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1 **Natural polymeric 3-alkylpyridinium salt affects vertebrate skeletal muscle**
2 **contractility by preferentially blocking neuromuscular transmission**

3
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15
16 **Highlights**

- 17 • nPoly-3-APS preferentially blocks the skeletal neuromuscular transmission
18 • nPoly-3-APS acts by a non-depolarizing mechanism
19 • In vivo toxicity of nPoly-3-APS mainly occurs through antagonist action on nAChRs

20
21 **Abstract**

22 The effects of natural polymeric alkylpyridinium salt (nPoly-3-APS), a potent
23 acetylcholinesterase inhibitor isolated from the marine sponge *Reniera sarai*, were studied on
24 isolated mouse phrenic nerve-hemidiaphragm muscle preparations using electrophysiological
25 approaches. nPoly-3-APS inhibited nerve-evoked isometric muscle twitch and tetanic
26 contraction in a concentration-dependent manner ($IC_{50} = 29.4 \mu M$ and $18.5 \mu M$, respectively)
27 and produced a 30-44% decrease of directly muscle-elicited twitch and tetanus amplitudes at
28 $54.4 \mu M$. Additionally, nPoly-3-APS ($9.1-27.2 \mu M$) markedly decreased the amplitude of
29 miniature endplate potentials, while their frequency was only affected at the highest
30 concentration used. Endplate potentials were also inhibited by nPoly-3-APS in a
31 concentration-dependent manner ($IC_{50} = 20.1 \mu M$), without significant change in the resting
32 membrane potential of muscle fibers (up to $54.4 \mu M$). In conclusion, our results show, for the
33 first time, that nPoly-3-APS preferentially blocks the neuromuscular transmission, *in vitro*, by
34 a non-depolarizing mechanism. This strongly suggests that the *in vivo* toxicity of nPoly-3-
35 APS mainly occurs through an antagonist action of the compound on nicotinic acetylcholine
36 receptors of skeletal muscles.

37
38 *Key words: Natural Poly-APS; Neuromuscular junction; AChE inhibitors; Motor endplate*
39 *potential; Skeletal muscle contraction*

40 **1 Introduction**

41 Natural polymeric 3-alkylpyridinium salt (nPoly-3-APS), isolated from the marine sponge
42 *Reniera sarai* (Sepčić et al., 1997), shows a broad spectrum of biological activities, including
43 antibacterial and antifouling activities, acetylcholinesterase (AChE) inhibition, cytotoxicity,
44 as well as hemolysis and selective toxicity against non-small cell lung cancer (NSCLC) tumor
45 cells *in vitro* and *in vivo* (Sepčić et al., 1997; Sepčić et al., 1998; Malovrh et al., 1999; Faimali
46 et al., 2003; Tucker et al., 2003; Chelossi et al., 2006; Paleari et al., 2006; Catassi et al., 2008;
47 Elerseck et al., 2008). nPoly-3-APS can also be used for the stable transfection of nucleated
48 mammalian cells (Tucker et al., 2003). Its likely application in medicine led to the synthesis
49 of a series of analogs with different degree of polymerization and length of alkyl chains
50 (Houssen et al., 2010). Synthetic analogs of Poly-3-APS, such as APS12-2, APS3 (Grandić et
51 al., 2011; Grandić et al., 2013), APS8 (unpublished data) and structurally related compounds,
52 are promising new chemotherapeutic agents that exhibit very low *in vivo* toxicity in
53 experimental animals. Recently, the effects of some of these synthetic analogs possessing
54 potent AChE-inhibitory properties were studied on neuromuscular transmission in skeletal
55 muscle (reviewed in Grandić and Frangež, 2014) to avoid unwanted peripheral side effects
56 that could appear in patients treated with some AChE inhibitors. APS12-2 acts as a potent
57 non-competitive AChE inhibitor with an inhibitory constant (K_i) of 34×10^{-3} nM (Houssen et
58 al., 2010). It exerts hemolytic, antibacterial and antifungal actions (Houssen et al., 2010).
59 Some nicotinic cholinergic antagonists, such as APS3 and APS8, have shown promise in lung
60 cancer treatment because of their selective cytotoxicity against NSCLC, the most common
61 form of lung cancer (Zovko et al., 2013). NSCLC cells express cholinergic signaling
62 mediators, including neuronal-type $\alpha 7$ -nicotinic acetylcholine receptors (Paleari et al., 2006;
63 Catassi et al., 2008). The inhibitory effects of the synthetic analogs APS12-2 and APS-3 on
64 neuromuscular transmission and nerve-evoked skeletal muscle contraction were previously
65 reported (Grandić et al., 2012; Grandić et al., 2013). It was demonstrated that the
66 neuromuscular blockade produced by these two compounds is due to their direct inhibitory
67 effect on muscle-type nicotinic acetylcholine receptors (nAChRs). Therefore, it was of
68 interest to determine whether the natural compound nPoly-3-APS shares the same biological
69 effects on neuromuscular transmission and skeletal muscle functioning. In particular, due to
70 the structural similarity between nPoly-3-APS and quaternary ammonium compounds that
71 have been reported to block muscle-type nAChRs, the aim of this study was to investigate the
72 effects and underlying mechanisms of the natural compound on neuromuscular transmission
73 and skeletal muscle contraction. We show, for the first time, that nPoly-3-APS affects the
74 skeletal muscle functioning, *in vitro*, by preferentially blocking the neuromuscular
75 transmission probably through an antagonist action on muscle-type nAChRs.

