.

The ability of NO to affect the amplitude of endplate potentials (quantal content) in the neuromuscular junction has been reported previously (Thomas & Robitaille, 2001). Therefore, the changes in the parameters associated with mEPCs were analysed only to avoid interference from the putative effects of NO on both mEPC amplitude and quantal content.

Based on the inhibition curve of AChE by NO *in vitro*, for electrophysiological recordings, SNAP was used at concentrations ranging from 20 μM to 2 mM. In controls, the mean mEPC amplitude and decay time constant (τ) were 2.77 ± 0.16 nA and 1.03 ± 0.06 ms, respectively (n = 20, Fig. 2). At the lowest concentration (20 μM), SNAP had no effect on mEPC parameters. Increasing the SNAP concentration to 200 μM enhanced mEPC amplitude by 13% (3.14 ± 0.09 nA vs. control, unpaired t-test, t_{38} = 2.04, P = 0.048, Fig. 2B) and had no effect on the decay time constant (Fig. 2C). SNAP at a concentration of 2 mM resulted in a dramatic increase in both mEPC amplitude (47% increase, 4.08 ± 0.16 nA vs. control, unpaired t-test, t_{38} = 5.85, P < 0.00001, Fig. 2B) and decay time constant (89% increase, 1.95 ± 0.10 ms vs. control, unpaired t-test, t_{38} = 7.71, P < 0.000001, Fig. 2C). This effect was fully reversible when SNAP was removed from the bathing solution (data not shown).

To examine whether the effects of SNAP on mEPC parameters are associated with inhibition of AChE, mEPC measurements were performed in the presence of the traditional anti-AChE compound, paraoxon. As expected, application of 10 μM paraoxon dramatically increased mEPC amplitude (4.93 ± 0.10 nA vs. control, unpaired t-test, t_{42} = 11.89, P < 0.00001, Fig. 3A) and prolonged the decay time constant by 299% (4.10 ± 0.13 ms vs. control, unpaired t-test, t_{42} = 20.50, P < 0.00001, Fig. 3B). Once paraoxon has exerted its influence on the mEPC amplitude and decay time constant, no further significant increase in mEPC parameters was observed when 2 mM SNAP was applied thereafter (amplitude: paraoxon + SNAP 5.13 ± 0.20 nA, paraoxon 4.93 ± 0.10 nA, unpaired t-test, t_{38} = 1.02, P = 0.31; τ of decay: paraoxon + SNAP 3.67 ± 0.10 ms, paraoxon 4.10 ± 0.13 ms, unpaired t-test, t_{38} = 1.66, P = 0.105).

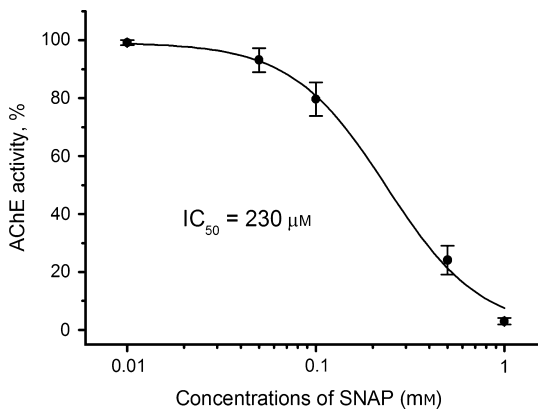

FIG. 1. The NO donor, SNAP, reduces AChE activity in a dose-dependent manner in rat EDL homogenates. AChE activity was estimated using Ellman's method (Ellman *et al.*, 1961). Control values were obtained in the absence of SNAP. Experimental values are presented as % control. Using Hill's equation, the estimated IC_{50} for SNAP was 230 μM. Data are presented as mean ± SEM (n = 3 muscles). Note: log-linear axis.

The question arises whether a dose-dependent increase in mEPC amplitude and a prolongation of the decay time, which are typical of AChE inhibition, are the result of action by NO molecules or of SNAP-mediated depression of AChE activity. To address this question, SNAP (2 mM) was added to haemoglobin (30 μM), an NO scavenger (Mukhtarov *et al.*, 2000; Thomas & Robitaille, 2001). Haemoglobin *per se* did not influence mEPC amplitude and duration (Fig. 3). However, the effects of SNAP on mEPC amplitude and duration were less pronounced in the presence of haemoglobin. The mEPC amplitude increased by only 22% (haemoglobin + SNAP, 3.37 ± 0.18 nA; haemoglobin, 2.76 ± 0.15 nA, unpaired t-test, t_{41} = 2.65, P = 0.011, Fig. 3A) and the decay time constant increased by only 24% (haemoglobin + SNAP, 1.39 ± 0.06 ms; haemoglobin, 1.13 ± 0.06 ms, unpaired t-test, t_{41} = 3.18, P = 0.003, Fig. 3B).

Therefore, the effects of the NO donor on mEPC amplitude and decay time were similar to the effects typically seen with AChE inhibition. Further, the absence of these effects after preincubation with an anti-AChE and significant attenuation of these effects in the presence of an NO scavenger support the hypothesis that exogenous NO molecules can depress AChE activity in the mammalian neuromuscular junction.

Activation of N-methyl-D-aspartate receptors increases miniature endplate current size through endogenous nitric oxide production

To address whether NMDA receptor activation triggers sufficient endogenous NO production to depress AChE activity, 100 μM glutamate was added to the bathing solution. Glutamate enhanced mEPC amplitude, but the effect became significant only 30–40 min after the addition of glutamate. Incubation for 1 h with glutamate increased the mEPC size by 9% (3.53 ± 0.10 nA vs. control: 3.23 ± 0.07 nA, unpaired t-test, t_{60} = 2.41, P = 0.02, Fig. 4). There was no difference in the effects of glutamate when the NMDA receptor co-agonist, glycine, was added to the solution at a low concentration (100 μM, data not shown). However, a higher concentration of glycine (700 μM) significantly enhanced the effect of glutamate, leading to an increase in mEPC amplitude of 19% (3.87 ± 0.11 nA vs. control, unpaired t-test, t_{75} = 5.08, P < 0.000001, Fig. 4). Glycine addition at any concentration did not change the duration of mEPCs. A further increase in glutamate concentration (up to 500 μM, in the presence of 700 μM glycine) did not amplify the effects on mEPC amplitude (data not shown). Finally, 700 μM glycine alone did not alter either the mEPC amplitude or duration (Fig. 4). A representative example of changes in mEPC amplitude during the combined application of glutamate and glycine is shown in Fig. 4 (insert).

