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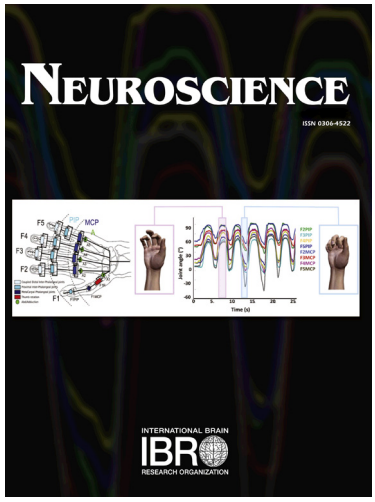

Research Article

Adenosine promotes endplate nAChR channel activity in adult mouse skeletal muscle fibres via low affinity P1 receptors

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Abstract

Adenosine is a powerful modulator of skeletal neuromuscular transmission, operating *via* inhibitory or facilitatory purinergic-type P1 receptors. To date, studies have been focused mainly on the effect of adenosine on presynaptic P1 receptors controlling transmitter release. In this study, using two-microelectrode voltage clamp and single channel patch-clamp recording techniques, we have explored potential postsynaptic targets of adenosine and their modulatory effect on nicotinic acetylcholine receptor (nAChR)-mediated synaptic responses in adult mouse skeletal muscle fibres *in vitro*.

In the whole-mount neuromuscular junction preparation, adenosine (100 μM) significantly reduced the frequency of the miniature endplate currents (MEPCs) and slowed their rising and decay time. Consistent with a postsynaptic site of action, adenosine and the potent P1 receptor agonist NECA significantly increased the open probability, the frequency and the open time of single nAChR channels, recorded at the endplate region. By using specific ligands for the P1 receptor subtypes, we found that the low affinity P1 receptor subtype A_{2B} was responsible for mediating the effects of adenosine on the nAChR channel openings. Our data suggest that at the adult mammalian neuromuscular junction, adenosine acts not only presynaptically to modulate acetylcholine transmitter release, but also at the postsynaptic level, to enhance the activity of nAChRs. Our findings open a new scenario in understanding of purinergic regulation of nAChR activity at the mammalian endplate region.

Highlights

- Adenosine enhances the activity of endplate nAChR channels via low affinity purinergic P1-type receptors.
- The P1 receptor A_{2B} subtype was found to be the main candidate of the adenosine-mediated modulation of muscle nAChRs.
- A new role of postsynaptic P1-type receptors at the neuromuscular junction is proposed.

Key words: adenosine, adult skeletal muscle, MEPC, nAChR, P1 receptor

INTRODUCTION

Adenosine and ATP, acting extracellularly *via* purinergic-type receptors (Burnstock, 1972; Burnstock and Verkhratsky, 2009), are known to play important roles in numerous biological processes. Among them, adenosine represents an important autocrine modulator of nicotinic cholinergic synaptic activity (Cunha and Sebastião, 1993; Ribeiro et al., 1996; Todd et al., 2006). At the skeletal neuromuscular junction (NMJ), the extracellular concentration of adenosine is mainly controlled by ATP, co-released at the nerve terminals with acetylcholine (ACh) and converted into adenosine by ectoenzymes. Adenosine itself is also released into the extracellular compartment from muscle cells (Lynge et al., 2001). At rest, the extracellular concentration of adenosine at the NMJ is estimated to be around 10 nM, whereas during muscle contraction, it increases up to the μM range (Smith, 1991; Cunha and Sebastião, 1993). It is now widely accepted that adenosine modulates the release of ACh through the activation of P1-type receptors (P1Rs), known to be expressed presynaptically on cholinergic nerve terminals (Giniatullin and Sokolova, 1998; De Lorenzo et al., 2004; Baxter et al., 2005; Tomàs et al., 2014; Nascimento et al., 2014). Besides this, the presence of postsynaptic P1Rs, and specifically R_{A2B} and R_{A3} subtypes, has also been recently reported by immunocytochemistry and Western Blotting analysis at the postsynaptic side of the mouse neuromuscular junction (Garcia et al., 2013; 2014). This finding opens a new scenario, with adenosine as a potential endogenous modulator of cholinergic neurotransmission also acting at the *post*synaptic level. This intriguing aspect has yet to be investigated.

Adenosine can bind to four P1R subtypes, named A_1, A_{2A}, A_{2B} and A_3 (Fredholm et al., 2011), all G-protein coupled receptors, with a different affinity for the endogenous ligand. A_1 and A_{2A} are known as high affinity P1Rs (in rodent, A_1R: K_i = 73 nM, $A_{2A}R$: K_i = 150 nM), while the A_{2B} and A_3 are low affinity subtypes (in rodent, $A_{2B}R$: K_i = 5100 nM, A_3R: K_i = 6500 nM, reviewed in Fredholm et al., 2011). It is generally accepted that the activation of A_1R and A_3R inhibits the activity of adenylyl cyclase, while A_{2A} and A_{2B} subtypes increase the activity of the same enzyme. Moreover, all of them can trigger other unknown and often unpredictable signal pathways (Fredholm et al., 2011).

The activation of P1Rs modulates the activity of many ion channels and receptors (Sebastião and Ribeiro, 2009) and the recent detection of P1Rs at the postsynaptic level of the adult NMJ (Garcia et al., 2014) makes potentially possible an adenosine-mediated modulation of the nicotinic acetylcholine receptor (nAChR) channels located on the motor endplate under physiological conditions.

After innervation and the release of trophic factors, the embryonic nAChR ($\alpha_2\beta\gamma\delta$) expressed in immature skeletal muscle is replaced by the adult isoform by the substitution of the γ subunit with the ε (Steinbach, 1989). The developmental switch of the subunits confers to the channel a higher conductance and a reduction in the mean open time with respect to the embryonic isoform. Moreover, after innervation, the nAChRs become mainly clustered at the endplate region and not spread along the cell surface as in immature muscle (Kummer et al., 2006). The channel activity of the embryonic and the adult nAChR isoforms is differentially modulated by protein kinases and by second messenger signalling pathways (Huganir and Miles, 1989). In a previous study, we demonstrated that endogenous adenosine released by contracting myotubes in culture, increased the open probability and mean open time of the embryonic isoform of the nAChR-ion channel by acting via A_{2B} type receptors (Bernareggi et al., 2015). Therefore, adenosine, as well as ACh (Bandi et al., 2005), could play an important role to sustain mechanical activity and muscle trophism during the early stages of myogenesis, when nerve contact is not yet established.