76

77 **2 Materials and methods**

78 **2.1 Materials**

79 **2.1.1 Drugs**

80 nPoly-3-APS was isolated from the marine sponge *Reniera sarai* according to Sepčić et al.
81 (1997). Before use, the toxin was dissolved in a sterile 0.9% saline solution at a stock

82 concentration of 10 mg/mL and was stored at 4°C. Neostigmine methyl sulfate (Tokyo
83 Chemical Industry CO./LTD, Japan), 3,4-diaminopyridine (3,4-DAP; Sigma-Aldrich, USA)
84 and μ -conotoxin GIIIB (Bachem, Switzerland) were of the highest grade available.

85 **2.1.2 Experimental animals and neuromuscular preparations**

86 Adult male Balb/C mice were obtained from the animal breeding facility at the Veterinary
87 Faculty (University of Ljubljana, Slovenia). All the experiments followed ethical standards
88 and were approved by the administration of the Republic of Slovenia for food safety,
89 veterinary and plant protection with permit no. 34401-20/2009/30.

90 Mice were sacrificed by cervical dislocation followed by immediate exsanguination. The
91 diaphragm muscle along with both phrenic nerves was dissected and split into two
92 neuromuscular preparations. Each hemidiaphragm was maintained in oxygenated standard
93 Krebs-Ringer solution composed of the following: 154 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1
94 mM MgCl₂, 5 mM HEPES and 11 mM D-glucose (pH 7.4). All experiments were performed
95 at 22-24°C.

96 **2.2 Methods**

97 **2.2.1 Muscle twitch and tetanus recordings**

98 The hemidiaphragm was pinned to the organ bath on its lateral side. It was connected to an
99 isometric mechano-electrical transducer (Grass Instruments, West Warwick, RI, USA) by
100 silky thread *via* a stainless-steel hook, attached to its tendinous part. The motor nerve of the
101 isolated neuromuscular preparation was stimulated using a square pulse S-48 stimulator
102 (Grass Instruments, West Warwick, RI, USA) *via* a suction electrode with pulses of 0.1 ms
103 and supramaximal voltage (typically 5-10 V) applied at a 0.1-Hz frequency. Direct muscle
104 stimulation was evoked by electric field stimulation *via* a platinum electrode assembly in the
105 organ bath connected to the S-48 Grass stimulator and producing pulses of 0.1 ms and 50-120
106 V applied at a 0.1-Hz frequency. Directly- (*via* muscle stimulation) or indirectly- (*via* nerve
107 stimulation) evoked tetanic contraction was obtained with a pulse train duration of 1000 ms
108 and a stimulation rate of 80 Hz. To achieve the maximal contractile response upon nerve-
109 evoked stimulation, the stable resting tension for each neuromuscular preparation (typically
110 1.5-2.5 g) was adjusted approximately 20 min before starting the experiment. Muscle twitch
111 or tetanic tension was measured using a Grass FT03 force transducer (Grass Instruments,
112 West Warwick, RI, USA). Electrical signals were amplified using a P122 strain gauge
113 amplifier (Grass Instruments, West Warwick, RI, USA) and digitized using a data acquisition
114 system (Digidata 1440A; Molecular Devices, Sunnyvale, CA, USA) at a sampling rate of 1
115 kHz. The inhibitory response of nPoly-3-APS was continuously measured during 60-90 min
116 after compound application. The muscle twitch or tetanic tension blockade produced by
117 nPoly-3-APS was expressed as the percentage of the maximal response recorded before
118 compound application. The concentrations of nPoly-3-APS used were 9.1, 18.1, 27.2, 36.2,
119 45.3 and 54.4 μ M.