To test the hypothesis that the change in mEPC amplitude may result from a decrease in AChE activity, amino acids were applied after complete AChE inhibition by paraoxon. With paraoxon, the mean mEPC amplitude was 4.93 ± 0.10 nA (n = 24) and did not

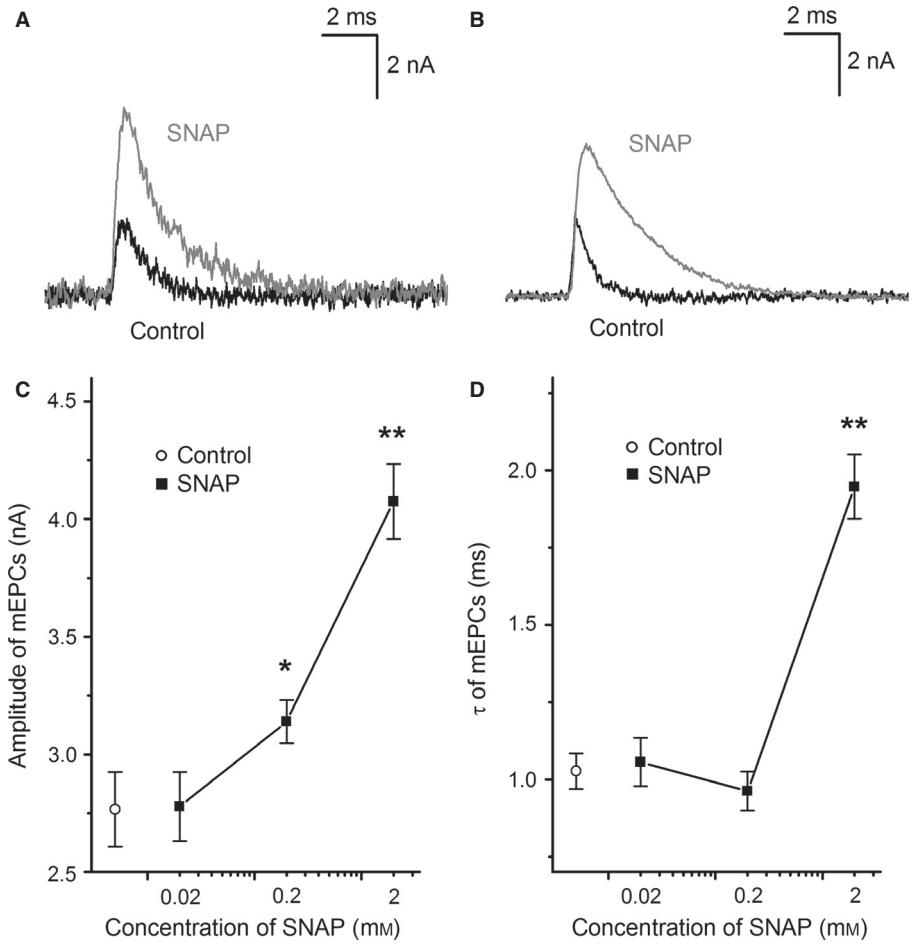

FIG. 2. SNAP increases the amplitude and decay time constant (τ) of mEPCs in a dose-dependent manner. (A) Representative individual mEPCs in a control (black line) and in the presence of 2 mM SNAP (gray line). (B) The average of 100 individual signals recorded at the same endplate. (C) A moderate enhancement of mEPC amplitude was observed at 200 μM of SNAP. Application of 2 mM SNAP dramatically increased the mEPC amplitude (n = 20 per concentration and control). (D) Decay time constant (τ) was significantly increased by 2 mM SNAP. Lower SNAP concentrations had no effect (n = 20 per concentration and control). Data are presented as mean ± SEM. Note: log-linear axis. Asterisks indicate a statistically significant difference (*P < 0.05 and **P < 0.0001, respectively) from the control value.

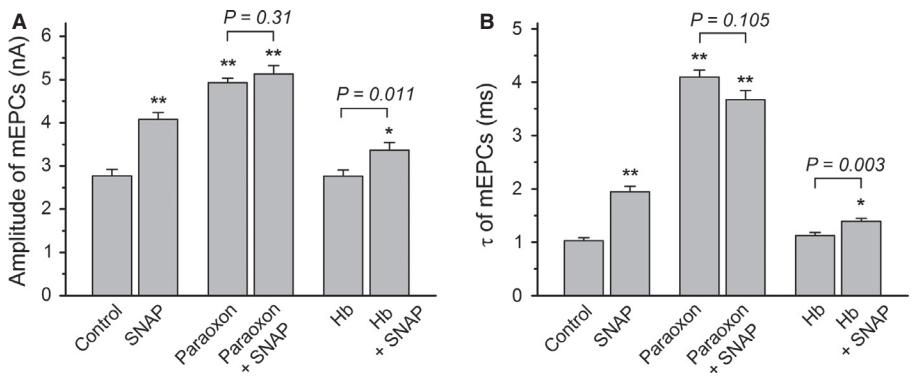

FIG. 3. The effects of SNAP on mEPC amplitude and decay time constant (τ) are associated with inhibition of synaptic AChE by released NO molecules. Incubation with the AChE inhibitor, paraoxon (10 μM), dramatically increased both mEPC amplitude (A) and τ of decay (B). The application of SNAP (2 mM) after preincubation with paraoxon did not affect either mEPC amplitude or τ of decay. The NO scavenger, haemoglobin (Hb) (30 μM), alone had no effect on mEPC parameters, whereas Hb significantly attenuated the effects of SNAP. Data are presented as mean ± SEM, pooled from four to five animals (19–24 endplates). Asterisks indicate a statistically significant difference (*P < 0.05 and **P < 0.0001, respectively) from the control value.

change significantly with application of glutamate and glycine (5.07 ± 0.14 nA vs. paraoxon, unpaired t-test, t_{47} = 0.85, P = 0.40, Fig. 4). In addition, AChE activity in EDL homogenates was not altered by application of glutamate or glycine, applied together or individually, in concentrations used for electrophysiological recordings (data not shown).