In the present work, the possible interplay between postsynaptic P1Rs and the *adult* isoform of the nAChRs was investigated, because a possible adenosinergic regulation of the cholinergic receptors at the mature endplate region of skeletal muscle fibres could result in a significant change of muscle activity. To address this issue, we recorded nAChR-mediated miniature endplate currents (MEPCs) at the mouse NMJ and nAChR-activated single channel currents at the motor end plate region of isolated mouse muscle fibres.

Our results reveal that adenosine enhances the activity of adult junctional nAChRs. To our knowledge, our finding represents the first evidence of a possible involvement of postsynaptic P1Rs in affecting adult mammalian neuromuscular synaptic transmission.

Experimental procedures

Ethical Approval

Animal care and experiments were carried out in accordance with the European Communities Council Directive 86/609/EEC approved by the Ethical Committee of Kazan Medical University and the European Directive 2010/63/UE and Italian legislation for research involving animals (D.Lv.26/14). All efforts were made to minimize animal suffering.

Innervated diaphragm preparation

All experiments were performed on the isolated diaphragm muscle of 2-3 months male B&/SJL mice (21 animals) with a body weight of 22–25 g. Animals were previously anaesthetized and then killed by decapitation, the chest immediately opened and the muscle quickly excised. The diaphragm muscle with an attached phrenic nerve was pinned to the bottom of a glass chamber lined with Sylgard and continuously superfused during the experiment with mammalian physiological saline containing (in mM): NaCl 130, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, glucose 11, $NaHPO_4$ 1, $NaHCO_3$ 11, gassed with 95% O_2/5% CO_2, starting usually 1 h before the experiment, pH 7.3-7.4, at 25 °C. One animal was used for each preparation.

Isolated myofibres

Freshly dissociated adult mouse skeletal myofibres (20 animals) were obtained from dissection and dissociation of the *Flexor Digitorium Brevis* (*FDB*) as described in detail elsewhere (Grohovaz et al., 1993). The *FDB* muscles were removed from both feet of 1 up to 2 months old C57B lack male mice, killed by cervical dislocation. Briefly, immediately after the isolation, *FDB* muscles were digested in 3 mg/ml collagenase solubilized in culture medium, in a physiological saline containing (in mM): NaCl 137, KCl 2.7, $MgCl_2$1, $CaCl_2$ 1.8, Na_2HPO_4 0.35, $NaHCO_3$ 12, HEPES 25.2, D-glucose 5.5, pH 7.4 NaOH plus 100 U/ml penicillin, 100 µg/ml streptomycin and 10% Foetal Bovine Serum. Muscle fibres were then mechanically dissociated, washed and seeded into 35 mm Petri dishes coated with Matrigel (1 mg/ml, Biosciences). Cultures were maintained in the physiological saline as above at 37 °C in a humid air atmosphere containing 5% CO_2. Electrophysiological recordings were performed the day after the isolation, when the endplate region was identifiable by phase-contrast microscopy as a distinct roughness of the cell surface (Grohovaz et al., 1993).

Electrophysiological recordings

Recordings of MEPCs from the mouse diaphragm muscle supplied by a phrenic nerve were performed using a two-microelectrode voltage-clamp technique, as previously described (Giniatullin et al., 2006). Signals were acquired with glass microelectrodes (resistance of 3-5 MΩ, filled with 2.5 M KCl) using a home-made low-noise recording amplifier (voltage-clamp gain x 10,000, band-pass frequency 0-10 kHz). MEPCs were recorded at 25 °C for 10-15 minutes before (Ctrl) and after 100 μM adenosine, at a holding potential of -60 mV. The electrode resistance was checked continuously and only the recordings in which it was stable were considered. Adenosine was applied via gravity-driven superfusion. For analysis, at least 300 MEPCs were considered for each preparation, both before and after adenosine application. Each MEPC was visually inspected, to exclude any electrical disturbance interfering with the analysis. MEPCs were digitized at 50 kHz. Data were collected and processed using a custom-developed program (https://github.com/AndreyZakharovExp/Elph), stored on a PC and analyzed off-line to calculate mean amplitudes, rise times (from 10 to 90% of the peak amplitude), decay time constants (Tau) and the inter-event intervals, using Origin software (Microcal Software, Northampton, MA).

The resting membrane potential was recorded at the junctional membrane regions of 150 different fibres (n = 3 animals) superfused for 15 minutes in the absence or presence of adenosine, by using intracellular sharp microelectrodes (resistance of 10 -15 MΩ, recording time 5-7 min). For the patch clamp experiments, mouse *FDB* fibres were kept in a bath solution containing (in mM): KCl 2.8, NaCl 140, $CaCl_2$ 2, Hepes 10, $MgCl_2$ 2, glucose 10, pH 7.4 NaOH. Single channel recordings in cell-attached configuration were performed at the endplate region at room temperature. The borosilicate patch pipettes were fire-polished to a final tip resistance of 5-8 MΩ and filled with the bath solution plus ACh 200 nM. The P1R ligands were generally added to the bath before the recordings at room temperature at the concentrations indicated in the Results section, with the exception of adenosine. Adenosine was applied by gravity-driven superfusion (for 15-20 min before the gigaseal formation) to counteract adenosine degradation and uptake and guarantee a constant nucleoside concentration. Corresponding controls were also superfused to provide similar experimental conditions. Single channel recordings were performed using an Axopatch 200 amplifier (Molecular Devices, Union City, CA, USA) after achieving a gigaseal, when the baseline was stable. Unstable patches were discarded. Signals were sampled at 50 kHz, filtered at 2 kHz with a low-pass Bessel filter and analysed by the pCLAMP 8.0 software package (Molecular Devices, Union City, CA, USA), using a threshold crossing criterion. The open channel probability (NP_o) was calculated by the program according to the formula:

$$NP_o = t_o/T$$

where t_o is the total open time over the time T, and N the number of channels in the patch, which was assumed to be unknown. The channel open frequency was calculated as the number of openings per second. Open time distribution was represented in semi-log plots binning of the channel open durations and best fitted with one exponential by maximum likelihood method. The life times of nAChR channel openings were expressed as the time constants (τ) of the exponential curves. At the low ACh concentration used in all our experiments (200 nM), in most of the recordings, only one level of channel amplitude was detected, most likely due to the low probability of channel openings. When multiple openings were observed, the entire recording was discarded. T (2-10 min) was set in order to acquire at least 300 events. Single channel conductance was estimated from the slope of the regression line obtained by plotting the current amplitude against the pipette potentials (Vp) at +40, +60, +80 mV and +100 mV.