120 **2.2.2 Membrane potential recordings**

121 Hemidiaphragm preparations were equilibrated for 30 min in standard Krebs-Ringer solution
122 added with 1.6 μ M μ -conotoxin GIIIB, which blocks Na_v1.4 muscle sodium channel subtypes

123 (Cruz et al., 1985; Hong and Chang, 1989) and allows the recording of the full-sized
124 amplitude of the endplate potentials (EPPs). Miniature endplate potentials (MEPPs), EPPs
125 and the resting membrane potential (rV_m) were recorded from superficial hemidiaphragm
126 muscle fibers using intracellular borosilicate glass microelectrodes and Axoclamp 900A
127 microelectrode amplifier (Molecular Devices, Sunnyvale, CA, USA). Microelectrodes were
128 pulled using a P-97 Flaming/Brown micropipette puller (Sutter Instruments, Novato, CA,
129 USA) and were filled with 3 M KCl solution. Only microelectrodes with resistance of 10-20
130 M Ω were used. Endplate regions, where EPPs were recorded, were indicated by the presence
131 of MEPPs. EPPs were evoked by stimulating the phrenic nerve *via* the bipolar suction
132 electrode with supramaximal square pulses of 0.1 ms at 0.5 Hz using the S-48 Grass
133 stimulator. MEPPs and EPPs were digitized at 25 kHz using Digidata 1440A and pCLAMP
134 10 (Molecular Devices, Sunnyvale, CA, USA) and were stored for later analysis using
135 pCLAMP-Clampfit 10 software (Molecular Devices, Union City, CA, USA). The recordings
136 were performed before treatment, 45 and 90 min after the application of nPoly-3-APS and 15
137 min after the compound wash-out. MEPP and EPP amplitudes were normalized to a rV_m of -
138 70 mV using the formula $V_c = V_o \times (-70)/rV_m$, where V_c is the normalized amplitude of
139 MEPPs and EPPs, and V_o is their recorded amplitude (Pardo et al., 2006). The concentrations
140 of nPoly-3-APS used were 1.8, 9.1, 18.1, 27.2, 36.2 and 54.4 μ M.

141 **2.2.3 Data analysis and statistics**

142 The results are presented as the means \pm S.E. The nPoly-3-APS concentrations producing
143 50% inhibition of initial responses (IC_{50}) were determined by fitting the concentration-
144 response relationships using the four-parameter nonlinear regression model (GraphPad Prism
145 version 6.00). The data were statistically analyzed using Sigma Plot for Windows version
146 12.5 (Systat Software Inc., USA). Student's two-tailed *t*-test was used for statistical analysis
147 of the data. Statistical significance was set at a *P* value \leq 0.05.

148

149 **3 Results**

150 **3.1 nPoly-3-APS blocks muscle contraction *in vitro***

151 The effects of natural nPoly-3-APS (9.1-54.4 μ M) were first determined on nerve-evoked and
152 directly muscle-elicited isometric twitch and tetanic contraction in mouse hemidiaphragm
153 preparations. A representative example of the time-course of nPoly-3-APS effects on muscle
154 contraction is shown in Fig. 1A. The compound (36.2 μ M) blocked nerve-evoked twitch and
155 tetanic contraction within 90 min (Fig. 1A and B). This effect was in part reversible since,
156 after about 30 min of thorough washing of the preparation with standard physiological
157 solution, the nerve-evoked muscle twitch and tetanic contraction were restored partially (Fig.
158 1A). In contrast, directly muscle-elicited twitch and tetanic contraction were much less
159 affected by a similar compound concentration (Fig. 1A and C). The nPoly-3-APS-induced
160 block of muscle contraction occurred in a concentration-dependent manner (Fig. 1D and E).
161 The IC_{50} values, calculated from the concentration-response curves, were 18.5-29.4 μ M for
162 nerve-evoked muscle contraction, and 56-90 μ M for directly muscle-elicited contraction (Fig.
163 1E). Therefore, nPoly-3-APS was more than 3 times more efficient to block nerve-evoked
164 than directly muscle-elicited twitch and tetanic contraction. It is worth noting that no decrease

165 of muscle contraction amplitude occurred during 90-min control experiments during which
166 neuromuscular preparations were only bathed with Krebs-Ringer solution (data not shown).