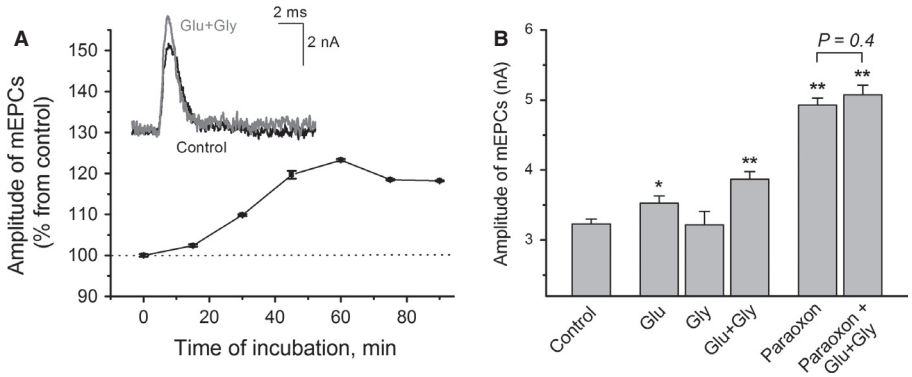

FIG. 4. Effects of glutamate (Glu) and glycine (Gly) on mEPC amplitude are associated with inhibition of synaptic AChE. (A) Representative example illustrating the time-course of mEPC amplitude enhancement during combined application of Glu and Gly in one typical experiment (each point represents the mean amplitude from ~200 mEPCs). The insert shows the average of 100 mEPCs recorded in the same endplate in a control (black line) and after incubation for 1 h with 100 μM Glu and 700 μM Gly (gray line). (B) Incubation for 1 h with 100 μM Glu resulted in a 9% increase in mEPC amplitude. The combined action of Glu (100 μM) and Gly (700 μM) caused even greater enhancement of mEPC amplitude (19% increase). The application of amino acids after pretreatment with the AChE inhibitor, paraoxon (10 μM), did not effect mEPC amplitude. Data are presented as mean ± SEM, pooled from four to seven animals (21–40 endplates). Asterisks indicate a statistically significant difference (*$P < 0.05$ and **$P < 0.0001$, respectively) from the control value.

Therefore, it is likely that the amino acids, glutamate and glycine, act on mEPC amplitude through partial and indirect depression of AChE activity. Based on the results of this study, other potential mechanisms for this activity (e.g. an increase in quantum size or changes in acetylcholine receptor affinity) are less likely.

To investigate the hypothesis that glutamate-mediated NO production leads to depression of AChE activity via NMDA receptor activation, mEPCs were recorded in muscles preincubated with either the competitive NMDA receptor antagonist, AP-5, or the NO synthase inhibitor, L-NAME. Preincubation with AP-5 (25 μM) alone did not change mEPC amplitude (AP-5, 2.86 ± 0.11 nA; control, 3.09 ± 0.10 nA; unpaired *t*-test, $t_{47} = 1.56$, $P = 0.12$). After pretreatment with AP-5, the effect of glutamate and glycine on mEPC amplitude was eliminated (glutamate + glycine + AP-5, 2.82 ± 0.10 nA; AP-5, 2.86 ± 0.11 nA; unpaired *t*-test, $t_{46} = 0.23$, $P = 0.82$). The incubation of muscles with the NO synthase inhibitor, L-NAME (100 μM), resulted in a small but statistically significant decrease (8% decrease) in mEPC amplitude (2.96 ± 0.08 nA, control: 3.23 ± 0.07 nA, unpaired *t*-test, $t_{72} = 2.51$, $P = 0.014$, Fig. 5). The application of amino acids after incubation with L-NAME did not increase mEPC amplitude (glutamate + glycine + L-NAME, 2.79 ± 0.08 nA; L-NAME, 2.96 ± 0.08 nA; unpaired *t*-test, $t_{57} = 1.51$, $P = 0.14$, Fig. 5).

In additional experiments we attempted to attenuate the effect of amino acids on mEPC amplitude using the membrane-non-permeable NO scavenger haemoglobin (30 μM). Indeed, we did not observe any significant increase in mEPC amplitude after combined application of haemoglobin and amino acids (haemoglobin, 2.79 ± 0.11 nA; glutamate + glycine + haemoglobin, 2.99 ± 0.18 nA; unpaired *t*-test, $t_{51} = 1.02$, $P = 0.31$).

These results suggest that NMDA receptor activation triggers a long-term Ca^{2+}-dependent pathway, which leads to enhancement of NO production and partial depression of AChE activity.

Increase in miniature endplate current amplitude with N-methyl-D-aspartate receptor activation is associated with phosphatase activity, rather than de novo protein synthesis

Two of the mechanisms most likely to explain the delayed effect of glutamate and glycine on the mEPC time-course are: (i) NMDA receptor activation enhances the synthesis of new proteins (Skinner *et al.*, 2008) and, perhaps, enhances synthesis of NO synthase and (ii) Ca^{2+} entry through NMDA receptors initiates a Ca^{2+}-dependent pathway that regulates NO synthase by changing its level of phosphorylation (Rameau *et al.*, 2004).