Chemicals

Foetal bovine serum was purchased from Life Technologies (Monza, Italy). P1 receptors ligands were from Tocris Bioscience (Bristol, UK), ADA from Roche S.p.A. (Milano, Italy), Adenosine and NYK80 from Sigma (St. Louis, MO, USA).

Statistical analysis

Data were analysed using Microcal Origin 6.0 (OriginLab Corporation, Northampton, MA, USA). In the text, the data are expressed as means ± SEM with *n* being the number of fibres tested. The data presented in this study passed the normality test. For the statistic of the MEPCs, test conditions were normalized to Ctrl (before Ado treatment of the same preparation). In this case a paired Student's *t*-test was used (GraphPad Prism 4 software, San Diego, USA). Patch clamp recordings in control and test conditions were usually preformed in fibres isolated from the same animal. Statistical significance for each of the single channel parameters, before and during drug tests, was evaluated by Nested ANOVA, wherein data from each animal were nested within test condition. Post hoc Tukey HSD test (JMP version 13; SAS Institute, Cary, NC) was used for multiple comparisons of groups. Differences were considered significant when $P < 0.05$.

RESULTS

Adenosine modulates MEPCs at the neuromuscular junction

To test a possible adenosine-mediated modulation of junctional nAChRs, the effect of adenosine was first studied on the MEPCs recorded in the whole-mount muscle preparation, innervated by a phrenic nerve. The frequency, amplitude, rise time (R.t) and time constant decay (Tau) values of nAChR-mediated MEPCs recorded in control conditions were similar to those already reported ($0.9 \pm 0.3\ s^{-1}$; 2.1 ± 0.46 nA; 352 ± 20 μs and 646 ± 49 μs respectively; Robertson and Wann, 1984). Moreover, in agreement with previous studies, adenosine significantly affected the frequency of MEPCs depending on its concentration. Specifically, 0.1 and 1 μM adenosine increased the frequency, while 10 and 100 μM adenosine decreased it (Fig. 1A). This concentration-dependent effect was ascribable to the activation of adenosine receptors located at the presynaptic level. In the presence of lower concentrations (0.1 and 1 μM), a similar effect was already described as the result of an interplay between presynaptic $A_1R/A_{2A}Rs$ (Pousinha et al., 2010). At higher concentrations (10 and 100 μM), the effect was likely mediated by the prevalent activation of the presynaptic A_1R subtype (Correia-de-Sá et al., 1991; Giniatullin and Sokolova, 1998; De Lorenzo et al., 2004).

Interestingly, higher adenosine concentrations significantly affected the kinetics of MEPCs, while MEPC amplitudes were unchanged (Fig.1A-E). At 10 and 100 μM the rise time of MEPCs was significantly increased (Fig.1 C,F). Likewise, the decay time constant (Tau), which reflects the kinetics of postsynaptic nAChR channels (Katz and Miledi, 1973), was also increased (Fig. 1 D,F). All these effects were fully recovered after washing.

Additional experiments, excluded any effect of adenosine on the resting membrane potential (Ctrl: -73.4 ± 0.9 mV, $n = 3$ animals, 150 fibres; Ado: -72 ± 0.5 mV, $n = 3$ animals, 150 fibres; *ns* unpaired t-test). As a whole, these results suggested an adenosinergic modulation of the nAChR channel activity at the postsynaptic level.

Adenosine modulates nAChR-channel activity at the motor endplate region

To analyse the effect of adenosine at the postsynaptic level of the NMJ, the activity of single nAChR channels was recorded at the motor endplate region of isolated *FDB* mouse muscle fibres, which is a common and suitable model to perform single channel recordings of junctional nAChR activity (Henderson et al., 1987). In freshly dissociated skeletal muscle fibres and up to 24 hours after isolation, the channel activity was detectable in membrane patches at the level of the endplate region, identifiable by phase-contrast microscopy as a roughness of the cell surface (see also Grohovaz et al., 1993; Fig. 2A). To avoid agonist-induced channel desensitization and

multiple channel openings, a low concentration of ACh (200 nM) was used in the recording pipette solution. Single ACh channels were recorded at a pipette potential of +60 mV (Fig. 2B). The unitary current amplitude distribution was best fitted by a single Gaussian curve (Fig. 2C) and the open time distribution was fitted by a single exponential (Fig. 2D), revealing the presence of only one type of nAChR channel. Mean single channel conductance and time constant were 56.98 ± 4.77 pS (*n* = 18) and 1.57 ± 0.036 ms (*n* = 72, 20 animals), respectively. These data confirmed the presence of the adult junctional nAChR isoform (Henderson et al., 1987).

A set of experiments was then performed to investigate whether the activation of P1Rs could have some effect on the junctional nAChR channel activity. When cells were exposed to 100 μM adenosine (15-20 min) the conductance of the channel (Ado: 57.35 ± 4.5 pS; Ctrl: 56.3 ± 0.7) as well as the reversal potential (Ado: - 59.78 ± 3.65 mV; Ctrl: - 57.71 ± 3.72 mV, *ns* unpaired *t*-test; Fig. 3B) did not significantly change. However, the NP_o significantly increased (Fig. 3C). Such an effect was the result of an increase in the channel open frequency (Fig. 3D) and in the duration of channel openings (Fig. 3E). In addition, the effect of adenosine on NPo could be also a consequence of the recruitment of previously unresponsive/unavailable channels. In the presence of adenosine, the open time distribution could be best fitted by a single exponential as in controls (Fig. 3F). These results confirmed the presence of an adenosine-mediated modulation on the nAChR activity at the endplate region of adult muscle fibres.

Low affinity P1Rs mediate the adenosinergic effects on nAChR-channel activity

The postsynaptic low affinity P1R subtypes (A_{2B} and A_3) expressed in mouse are activated by micromolar concentrations of the agonist NECA (5'-*N*-ethylcarboxamidoadenosine) (Suh et al., 2001) and are selectively inhibited by nanomolar concentrations of the antagonists MRS 1754 (A_{2B} antagonist, Auchampach et al., 2009; Bernareggi et al., 2015) and MRS 1334 (A_3 antagonist, Garcia et al., 2013). The possible role of the low affinity subtypes in the modulation of nAChRs was explored by activating the P1Rs with NECA and in combination with the specific antagonists. The non-hydrolyzable adenosine analogue NECA was used to guarantee a stable activation of P1Rs.