167 Figure 1 near here

168 During the development of the contraction block, obtained 30 min after the application of
169 36.2 μM nPoly-3-APS (*i.e.* $33 \pm 4.4\%$, $n = 4$), 3 μM neostigmine, which is known to inhibit
170 AChE (Alderdice, 1982), did not modify muscle twitch and tetanic contraction (data not
171 shown). Additionally, 300 μM 3,4-diaminopyridine, a well-known potassium channel blocker
172 in nerve terminals (Molgò et al., 1980), significantly increased the twitch and tetanic
173 contraction amplitudes to approximately $120 \pm 11\%$ ($n = 4$) of their initial value (data not
174 shown).

175 **3.2 Effects of nPoly-3-APS on rV_m , MEPPs and EPPs**

176 In a second set of experiments, we tested whether nPoly-3-APS has some effects on rV_m ,
177 MEPPs and EPPs since the compound was shown to block preferentially nerve-evoked
178 muscle twitch and tetanic contraction.

179 **3.2.1 nPoly-3-APS does not affect rV_m**

180 nPoly-3-APS (1.8-54.4 μM), *i.e.* at concentrations that partially or completely block nerve-
181 evoked muscle twitch and tetanic contraction in mouse hemidiaphragms (see Fig. 1D and E),
182 did not significantly affect the rV_m of muscle fibers recorded in the endplate regions of the
183 surface fibers after 45 and 90 min of compound application (Fig. 2). In particular, the mean
184 rV_m was -65.5 ± 1.7 mV under control conditions (*i.e.* at time zero, before compound
185 application), and -64.3 ± 1.5 mV and -61.2 ± 0.6 mV, 45 and 90 min after exposure to 54.4
186 μM nPoly-3-APS, respectively.

187 Figure 2 near here

188 **3.2.2 nPoly-3-APS reduces MEPP and EPP amplitudes**

189 The effects of nPoly-3-APS on the MEPP amplitude and frequency are shown in Fig. 3.
190 nPoly-3-APS (9.1-27.2 μM), in a concentration-dependent manner, significantly reduced the
191 amplitude of MEPPs recorded in superficial muscle fibers of the mouse hemidiaphragm (Fig.
192 3A and B), without significant decrease of their frequency except at the highest compound
193 concentration (Fig. 3C). At higher concentrations (36.2-54.4 μM), nPoly-3-APS completely
194 abolished MEPPs (Fig. 3A). No significant change in the EPP half-decay time was observed
195 among the concentrations studied (Fig. 3A).

196 Figure 3 near here

197 Electrophysiological recordings of EPPs from the endplates of the mouse hemidiaphragm
198 exposed to nPoly-3-APS (1.8-54.4 μM) for 90 min revealed that the compound reduced their
199 amplitude in a concentration-dependent manner (Fig. 4). The IC_{50} calculated value for the
200 nPoly-3-APS-induced reduction of the EPP amplitude was 20.1 μM (Fig. 4B). No significant
201 change in the EPP half-decay time was observed among the concentrations studied (Fig. 4A).

202 Figure 4 near here

203

204 4. Discussion

205 This study shows, for the first time, the effects of natural alkylpyridinium polymer (nPoly-3-
206 APS), produced by the marine sponge *Reniera sarai*, on neuromuscular transmission and
207 skeletal muscle functioning. In a previous study, it was shown that nPoly-3-APS after *i.v.*
208 administration at a sublethal dose to male Wistar rats increased the residual respiratory
209 volume, prolonged expiration, produced bradypnea; at a lethal dose, nPoly-3-APS causes
210 respiratory arrest (Bunc et al., 2000). However, nPoly-3-APS showed many interesting
211 biological activities (reviewed in Turk et al., 2007). Its application in medicine led to the
212 synthesis of a series of synthetic analogs with different degrees of polymerization and
213 different lengths of alkyl chains (Houssen et al., 2010). Recently, the effects of some of these
214 synthetic analogs due to their AChE inhibitory potency were studied on the skeletal
215 neuromuscular transmission (reviewed in Grandic and Frangez, 2014). Experiments on
216 skeletal neuromuscular preparations revealed that APS12-2, a non-competitive AChE
217 inhibitor which was previously shown to be selectively cytotoxic toward NSLC cells *in vitro*,
218 is a potent antagonist of skeletal muscle nAChRs (Grandic et al., 2012). Later, it was shown
219 that the synthetic polymeric 3-alkylpyridinium salt (APS3) *in vitro* inhibits nerve-evoked
220 muscle contraction of the mouse hemidiaphragm due to specific block of muscle-type
221 nAChRs with an IC₅₀ of 20.3 μM (Grandic et al., 2012). Therefore, it was of interest to
222 examine the natural molecule nPoly-3-APS for similar biological effects on skeletal
223 neuromuscular transmission and to propose different mechanistic hypotheses to explain its
224 underlying molecular mechanism of action.