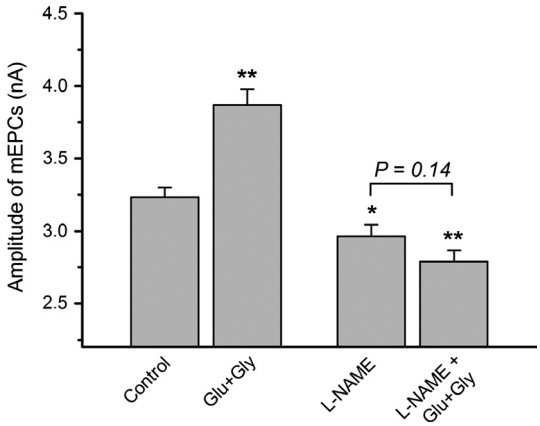

FIG. 5. Effect of amino acids [glutamate (Glu) and glycine (Gly)] on mEPC amplitude is mediated via NO synthase activity. The combined action of Glu (100 μM) and Gly (700 μM) resulted in a 19% increase in mEPC amplitude. The NO synthase inhibitor, L-NAME (100 μM), slightly decreased mEPC amplitude (8% decrease) and fully prevented the effects of Glu and Gly. Data are presented as mean ± SEM, pooled from four to six animals (21–37 endplates). Asterisks indicate a statistically significant difference (*$P < 0.05$ and **$P < 0.0001$, respectively) from the control value.

To investigate whether NMDA receptor activation enhances the *de novo* protein synthesis and/or enhances synthesis of NO synthase, the selective protein synthesis inhibitor, cycloheximide, was applied. Incubation for 180 min with cycloheximide (350 μM) increased mEPC amplitude (cycloheximide, 3.59 ± 0.09 nA; control, 3.23 ± 0.07 nA, unpaired *t*-test, $t_{69} = 3.21$, $P = 0.002$). After incubation for the same time (180 min), the effect of glutamate and glycine on mEPC amplitude was preserved in the cycloheximide-containing bathing solution (cycloheximide + glutamate + glycine, 4.05 ± 0.14 nA; cycloheximide, 3.59 ± 0.09 nA, unpaired *t*-test, $t_{56} = 2.78$, $P = 0.007$, Fig. 6). Therefore, it is likely that *de novo* protein synthesis is not involved in the enhancement of NO production caused by glutamate.

Rameau *et al.* (2004) showed that delayed enhancement (40–60 min) of NO production after NMDA receptor activation can be

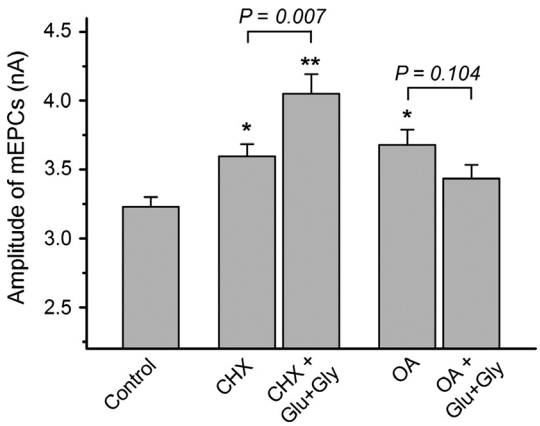

FIG. 6. The inhibition of PP1 and PP2A phosphatases, but not inhibition of protein synthesis, prevents the increase in mEPC amplitude induced by glutamate (Glu) and glycine (Gly). Preincubation with the protein synthesis inhibitor, cycloheximide (CHX) (350 μM), did not prevent the increase in mEPC amplitude in the presence of Glu (100 μM) and Gly (700 μM). Application of the PP1 and PP2A phosphatase inhibitor, OA (1 μM), completely prevented the increase in mEPC amplitude associated with Glu and Gly. Data are presented as mean ± SEM pooled from four to seven animals (21–40 endplates). Asterisks indicate a statistically significant difference (*$P < 0.05$ and **$P < 0.0001$, respectively) from the control value.

mediated by dephosphorylation of NO synthase by the serine–threonine protein phosphatases 1 and 2A (PP1 and PP2A). The possibility that PP1 and PP2A are involved in the action of glutamate on mEPC amplitude was investigated using OA (1 μM), an inhibitor of PP1 and PP2A. The mean amplitude of mEPCs after pretreatment with OA was increased 14% compared with control (3.68 ± 0.11 nA, unpaired *t*-test, $t_{68} = 3.62$, $P = 0.0006$). Furthermore, OA fully prevented the action of glutamate and glycine on mEPCs (OA, 3.68 ± 0.11 nA; OA + glutamate + glycine, 3.43 ± 0.10 nA; unpaired *t*-test, $t_{58} = 1.65$, $P = 0.104$, Fig. 6). Therefore, the activation of NMDA receptors by exogenous glutamate induces inhibition of AChE activity via a pathway involving NO synthase dephosphorylation by a calcium-dependent mechanism.

Activation of N-methyl-D-aspartate receptors by endogenous glutamate results in an increase in miniature endplate current amplitude during high-frequency nerve stimulation

To investigate whether the changes in mEPC parameters can occur during rhythmic high-frequency stimulation, an analysis was conducted of the amplitude and temporal parameters of mEPCs recorded during interstimulus intervals. High-frequency (10 Hz) stimulation was chosen, as this frequency allowed for recording of a sufficient number of mEPCs during the interstimulus intervals. As mEPCs differ significantly between endplates, the parameters of mEPCs recorded at the same endplate at rest and during a high-frequency train of stimulation were compared, in both the absence and presence of glycine. In glycine-free solution, the mEPC amplitude and duration did not change before and during 10 Hz stimulation (before, 2.86 ± 0.16 nA; during, 2.84 ± 0.18 nA; paired *t*-test, $t_{17} = 0.36$, $P = 0.72$, Fig. 7). However, in the presence of glycine (700 μM), mEPC amplitude increased by 108.7 ± 2.9% during high-frequency stimulation (before vs. during, paired *t*-test, $t_{16} = 2.33$, $P = 0.03$, Fig. 7).