Cell incubation (30 min) with NECA affected the nACh-channel openings similarly to what was observed in the presence of adenosine (Fig. 4). In NECA, ion channel conductance and reversal potential remained similar to controls (NECA: 54.81 ± 3.37 pS, - 67.22 ± 6.9 mV; Ctrl: 50.61 ± 4.31 pS, - 61.23 ± 3.96 mV; Fig. 4B). In addition, as in adenosine, in NECA, the open time distribution was best fitted by a single exponential as in controls (Fig. 4F).

The co-application of NECA with both antagonists of the low-affinity P1R (20 nM MRS 1754 + 50 nM MRS 1334), or with A_{2B}R antagonist alone (MRS 1754), invariably prevented the effect of NECA. Indeed, the nAChR channel activity recorded in the presence of the two antagonists appeared significantly reduced with respect to NECA (Fig. 5). In the presence of the A_{2B} antagonist, a similar effect was observed being NP_o, channel open frequency and τ reduced (Fig. 5). When the A_3 antagonist MRS 1334 was used, a smaller but not significant reduction of the NECA effect was detected (Fig. 5).

We also tested the effect of MRS 1754 and MRS1334 administered alone. The two antagonists did not significantly affect the nAChR channel activity with respect to control conditions (Ctrl: NP_o = 0.004 ± 0.001, open frequency = 1.9 ± 1.2 Hz, τ = 1.45 ± 0.14 ms, *n* = 4; MRS 1754: NP_o = 0.0035 ± 0.0012, open frequency 1.41 ± 0.43 Hz, τ = 1.68 ± 0.31 ms, *n* = 4; MRS 1334: NP_o = 0.0037 ± 0.002, open frequency = 1.75 ± 0.77 Hz, τ = 1.45 ± 0.19 ms, *n* = 6). Moreover, also the single channel conductance remained unaltered (Ctrl: 59.7 ± 5.14 pS, *n* = 4; MRS 1754: 61 ± 0.63 pS, *n* = 3; MRS 1334: 55.68 ± 2.74 pS, *n* = 4) excluding effects of the two antagonists *per se*. On the whole, our results indicate the prevalent role of the A_{2B} over A_3 subtype on the modulation of nAChR activity.

The role of adenylyl cyclase in the adenosine-mediated modulation of the nAChR-channel

The activation of the A_{2B} P1-receptor subtype is associated with an increase in intracellular adenylyl cyclase (AC) activity in many cell types, including skeletal muscle cells (Lynge et al., 2003; Bernareggi et al., 2015). To verify the possible involvement of AC activity in the adenosine-mediated modulation of the nAChR-channels, a series of experiments was carried out in the presence of NKY80, an inhibitor of AC able to reduce the production of cAMP also in mouse skeletal muscle fibres (Menezes-Rodrigues et al., 2013). Cell pre-incubation with 100 μM NKY80 for 15 min was enough to prevent the effect of 100 μM NECA on NP_o, channel open frequency and τ (Fig. 6). When applied alone, NYK80 had no effect on the nAChR channel activity (Fig. 6). This finding indicated that AC activity was required for the modulatory effect of the Ado receptor agonist on the nAChR channels.

DISCUSSION

Recent reports have clearly demonstrated the presence of postsynaptic P1Rs at the adult mammalian NMJ (Garcia et al., 2013; 2014). However, their functional role (if any) has so far remained unknown. In this study, we provide the first evidence in favour of a distinct postsynaptic modulatory effect of adenosine mediated *via* P1Rs. Our data also suggest the A_{2B} subtype as the major contributor in the adenosine-mediated modulation of nAChR channel activity in mammalian skeletal muscle fibres.

There is a general consensus in considering adenosine as an important neuromodulator of synaptic transmission in the central nervous system, where it elicits its effects by binding to P1Rs expressed both at the presynaptic and postsynaptic level as well as on glial cells (Burnstock, 2015). At the level of the NMJ, pre- and post-synaptic actions of adenosine might also occur. The presynaptic effects are well established in many species, included rodents, where adenosine acts on high affinity P1Rs at the cholinergic nerve endings (Ginsborg and Hirst, 1972; Ribeiro and Sebastião, 1987; Ribeiro and Sebastião, 1988; Sebastião et al., 1990; Silinsky, 2004; De Lorenzo et al., 2004; Garcia et al., 2013). The *post*synaptic effects of adenosine however, are only now starting to be identified (Garcia et al., 2013; 2014). The main result of the present work is the observation that, at the adult NMJ, adenosine significantly modulates the activity of nAChRs also at the *post*synaptic level. We provide two pieces of experimental evidence in favour of this novel mechanism.

The first evidence is at the mouse diaphragm NMJ where a change of MEPC kinetics was observed in the presence of adenosine. This is in addition to the modulation of MEPC frequency due to the known presynaptic effect, well described at the rat (Ginsborg and Hirst, 1972; Correia-de-Sá et al., 1991; Pousinha et al., 2010), frog (Giniatullin and Sokolova, 1998) and mouse (Silinsky, 2004; De Lorenzo et al., 2004; Garcia et al., 2013) NMJs.

The second evidence comes from the results obtained by patch-clamp recordings carried out at the endplate region of isolated mouse *FDB* fibres, where adenosine and the P1R agonist NECA significantly affected the NP_o, the open frequency and the time constant τ of the adult nAChR channels. It is generally assumed that the kinetics of the MEPCs reflects the channel activity of the postsynaptic nAChRs (Katz and Miledi, 1973; Lu et al., 1993; Van der Kloot et al., 1994; Ahmed and Ali, 2016). In our control conditions, the rise and decay time of MEPCs were similar to those already reported elsewhere (Robertson and Wann, 1984; Tanzi and D Angelo, 1995; Petrov et al., 2009) and both values were significantly increased in the presence of adenosine.

There was an apparent discrepancy between MEPC decay time and τ values of nAChR channels reported in our study (0.67 *vs.* about 1.6 ms respectively). One of the reasons could be the

amount of neurotransmitter released by the nerve terminal responsible for the MEPC, estimated to be from around 300-500 μM (Steinbach and Stevens, 1976) up to 1 mM (see in Scimemi and Beato, 2009). Unfortunately, this range of concentrations induces multiple channel openings, making it difficult to analyse the kinetic of the nAChR activity at the single channel level (Franke et al., 1991). In addition, the single channel analysis was performed at a pipette voltage of +60 mV, corresponding to a membrane potential of ~ -120 mV, while the MEPC analysis was carried out at a membrane potential of -60 mV. Because the lifetime of nAChR openings increases when the membrane is more hyperpolarized (Auerbach et al., 1996), it seems likely that the observed difference between MEPC decay time and τ values of nAChR channels could be also due to the different experimental conditions. Moreover, we cannot exclude that some of the kinetic properties of the MEPCs could be affected not only by postsynaptic but also by presynaptic factors, such as the amount of ACh released per quantum, a heterogenous fraction of synaptic vesicles and a variable efficiency of vesicle fusion (Kovyazina et al., 2003). Nevertheless, both experimental approaches are in line with the idea of a functional adenosinergic modulation of nAChRs at the adult motor endplate.

At the NMJ, the most important supplier of endogenous adenosine is the nerve ending. Adenosine derives from the degradation of ATP, co-released with ACh (Ribeiro et al., 1996). In addition, both adenosine and ATP are also released by muscle itself (Lin et al., 1985; Lynge et al 2001). Although it is well known that adenosine in the synaptic cleft reaches high levels such as 100 μM (Smith, 1991), the role of the nucleoside at the motor endplate has so far remained unclear. The presence of the high or low affinity P1Rs at the endplate region is still controversial. Garcia et al. (2013) excluded the presence of the A_1 subtype at the motor endplate region, while it is expressed extrajunctionally (Lynge et al., 2003).

Recently, it has been reported that a co-localization of A_{2B} and A_3Rs and nAChRs exists at the mouse endplate (Garcia et al., 2014), which opens the intriguing possibility of a functional interaction with the nAChRs. In line with the latter hypothesis, we investigated if the adenosine-mediated modulation of the kinetics and NP_o of the nAChR channel activity involved A_{2B}Rs and/or A_3Rs. The effects observed using the specific A_{2B}R and A_3R antagonists, confirmed the role of low-affinity P1Rs in the modulation of the nAChRs at the mouse NMJ, where the A_{2B}Rs appeared to be the predominant between the two low-affinity subtypes in controlling the nAChR activity. In accord with this, our data on NMJ showed that adenosine modulated the Tau of MEPCs only at higher doses.

Interestingly, these results indicate that adenosine-modulation of the nAChR-channel activity mediated by the A_{2B}Rs, as previously observed in *developing* mouse muscle cells (Bernareggi et al., 2015), is conserved in fully differentiated skeletal muscle cells, despite the change in nAChR

subunit composition and endplate localisation of the nAChRs after innervation (Steinbach, 1989; Kummer et al., 2006).

The activation of the A_{2B}R subtype triggers different intracellular metabolic pathways, often linked to the activation of adenylyl cyclase and increased levels of intracellular cAMP (Lynge et al., 2003; Bernareggi et al., 2015). The nAChR-channel phosphorylation mediated by the cAMP/PKA pathway was reported to promote an increase in the open probability and duration of the nAChR-channel (Ferrer-Montiel et al., 1991; Lu et al., 1993). Here we found that the adenylyl cyclase activity is required for the modulatory effect on the nAChR channel activity, indicating cAMP as the key-signalling molecule responsible for the effect mediated by A_{2B}Rs. It has been already shown that the cAMP/PKA pathway is involved in trafficking, clustering and stability of the nAChR on the membrane surface (Reynolds et al., 2008; Martinez-Pena y Valenzuela et al., 2013; see also Berdeaux and Steward, 2012). The current study suggests that in addition to possible functions related to the stability of nAChRs at the endplate region, the activation of A_{2B}Rs by adenosine and the downstream signalling, could be implicated in the control of the nAChR channel activity.

Taking into account the crucial role of nAChRs at the NMJ, the adenosine-mediated modulation of the nAChR activity *via* A_{2B}Rs discloses a novel potential functional effect of the nucleoside on the regulation of synaptic transmission. Synaptic adenosine could be generated due to the breakdown of ATP (Ribeiro and Sebastiao, 1987; Redman and Silinsky, 1994) co-released with ACh from motor nerve endings (Silinsky and Redman, 1996). Notably, both ATP and adenosine can inhibit transmitter release (Giniatullin and Sokolova, 1998), which is the major modulatory effect of these two purines. Given that ATP acts via excitatory P2Y1 (Choi et al., 2001) or P2Y2 receptors (Buvinic et al., 2009) at the postsynaptic level, the current study suggests that adenosine might act in concert with the parent compound ATP at the skeletal muscle level.

Taken as a whole, our present observations reveal a novel complex modulatory system of the adult isoform of the nAChR controlled by adenosine, that could have a significant physiological relevance when adenosine achieves an effective level in the synaptic cleft, either under normal or pathological conditions. In this perspective, our findings open new scenarios in the understanding of the regulation mechanisms of nAChRs at the synaptic level and suggest new potential target molecules for controlling the activity of the nAChR ion channel in skeletal muscle.

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Conflict of interest

The authors report no conflict of interest.

References

Ahmed KT, Ali DW (2016), Nicotinic acetylcholine receptors (nAChRs) at zebrafish red and white muscle show different properties during development. Dev Neurobiol 76: 916-36.

Auchampach JA, Kreckler LM, Wan TC, Maas JE, van der Hoeven D, Gizewski E, Narayanan J, Maas GE (2009), Characterization of the A_{2B} adenosine receptor from mouse, rabbit, and dog. J Pharmacol Exp Ther 329: 2-13.

Auerbach A, Sigurdson W, Chen J, Akk G (1996), Voltage dependence of mouse acetylcholine receptor gating: different charge movements in di-, mono- and unliganded receptors. J Physiol 494: 155-170.

Bandi E, Bernareggi A, Grandolfo M, Mozzetta C, Augusti-Tocco G, Ruzzier F, Lorenzon P (2005), Autocrine activation of nicotinic acetylcholine receptors contributes to Ca^{2+} spikes in mouse myotubes during myogenesis. J Physiol 568: 171-180.

Baxter RL, Vega-Riveroll LJ, Deuchars J, Parson SH (2005), A_{2A} adenosine receptors are located on presynaptic motor nerve terminals in the mouse. Synapse 57: 229-234.