The mEPC duration did not change. Immediately following cessation of the stimulation, the mEPC amplitude tended to decline and was not statistically different from baseline at 5 min after cessation (data not shown).

When nerve–muscle preparations were preincubated with either AP-5 (25 μM) or L-NAME (100 μM), the mEPC amplitude did not change during 10 Hz stimulation performed in a glycine-containing bathing solution. Therefore, it is likely that, during normal synaptic functioning, the increase in synaptic signals due to NO-mediated partial AChE inhibition occurs via NMDA activation by glutamate and glycine. In contrast to the slowly developing effects of exogenous amino acids, the effect of endogenous glutamate appeared immediately after the onset of high-frequency nerve stimulation.

Discussion

The gaseous mediator, NO, modulates many functions in the organism, including synaptic transmission (Schuman & Madison, 1994; Ohkuma & Katsura, 2001) and a number of synaptic proteins can serve as targets for NO. The results of this study suggest that AChE serves as an additional target of NO molecules.

S-nitroso-N-acetyl-DL-penicillamine inhibits acetylcholinesterase activity both in vitro and ex vivo

In agreement with previously reported biochemical evidence suggesting that another NO donor, spermine NONOate, can depress AChE activity *in vitro* (Udayabanu *et al.*, 2008), the current results show that the NO donor, SNAP, inhibits the activity of AChE in muscle homogenates. SNAP increases the amplitude and prolongs the decay time of endplate currents, characteristic of AChE inhibition in the neuromuscular junction (in the range of concentrations inhibiting AChE *in vitro*). The absence of these SNAP effects after preincubation with paraoxon (an irreversible inhibitor of AChE) supports the assertion that, in the presence of an NO donor, the changes in the amplitude and time parameters of postsynaptic responses are caused by depression of AChE activity. Furthermore, the NO scavenger, haemoglobin (Mukhtarov *et al.*, 2000; Thomas & Robitaille, 2001), markedly attenuated the effects of SNAP on mEPCs. Therefore, it is likely that the inhibition of AChE is caused primarily by NO molecules, rather than by the direct action of SNAP.

Nitric oxide donors are widely used in medical practice to relax the smooth muscle of blood vessels (for review, see Katsumi *et al.*, 2007; Wimalawansa, 2008). In addition, NO donors are considered a potential pharmacological tool in the treatment of Alzheimer's disease (Thatcher *et al.*, 2006). However, their ability to down-regulate AChE activity has not been investigated prior to the current study. In addition to neurotransmission, acetylcholine can regulate some non-synaptic functions (Kawashima & Fujii, 2008). Therefore, partial inhibition of AChE activity in the presence of NO donors may influence a number of different physiological processes, a point that must be considered in therapeutic practice.

The production of endogenous nitric oxide, triggered by N-methyl-D-aspartate receptor activation, is sufficient for partial inhibition of acetylcholinesterase activity

It remains unclear, however, whether the inhibitory effect of NO takes place only during the application of NO donors, in pathological conditions (when NO production is enhanced), or if the NO radical can influence AChE activity under normal physiological conditions. The co-localization of NO synthase with NMDA receptors in the mammalian neuromuscular junction has been established (Grozdanovic & Gossrau, 1998; Luck *et al.*, 2000). NMDA receptors exhibit relatively high calcium permeability and are located directly on the postsynaptic sarcolemma (in the secondary

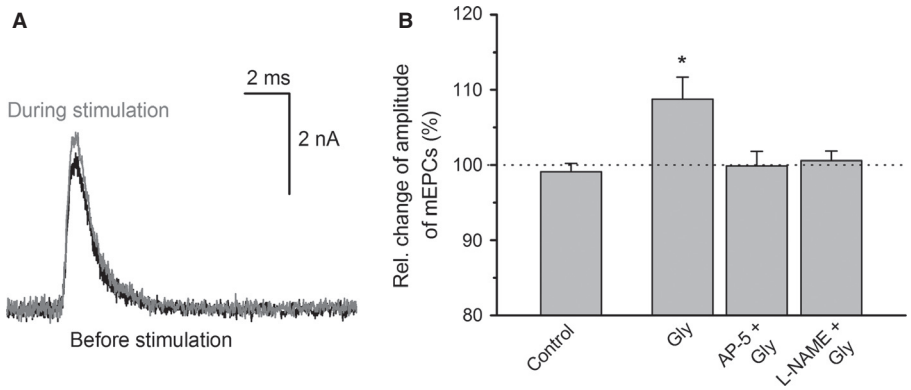

FIG. 7. High-frequency nerve stimulation (10 Hz) increases mEPC amplitude, mediated by endogenous glutamate-induced NMDA receptor activation, with a subsequent elevation in NO production. (A) The average of 100 mEPCs recorded at the same endplate prior to stimulation onset (black line) and during 10 Hz stimulation (gray line) after a 30 min preincubation with 700 μM glycine (Gly). (B) In untreated endplates, mEPC amplitude was unchanged during 5 min of 10 Hz stimulation. Addition of Gly (700 μM) to the bathing solution resulted in a relative increase (9% increase) in mEPC amplitude recorded during interstimulus intervals. Preincubation with either the NMDA receptor blocker, AP-5 (25 μM), or the NO synthase inhibitor, L-NAME (100 μM), eliminated the effects associated with addition of Gly. Control values were recorded prior to stimulus onset. Data are expressed as % control, mean ± SEM, in the same 16–20 endplates (from four animals). Asterisk indicates a statistically significant difference from the control value (paired *t*-test, $*P < 0.05$).