Berdeaux R, Stewart R (2012), cAMP signaling in skeletal muscle adaptation: hypertrophy, metabolism, and regeneration. Am J Physiol Endocrinol Metab 303: E1-17.

Bernareggi A, Luin E, Pavan B, Parato G, Sciancalepore M, Urbani R, Lorenzon P (2015), Adenosine enhances acetylcholine receptor channel openings and intracellular calcium 'spiking' in mouse skeletal myotubes. Acta Physiol (Oxf) 214: 467-480.

Burnstock G (2015), Purinergic signalling in neuroregeneration. Neural Regen Res 10: 1919.

Burnstock G, Dale N (2015), Purinergic signalling during development and ageing. Purinergic Signal 11: 277-305.

Burnstock G, Verkhratsky A (2009), Evolutionary origins of the purinergic signalling system. Acta Physiol (Oxf) 195: 415-447.

Burnstock G (1972), Purinergic nerves. Pharmacol Rev 24: 509-581.

Buvinic S, Almarza G, Bustamante M, Casas M, López J, Riquelme M, Sáez JC, Huidobro-Toro JP, Jaimovich E (2009), ATP released by electrical stimuli elicits calcium transients and gene expression in skeletal muscle. J Biol Chem 11: 284:34490-505.

Choi RC, Man ML, Ling KK, Ip NY, Simon J, Barnard EA, Tsim KW (2001), Expression of the P2Y1 nucleotide receptor in chick muscle: its functional role in the regulation of acetylcholinesterase and acetylcholine receptor. J Neurosci 21: 9224-9234.

Correia-de-Sá P, Sebastião AM, Ribeiro JA (1991), Inhibitory and excitatory effects of adenosine receptor agonists on evoked transmitter release from phrenic nerve ending of the rat. Br J Pharmacol 103: 1614-1620.

Cunha RA, Sebastião AM (1993), Adenosine and adenine nucleotides are independently released from both the nerve terminals and the muscle fibres upon electrical stimulation of the innervated skeletal muscle of the frog. Pflugers Arch 424: 503-510.

De Lorenzo S, Veggetti M, Muchnik S, Losavio A (2004), Presynaptic inhibition of spontaneous acetylcholine release induced by adenosine at the mouse neuromuscular junction. Br J Pharmacol 142: 113-124.

Fredholm BB, IJzerman AP, Jacobson KA, Linden J, Müller CE (2011), International Union of Basic and Clinical Pharmacology. LXXXI. Nomenclature and classification of adenosine receptors-an update. Pharmacol Rev 63: 1-34.

Franke C, Hatt H, Parnas H, Dudel J (1991), Kinetic constants of the acetylcholine (ACh) receptor reaction deduced from the rise in open probability after steps in ACh concentration. Biophys J. 60:1008-1016.

Garcia N, Priego M, Hurtado E, Obis T, Santafe MM, Tomàs M, Lanuza MA Tomàs, J. (2014), Adenosine A_{2B} and A_3 receptor location at the mouse neuromuscular junction. J Anat 225: 109-117.

Garcia N, Priego M, Obis T, Santafe MM, Tomàs M, Besalduch N, Lanuza MA Tomàs J (2013), Adenosine A_1 and A_{2A} receptor-mediated modulation of acetylcholine release in the mice neuromuscular junction. Eur J Neurosci 38: 2229-2241.

Giniatullin AR, Darios F, Shakirzyanova A, Davletov B, Giniatullin R (2006), SNAP25 is a pre-synaptic target for the depressant action of reactive oxygen species on transmitter release. J Neurochem 98: 1789-1797.

Giniatullin RA, Sokolova EM (1998), ATP and adenosine inhibit transmitter release at the frog neuromuscular junction through distinct presynaptic receptors. Br J Pharmacol 124: 839-844.

Ginsborg BL, Hirst GD (1972), The effect of adenosine on the release of the transmitter from phrenic nerve of the rat. J Physiol 224: 629-645.

Grohovaz F, Lorenzon P, Ruzzier F, Zorec R (1993), Properties of acetylcholine receptors in adult rat skeletal muscle fibers in culture. J Membr Biol 136: 31-42.

Henderson PD, Lechleiter JD, Brehm P (1987), Single channel properties of newly synthesized acetylcholine receptors following denervation of mammalian skeletal muscle. J Gen Physiol 89: 999-1014.

Huganir RL, Miles K (1989), Protein phosphorylation of nicotinic acetylcholine receptors. Crit Rev Biochem Mol Biol 24: 183-215.

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

Kovyazina IV, Nikolsky EE, Giniatullin RA, Adámek S, Vyskocil F (2003), Dependence of miniature endplate current on kinetic parameters of acetylcholine receptors activation: a model study. Neurochem Res 28: 443-448.

Kummer TT, Misgeld T, Sanes JR (2006), Assembly of the postsynaptic membrane at the neuromuscular junction: paradigm lost. Curr Opin Neurobiol 16: 74-82.

Li J, King NC, Sinoway LI (1985), ATP concentrations and muscle tension increase linearly with muscle contraction. J Appl Physiol 95: 577-583.

Lu B, Fu W, Greengard P, Poo MM *(*1993), Calcitonin gene-related peptide potentiates synaptic responses at developing neuromuscular junction. Nature 363*:* 76-79.

Lynge J, Schulte G, Nordsborg N, Fredholm BB, Hellsten Y (2003), Adenosine A_{2B} receptors modulate cAMP levels and induce CREB but not ERK1/2 and p38 phosphorylation in rat skeletal muscle cells. Biochem Biophys Res Commun 307: 180-187.

Lynge J, Juel C, Hellsten Y (2001), Extracellular formation and uptake of adenosine during skeletal muscle contraction in the rat: role of adenosine transporters. J Physiol 537: 597-605.

Martinez-Pena y Valenzuela I, Pires-Oliveira M, Akaaboune M (2013), PKC and PKA regulate AChR dynamics at the neuromuscular junction of living mice. PLoSOne 8: e81311.

Menezes-Rodrigues FS, Pires-Oliveira M, Duarte T, Paredes-Gamero EJ, Chiavegatti T, Godinho RO (2013), Calcium influx through L-type channels attenuates skeletal muscle contraction via inhibition of adenylyl cyclases. Eur J Pharmacol.720: 326-334.

Nascimento F, Pousinha PA, Correia AM, Gomes R, Sebastião AM, Ribeiro JA (2014), Adenosine A_{2A} receptors activation facilitates neuromuscular transmission in the pre-symptomatic phase of the SOD1(G93A) ALS mice, but not in the symptomatic phase. PLoSOne 9: e104081.