folds where the main portion of AChE is localized) (Mays *et al.*, 2009; Bernard *et al.*, 2011; Malomouzh *et al.*, 2011). The voltage sensitivity of NMDA receptor-mediated currents may explain the function underlying the co-localization of these receptors with voltage-gated sodium channels in the folds (i.e. in this location, the flow of current induced by opening acetylcholine-gated channels has the greatest depolarizing effect on the membrane) (Wood & Slater, 2001). Glutamate is recognized as a co-mediator of acetylcholine (Vyas & Bradford, 1987; Israel *et al.*, 1993; Meister *et al.*, 1993; Waerhaug & Ottersen, 1993; Pinard *et al.*, 2003; Nishimaru *et al.*, 2005). Considering previous experimental evidence that NMDA receptor activation resulted in an increase in NO production (Malomouzh *et al.*, 2003, 2005), the possibility of a glutamate-induced, NO-mediated pathway of AChE inhibition was suggested. Application of glutamate and glycine enhanced the amplitude of endplate currents, but the effect was eliminated after NMDA receptor blockade and inhibition of NO synthase or AChE. In addition, biochemical assays were used to verify the absence of direct AChE inhibition by glutamate and glycine. These results support the idea of an NMDA receptor/NO synthase-mediated pathway that down-regulates AChE activity. Finally, this study showed that protein dephosphorylation (most likely, dephosphorylation of NO synthase) can play the key role in this 'tonic' regulation of NO production in the mammalian neuromuscular junction.

An additional series of experiments was conducted to investigate the putative action of endogenous glutamate on mEPC parameters. High-frequency (10 Hz) motor nerve stimulation was performed in both glycine-containing and glycine-free bathing solutions and mEPCs were recorded in the interstimulus intervals. The high-frequency stimulation increased the amplitude of mEPCs in the presence of glycine. However, no significant changes in mEPC amplitude were observed in glycine-free solution, with NMDA receptor blocked, or with NO synthase inhibition. Therefore, the mEPC amplitude enhancement during high-frequency stimulation was mediated by endogenous glutamate-induced, NMDA receptor activation, with a subsequent rise in NO production and partial inhibition of synaptic AChE activity. Interestingly, the increase in mEPC amplitude during nerve stimulation started immediately after stimulation onset (i.e. was not delayed) and remained at the same level during the entire 5 min recording period. This suggests that, in the case of endogenous glutamate released during high-frequency trains, the rise of NO production might be caused by the 'fast' direct activation of NO synthase by Ca^{2+} without involving the NO synthase dephosphorylation pathway.

The properties and functions of NMDA receptors in the vertebrate neuromuscular junction have not yet been well studied. Specifically, the dosage of agonists and co-agonists, pH and voltage sensitivity of NMDA receptor in the neuromuscular junction remain unknown. The contributions of glutamate and glycine transporters in limiting diffusion of these amino acids to the deep secondary folds also remain unclear. Therefore, it is likely that the *in vivo* effects of NMDA-mediated NO production on AChE activity may be greater than those estimated in this study. This study presents the first evidence supporting the down-regulation of synaptic AChE activity by an endogenous substance. The possibility remains that alternative pathways of NO synthase activation (apart from NMDA receptor activation) and other endogenous modulators of AChE activity (in addition to NO) exist.

Conclusion

Traditionally, the mechanisms underlying synaptic plasticity include factors altering the number of neurotransmitter quanta released, the synchrony of their secretion, or the sensitivity of postsynaptic receptors to the agonists (Van der Kloot & Molgo, 1994; Glavinovic, 1995; Atwood & Karunanithi, 2002; Lin & Faber, 2002; Nikolsky *et al.*, 2004; Taschenberger *et al.*, 2005). This study suggests the existence of another method for modulation of synaptic transmission via regulation of the timing of acetylcholine action in the synaptic cleft. Assuming that partial inhibition of AChE activity is an effective way to elevate the amplitude of synaptic responses, the possibility is raised that down-regulation of AChE activity occurs via endogenous substances to compensate for decreased quantal content (e.g. during synaptic fatigue) or reduced sensitivity of the postsynaptic membrane (in pathological conditions). The down-regulation of AChE activity may not only compensate for a pathological reduction in the safety factor, it may also serve as a regulatory mechanism. For example, in the brain, activation of different types of cholinoreceptors can induce long-term potentiation, but can also convert long-term potentiation to depression (Drever *et al.*, 2011). More importantly, in the context of this study, these effects depend not only on the type and location of receptors, but also on the duration

of action of the agonist. The effects of exogenous AChE inhibitors indirectly support the existence of regulatory pathways for synaptic transmission via fine-tuning of AChE activity. For example, AChE inhibition by physostigmine induces long-term depression in both wild-type and mutant mice, in a model of Alzheimer's disease (Goto *et al.*, 2008).

Moreover, there are indications that acetylcholine may be volume-transmitted in some brain structures (Umbriaco *et al.*, 1995; Sarter *et al.*, 2009; Yamasaki *et al.*, 2010). This model assumes that acetylcholine spills over into the extrasynaptic space. The partial depression of AChE activity by endogenous factors is the most straightforward way of enhancing the effectiveness of this mode of transmission.

Acknowledgements

The authors are grateful to Professor Lev Magazanik (Sechenov Institute of Evolutionary Physiology and Biochemistry RAS, Saint Petersburg, Russia), Professor Graham Barrett (University of Melbourne, Australia), and Professor Philippe Ascher (Université Paris Descartes, France) for reading the manuscript and for their helpful comments and discussion. The study was supported by grants from the RFBR (project nos. 11-04-12102, 11-04-01188, and 11-04-01471) and program of the President of the Russian Federation (Science School no. 2669.2012.7). E.K. and K.A.P. were supported by AFM grant no. 14332.

Abbreviations

AChE, acetylcholinesterase; AP-5, DL-2-amino-5-phosphopentanoic acid; EDL, *extensor digitorum longus*; IC_{50}, half-maximal inhibition; L-NAME, N^{ω}-nitro-L-arginine methyl ester; mEPC, miniature endplate current; NMDA, *N*-methyl-D-aspartate; NO, nitric oxide; OA, okadaic acid; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; SNAP, *S*-nitroso-*N*-acetyl-DL-penicillamine.

References

Anglister, L., Stiles, J.R. & Salpeter, M.M. (1994) Acetylcholinesterase density and turnover number at frog neuromuscular junctions, with modeling of their role in synaptic function. *Neuron*, **12**, 783–794.