Petrov K, Kovyazina I, Zobov V, Bukharaeva E, Nikolsky EE, Vyskocil F (2009), Different Sensitivity of Miniature Endplate Currents in Rat External and Internal Intercostal Muscles to the Acetylcholinesterase Inhibitor C-547 as Compared with Diaphragm and Extensor Digitorum Longus. Physiol Res 58: 149-153.

Pousinha PA, Correia AM, Sebastião AM, Ribeiro JA (2010), Predominance of adenosine excitatory over inhibitory effects on transmission at the neuromuscular junction of infant rats. J Pharmacol Exp Ther 332: 153-163.

Redman RS, Silinsky EM (1994), ATP released together with acetylcholine as the mediator of neuromuscular depression at frog motor nerve endings. J Physiol 47: 117-127.

Reynolds JG, McCalmon SA, Donaghey JA, Naya FJ (2008), Deregulated protein kinase A signaling and myospryn expression in muscular dystrophy. J Biol Chem 283: 8070-8074.

Ribeiro JA, Cunha RA, Correia-de-Sá P, Sebastião AM (1996), Purinergic regulation of acetylcholine release. Prog Brain Res 109: 231-141.

Ribeiro JA, Sebastião AM (1988),. Subtypes of adenosine receptors. Trends Pharmacol Sci. 9:279-80.

Ribeiro JA, Sebastião AM (1987), On the role, inactivation and origin of endogenous adenosine at the frog neuromuscular junction. J Physiol 384: 571-585.

Robertson B, Wann KT (1984), The effect of temperature on the growth and decay times of miniature end-plate currents in the mouse diaphragm. Brain Res 294: 346-349.

Scimemi A, Beato M (2009), Determining the neurotransmitter concentration profile at active synapses. Mol Neurobiol 40: 289-306.

Sebastião AM, Ribeiro JA (2009), Tuning and fine-tuning of synapses with adenosine. Curr Neuropharmacol 7: 180-194.

Sebastião AM, Stone TW, Ribeiro JA (1990), The inhibitory adenosine receptor at the neuromuscular junction and hippocampus of the rat: antagonism by 1,3,8-substituted xanthines. Br J Pharmacol 101: 453-459.

Sebastião AM, Ribeiro JA (1988), On the adenosine receptor and adenosine inactivation at the rat diaphragm neuromuscular junction. Br J Pharmacol 94: 109-120.

Silinsky EM (2004), Adenosine decreases both presynaptic calcium currents and neurotransmitter release at the mouse neuromuscular junction. J Physiol 558: 389-401.

Smith DO (1991), Sources of adenosine released during neuromuscular transmission in the rat. J Physiol 432: 343-354.

Silinsky EM, Redman RS *(*1996), Synchronous release of ATP and neurotransmitter within milliseconds of a motor nerve impulse in the frog. J Physiol 492*:* 815-822.

Steinbach JH (1989), Structural and functional diversity in vertebrate skeletal muscle micotinic acetylcholine receptors. Annu Rev Physiol 51: 352-365.

Steinbach JH, Stevens CF (1976) Neuromuscular Transmission In: Frog Neurobiology, a handbook (Llinas R, Precht W, eds), pp. 33–92. Springer-Verlag-Berlin-Heidelberg-New York.

Suh BC, Kim TD, Lee JU, Seong JK, Kim KT (2001), Pharmacological characterization of adenosine receptors in PGT-β mouse pineal gland tumour cells. Br J Pharmacol 134: 132-142.

Tanzi F, D'Angelo E (1995), Miniature Endplate Current Kinetics at the Mouse Neuromuscular Junction: Effects of Temperature and Medium Viscosity. Eur J Neurosci 7: 1926-1933.

Todd KJ, Robitaille R (2006), Purinergic modulation of synaptic signalling at the neuromuscular junction. Pflugers Arch 452: 608-614.

Tomàs J, Santafé MM, Garcia N, Lanuza MA, Tomàs M, Besalduch N, Obis T, Priego M, Hurtado E (2014), Presynaptic membrane receptors in acetylcholine release modulation in the neuromuscular synapse. J Neurosci Res 92: 543-554.

Van der Kloot W, Balezina OP, Molgó J, Naves LA (1994), The timing of channel opening during miniature endplate currents at the frog and mouse neuromuscular junctions: effects of fasciculin-2, other anti-cholinesterases and vesamicol. Pflugers Arch 428: 114-126.

Legends

Figure 1. Adenosine-mediated modulation of MEPCs at the mouse neuromuscular junction.

Effect of adenosine (0.1, 1, 10 and 100 μM) on MEPC frequency (**A**), amplitude (**B**), rise time (R.t, **C**) and decay time constant of MEPCs (Tau, **D**). Data were normalized with respect to Ctrl (before Ado application), n = 18 animals (n = 4 for 0.1, 1 and 10 μM, and n = 6 for 100 μM of Ado). *P < 0.05, **P < 0.01, paired t-test. **E**, native MEPCs recorded at -60 mV at the mouse NMJ in control (Ctrl) and after 15 min of adenosine application (Ado, 100 μM). **F**, superimposed representative traces of native MEPCs in Ctrl and after 15 min of adenosine application (100 μM).

Figure 2. Single channel activity of nAChRs recorded from mouse muscle fibre endplate.

A, phase-contrast image of a skeletal muscle fibre isolated from the *FDB* of an adult mouse; the arrow indicates the motor endplate. Scale bar = 30 μm. **B**, representative single nAChR channel recordings evoked by 200 nM ACh at the endplate region. Channel openings appeared as downward deflections. Corresponding unitary current amplitudes (**C**) and open time distribution (**D**) recorded at a pipette potential of +60 mV are shown, with the best fit superimposed.

Figure 3. Adenosine-mediated modulation of nAChR-channels at the mouse endplate.