Atwood, H.L. & Karunanithi, S. (2002) Diversification of synaptic strength: presynaptic elements. *Nat. Rev. Neurosci.*, **3**, 497–516.

Bernard, V., Girard, E., Hrabovska, A., Camp, S., Taylor, P., Plaud, B. & Krejci, E. (2011) Distinct localization of collagen Q and PRiMA forms of acetylcholinesterase at the neuromuscular junction. *Mol. Cell. Neurosci.*, **46**, 272–281.

Drever, B.D., Riedel, G. & Platt, B. (2011) The cholinergic system and hippocampal plasticity. *Behav. Brain Res.*, **221**, 505–514.

Ellman, G.L., Courtney, K.D., Andres, V. & Featherstone, R.M. (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.*, **7**, 88–95.

Gergel', D. & Cederbaum, A.I. (1997) Interaction of nitric oxide with 2-thio-5-nitrobenzoic acid: implications for the determination of free sulfhydryl groups by Ellman's reagent. *Arch. Biochem. Biophys.*, **347**, 282–288.

Glavinovic, M.I. (1995) Decrease of quantal size and quantal content during tetanic stimulation detected by focal recording. *Neuroscience*, **69**, 271–281.

Goto, Y., Niidome, T., Hongo, H., Akaike, A., Kihara, T. & Sugimoto, H. (2008) Impaired muscarinic regulation of excitatory synaptic transmission in the APPswe/PS1dE9 mouse model of Alzheimer's disease. *Eur. J. Pharmacol.*, **583**, 84–91.

Grozdanovic, Z. & Gossrau, R. (1998) Co-localization of nitric oxide synthase I (NOS I) and NMDA receptor subunit 1 (NMDAR-1) at the neuromuscular junction in rat and mouse skeletal muscle. *Cell Tissue Res.*, **291**, 57–63.

Hermann, A. & Erxleben, C. (2001) Nitric oxide activates voltage-dependent potassium currents of crustacean skeletal muscle. *Nitric Oxide*, **5**, 361–369.

Hussain, S.N., El-Dwairi, Q., Abdul-Hussain, M.N. & Sakkal, D. (1997) Expression of nitric oxide synthase isoforms in normal ventilatory and limb muscles. *J. Appl. Physiol.*, **83**, 348–353.

Israel, M., Lesbats, B. & Bruner, J. (1993) Glutamate and acetylcholine release from cholinergic nerve terminals, a calcium control of the specificity of the release mechanism. *Neurochem. Int.*, **22**, 53–58.

Kapur, S., Bedard, S., Marcotte, B., Cote, C.H. & Marette, A. (1997) Expression of nitric oxide synthase in skeletal muscle: a novel role for nitric oxide as a modulator of insulin action. *Diabetes*, **46**, 1691–1700.

Katsumi, H., Nishikawa, M. & Hashida, M. (2007) Development of nitric oxide donors for the treatment of cardiovascular diseases. *Cardiovasc. Hematol. Agents Med. Chem.*, **5**, 204–208.

Katz, B. & Miledi, R. (1973) The binding of acetylcholine to receptors and its removal from the synaptic cleft. *J. Physiol.*, **231**, 549–574.

Kawashima, K. & Fujii, T. (2008) Basic and clinical aspects of non-neuronal acetylcholine: overview of non-neuronal cholinergic systems and their biological significance. *J. Pharmacol. Sci.*, **106**, 167–173.

Kobzik, L., Reid, M.B., Bredt, D.S. & Stamler, J.S. (1994) Nitric oxide in skeletal muscle. *Nature*, **372**, 546–548.

Koelle, G.B., Davis, R., Diliberto, E.J.Jr. & Koelle, W.A. (1974) Selective, near-total, irreversible inactivation of peripheral pseudocholinesterase and acetylcholinesterase in cats in vivo. *Biochem. Pharmacol.*, **23**, 175–188.

Lin, J.W. & Faber, D.S. (2002) Modulation of synaptic delay during synaptic plasticity. *Trends Neurosci.*, **25**, 449–455.

Luck, G., Hoch, W., Hopf, C. & Blottner, W. (2000) Nitric oxide synthase (NOS-1) coclustered with agrin-induced AchR-specializations on cultured skeletal myotubes. *Mol. Cell. Neurosci.*, **16**, 269–281.

Malomouzh, A.I., Mukhtarov, M.R., Nikolsky, E.E., Vyskocil, F., Lieberman, E.M. & Urazaev, A.K. (2003) Glutamate regulation of non-quantal release of acetylcholine in the rat neuromuscular junction. *J. Neurochem.*, **85**, 206–213.

Malomouzh, A.I., Nikolsky, E.E., Lieberman, E.M., Sherman, J.A., Lubischer, J.L., Grossfeld, R.M. & Urazaev, A.Kh. (2005) Effect of N-acetylaspartylglutamate (NAAG) on non-quantal and spontaneous quantal release of acetylcholine at the neuromuscular synapse of rat. *J. Neurochem.*, **94**, 257–267.

Malomouzh, A.I., Nurullin, L.F., Arkhipova, S.S. & Nikolsky, E.E. (2011) NMDA receptors at the endplate of rat skeletal muscles: precise postsynaptic localization. *Muscle Nerve*, **44**, 987–989.

Mays, T.A., Sanford, J.L., Hanada, T., Chishti, A.H. & Rafael-Fortney, J.A. (2009) Glutamate receptors localize postsynaptically at neuromuscular junctions in mice. *Muscle Nerve*, **39**, 343–349.

Meister, B., Arvidsson, U., Zhang, X., Jacobsson, G., Villar, M.J. & Hokfelt, T. (1993) Glutamate transporter mRNA and glutamate-like immunoreactivity in spinal motoneurones. *NeuroReport*, **5**, 337–340.

Mukhtarov, M.R., Urazaev, A.Kh., Nikolsky, E.E. & Vyskocil, F. (2000) Effect of nitric oxide and NO synthase inhibition on nonquantal acetylcholine release in the rat diaphragm. *Eur. J. Neurosci.*, **12**, 980–986.

Nikolsky, E., Vyskocil, F., Bukharaeva, E., Samigullin, D. & Magazanik, L. (2004) Cholinergic regulation of the quantal release at frog neuromuscular junction. *J. Physiol.*, **560**, 77–88.

Nishimaru, H., Restrepo, C.E., Ryge, J., Yanagawa, Y. & Kiehn, O. (2005) Mammalian motor neurons corelease glutamate and acetylcholine at central synapses. *Proc. Natl. Acad. Sci. USA*, **102**, 5245–5249.

Ohkuma, S. & Katsura, M. (2001) Nitric oxide and peroxynitrite as factors to stimulate neurotransmitter release in the CNS. *Prog. Neurobiol.*, **64**, 97–108.

Petrov, K.A., Yagodina, L.O., Valeeva, G.R., Lannik, N.I., Nikitashina, A. D., Rizvanov, A.A., Zobov, V.V., Bukharaeva, E.A., Reznik, V.S., Nikolsky, E.E. & Vyskočil, F. (2011) Different sensitivities of rat skeletal muscles and brain to novel anti-cholinesterase agents, alkylammonium derivatives of 6-methyluracil (ADEMS). *Br. J. Pharmacol.*, **163**, 732–744.

Pinard, A. & Robitaille, R. (2008) Nitric oxide dependence of glutamate-mediated modulation at a vertebrate neuromuscular junction. *Eur. J. Neurosci.*, **28**, 577–587.

Pinard, A., Levesque, S., Vallee, J. & Robitaille, R. (2003) Glutamatergic modulation of synaptic plasticity at a PNS vertebrate cholinergic synapse. *Eur. J. Neurosci.*, **18**, 3241–3250.

Pregelj, P., Trinkaus, M., Zupan, D., Trontelj, J.J. & Sketelj, J. (2007) The role of muscle activation pattern and calcineurin in acetylcholinesterase regulation in rat skeletal muscles. *J. Neurosci.*, **27**, 1106–1113.

Rameau, G.A., Chiu, L.Y. & Ziff, E.B. (2004) Bidirectional regulation of neuronal nitric-oxide synthase phosphorylation at serine 847 by the N-methyl-D-aspartate receptor. *J. Biol. Chem.*, **279**, 14307–14314.

Rotundo, R.L. (2003) Expression and localization of acetylcholinesterase at the neuromuscular junction. *J. Neurocytol.*, **32**, 743–766.

Rotundo, R.L., Ruiz, C.A., Marrero, E., Kimbell, L.M., Rossi, S.G., Rosenberry, T., Darr, A. & Tsoulfas, P. (2008) Assembly and regulation of

acetylcholinesterase at the vertebrate neuromuscular junction. *Chem. Biol. Interact.*, **175**, 26–29.
Sarter, M., Parikh, V. & Howe, W.M. (2009) Phasic acetylcholine release and the volume transmission hypothesis: time to move on. *Nat. Rev. Neurosci.*, **10**, 383–390.
Schuman, E.M. & Madison, D.V. (1994) Nitric oxide and synaptic function. *Annu. Rev. Neurosci.*, **17**, 153–183.
Skinner, C.B., Upadhya, S.C., Smith, T.K., Turner, C.P. & Hegde, A.N. (2008) Signal transduction and gene expression in cultured accessory olfactory bulb neurons. *Neuroscience*, **157**, 340–348.
Taschenberger, H., Scheuss, V. & Neher, E. (2005) Release kinetics, quantal parameters and their modulation during short-term depression at a developing synapse in the rat CNS. *J. Physiol.*, **568**, 513–537.
Thatcher, G.R., Bennett, B.M. & Reynolds, J.N. (2006) NO chimeras as therapeutic agents in Alzheimer's disease. *Curr. Alzheimer Res.*, **3**, 237–245.
Thomas, S. & Robitaille, R. (2001) Differential frequency-dependent regulation of transmitter release by endogenous nitric oxide at the amphibian neuromuscular synapse. *J. Neurosci.*, **21**, 1087–1095.
Udayabanu, M., Kumaran, D., Nair, R.U., Srinivas, P., Bhagat, N., Aneja, R. & Katyal, A. (2008) Nitric oxide associated with iNOS expression inhibits acetylcholinesterase activity and induces memory impairment during acute hypobaric hypoxia. *Brain Res.*, **1230**, 138–149.
Umbriaco, D., Garcia, S., Beaulieu, C. & Descarries, L. (1995) Relational features of acetylcholine, noradrenaline, serotonin and GABA axon terminals in the stratum radiatum of adult rat hippocampus (CA1). *Hippocampus*, **5**, 605–620.
Van der Kloot, W. & Molgo, J. (1994) Quantal acetylcholine release at the vertebrate neuromuscular junction. *Physiol. Rev.*, **74**, 899–991.
Vyas, S. & Bradford, H.F. (1987) Co-release of acetylcholine, glutamate and taurine from synaptosomes of Torpedo electric organ. *Neurosci. Lett.*, **82**, 58–64.
Waerhaug, O. & Ottersen, O.P. (1993) Demonstration of glutamate-like immunoreactivity at rat neuromuscular junctions by quantitative electron microscopic immunocytochemistry. *Anat. Embryol. (Berl.)*, **188**, 501–513.
Wimalawansa, S.J. (2008) Nitric oxide: new evidence for novel therapeutic indications. *Expert Opin. Pharmacother.*, **9**, 1935–1954.
Wood, S.J. & Slater, C.R. (2001) Safety factor at the neuromuscular junction. *Prog. Neurobiol.*, **64**, 393–429.
Yamasaki, M., Matsui, M. & Watanabe, M. (2010) Preferential localization of muscarinic M1 receptor on dendritic shaft and spine of cortical pyramidal cells and its anatomical evidence for volume transmission. *J. Neurosci.*, **30**, 4408–4418.