A, representative single channel openings evoked by ACh (200 nM) at the endplate of *FDB* fibres in control conditions (Ctrl) and in the presence of 100 μM adenosine (Ado). Channel openings appeared as downward deflections. **B**, current-voltage relationships obtained in control (n = 8) and in the presence of adenosine (n = 14). Comparison of NP_o (**C,** Ctrl: 0.006 ± 0.001; Ado: 0.012 ± 0.003, *P = 0.04, $F_{(1,6)}$ = 4.86, η^2 = 0.21), channel open frequency, (**D,** Ctrl: 2.44 ± 0.42 Hz; Ado: 4.74 ± 0.42 Hz, *P = 0.04, $F_{(1,6)}$ = 4.59, η^2 = 0.18), open time constants (**E,** Ctrl: 1.71 ± 0.09 ms; Ado: 1.99 ± 0.07 ms, *P = 0.025, $F_{(1,6)}$ = 5.59, η^2 = 0.19) recorded in Ctrl and adenosine-treated endplates (Ctrl, n = 19; Ado, n = 19, 4 animals, nested ANOVA). **F**, representative open time distributions are shown. Pipette potential = +60 mV.

Figure 4. NECA-mediated modulation of nAChR-channels at the mouse endplate.

A, representative single channel openings evoked by ACh (200 nM) at the endplate of *FDB* fibres in control conditions (Ctrl) and in the presence of the P1-receptor agonist NECA (100 μM). **B**, current-voltage relationships obtained in control (n = 6) and in the presence of NECA (n

= 7). Comparison of NP_o (**C,** Ctrl: 0.0046 ± 0.0009; NECA: 0.011 ± 0.0008, ***P < 0.001, $F_{(1,4)}$ = 33.4, η^2 = 0.77) channel open frequency (**D,** Ctrl: 2.74 ± 0.52 Hz; NECA: 4.96 ± 0.64 Hz, **P = 0.007, $F_{(1,4)}$ = 10.6, η^2 = 0.56), open time constants (**E,** Ctrl: 1.48 ± 0.083 ms; NECA: = 1.84 ± 0.11 ms, P = 0.051, $F_{(1,4)}$ = 4.68, η^2 = 0.29) values recorded in Ctrl and NECA-treated endplates (Ctrl, n = 8; NECA, n = 8, 3 animals, nested ANOVA). **F**, representative open time distributions are shown. Pipette potential = +60 mV.

Figure 5. Low affinity P1Rs modulate the nAChR-channel activity at the adult endplate. Comparison of NP_o (**A,** Ctrl: 0.005 ± 0.007**;** NECA: 0.01 ± 0.001; NECA+$Antag_{low}$: 0.004 ± 0.0006; NECA+$Antag_{A2B}$: 0.003 ± 0.0007; NECA+$Antag_{A3}$: 0.011 ± 0.002; Ctrl *vs* NECA **P = 0.006; NECA *vs* NECA+$Antag_{low}$ **P = 0.005; NECA *vs* NECA+$Antag_{A2B}$ **P = 0.0025; Ctrl *vs* NECA+$Antag_{A3}$ *P = 0.0165; NECA+$Antag_{low}$ *vs* NECA+$Antag_{A3}$ **P = 0.008; NECA+$Antag_{A2B}$ *vs* NECA+$Antag_{A3}$ **P = 0.004; $F_{(4,16)}$ = 7.87, η^2 = 0.35), channel open frequency (**B,** Ctrl: 3.05 ± 0.31 Hz; NECA: 5.05 ± 0.58 Hz; NECA+$Antag_{low}$: 2.22 ± 0.34 Hz; NECA+$Antag_{A2B}$: 2.35 ± 0.5 Hz; NECA+$Antag_{A3}$: 2.86 ± 0.36 Hz; Ctrl *vs* NECA **P = 0.003; NECA *vs* NECA+$Antag_{low}$ **P = 0.0015; NECA *vs* NECA+$Antag_{A2B}$ **P = 0.002; NECA *vs* NECA+$Antag_{A3}$ *P = 0.03; $F_{(4,16)}$ = 6.6, η^2 = 0.35), open time constants (**C,** Ctrl: 1.57 ± 0.051 ms**;** NECA: 1.85 ± 0.06 ms; NECA+$Antag_{low}$: 1.4 ± 0.1 ms; NECA+$Antag_{A2B}$: 1.46 ± 0.08 ms; NECA+$Antag_{A3}$: 1.58 ± 0.092 ms; Ctrl *vs* NECA *P = 0.02; NECA *vs* NECA+$Antag_{low}$ ***P =0.0005; NECA vs NECA+$Antag_{A2B}$ **P = 0.0044; $F_{(4,16)}$ = 6.17, η^2 = 0.37) recorded in isolated muscle fibres in: control conditions (Ctrl, n = 31, 6 animals), NECA (100 μM) alone (n = 27, 5 animals), in combination with $Antag_{low}$ (NECA 100 μM + MRS 1754 20 nM + MRS 1334 50 nM, n = 12, 3 animals) or with $Antag_{A2B}$ (NECA 100 μM + MRS 1754 20 nM, n = 11, 3 animals) or with $Antag_{A3}$ (NECA 100 μM + MRS 1334 50 nM, n = 10, 3 animals). *P < 0.05, **P < 0.01, ***P < 0.001 (nested ANOVA with Tukey HSD test). Pipette potential = +60 mV.

Figure 6. The inhibition of adenylate cyclase (AC) prevents the effect of NECA on the nAChR-channel activity. NP_o, channel open frequency and τ in Ctrl and in the presence of NKY80 alone (10 μM) (**A**, Ctrl: 0.0042 ± 0.0001; NKY80: 0.0001 ± 0.0002; **B**, Ctrl: 1.43 ± 0.27 Hz; NKY80: 0.65 ± 0.16 Hz; **C**, Ctrl: 1.42 ± 0.075 ms; NKY80: 1.39 ± 0.15 ms). NP_o, channel open frequency and τ in NECA (100 μM) and in NECA + NKY80 (10 μM) (**A**, NECA: 0.0096 ± 0.0023; NECA+NKY80: 0.002 ± 0.0006; **P = 0.0047, $F_{(1,4)}$ = 12.51, η^2 = 0.57; **B**, NECA: 3.24 ± 0.78 Hz; NECA+NKY80: 0.8 ± 0.16 Hz; *P = 0.02, $F_{(1,4)}$ = 7.04, η^2 = 0.43; **C**, NECA: 1.96 ± 0.11 ms; NECA+NKY80: 1.1 ± 0.07 ms; ***P < 0.001, $F_{(1,4)}$ = 37.59, η^2 = 0.79). Ctrl: n = 9;

NKY80: n = 6, 3 animals; NECA: n = 9; NKY80+NECA: n = 8, 3 animals. *P < 0.05, **P < 0.01, ***P < 0.001 (nested ANOVA). Pipette potential = +60 mV.