225 Our results obtained from phrenic nerve-hemidiaphragm preparations are summarized in
226 Table 1. They show that nPoly-3-APS, at concentrations ranging from 9.1 to 54.4 μM,
227 progressively blocked the maximal amplitude of nerve-evoked isometric muscle twitch,
228 sustained (tetanic) contraction and EPPs, without any noticeable change in their decay phase.
229 Additionally, relatively small decrease of maximal amplitude was observed on directly
230 muscle-elicited twitch and tetanic contraction. The natural compound, without notably
231 affecting the frequency (except a reduction observed in the presence of the highest compound
232 concentration) and the decay phase, decreased the MEPP amplitude measured in
233 hemidiaphragm superficial muscle fibers. Taking into account that the muscle fiber rV_m
234 remained unchanged even after the longest exposure to the highest concentration of nPoly-3-
235 APS used, these results indicate a preferential postsynaptic activity of the natural compound.
236 Indeed, they are exactly what is expected if nPoly-3-APS produces muscle-type nAChR
237 blockage (Table 1). This mechanistic hypothesis was previously proposed to explain the
238 effects of APS12-2 and APS3, two synthetic analogs of polymeric alkylpyridinium salts
239 isolated from *Reniera sarai*, which were similar to those of the natural compound (Grandic et
240 al., 2012; Grandic et al., 2013). Additionally, available information in the literature indicates
241 that 3-alkylpyridinium polymers from the marine sponge *Reniera sarai*, at approximately
242 200-2000 times lower concentrations than those used in the present study, possess strong
243 AChE activity, and a detailed mechanism of kinetics of AChE inhibition has been proposed
244 (Sepcic et al., 1997; Sepcic et al., 1998; reviewed in Turk et al., 2007). The lack of AChE
245 activity at the neuromuscular junction level allows acetylcholine (ACh) to accumulate in the
246 synaptic cleft and thus to activate repetitively nAChRs (Van der Kloot et al., 1994). It is
247 characterized, in particular, by muscle inability to sustain a tetanic contraction under

248 repetitive high frequency stimulation of the motor nerve, due to both ACh accumulation and
249 decreased rV_m , by enhancement of nerve-evoked muscle twitch, EPP and MEPP amplitudes,
250 as well as MEPP frequency, and by prolongation of their decay phase (Table 1; Chang et al.,
251 1986; Katz and Miledi, 1973). However, no physiological evidence was obtained in the
252 present study of an eventual anti-AChE effect of nPoly-3-APS since we did not observe any
253 of these AChE inhibition characteristics (Table 1). Furthermore, the nerve-evoked muscle
254 twitch block was not antagonized/augmented by 3 μ M neostigmine, which almost completely
255 inhibits AChE in the neuromuscular junctions of the mouse hemidiaphragm (Minic et al.,
256 2003). Possible involvement of anti-AChE activity of the natural compound is therefore
257 excluded in the present results. Different hypotheses can be proposed to explain the absence
258 of an observation of nPoly-3-APS-induced AChE inhibition at the level of mouse skeletal
259 neuromuscular junction. The first is that such an inhibition is masked by the nPoly-3-APS-
260 produced blockage of muscle-type nAChRs. However, none of the AChE inhibition
261 characteristics were observed at low concentrations (1.8-9.1 μ M) which produced no marked
262 nAChR blockage even if they were 100-1000 times higher than those reported previously to
263 inhibit AChE (Sepcic et al., 1997). The second hypothesis is the polycationic nature of nPoly-
264 3-APS, leading to limited ability of the natural compound to reach its binding site since its
265 AChE activity was previously shown to occur only in aqueous solutions (Sepcic et al., 1997),
266 which may also have an impact on its activity in biological systems. Finally, it is worth noting
267 that these authors reported only inhibition of recombinant insect, electric eel and human
268 erythrocyte AChE, as well as equine BuChE, without any information on mammal neuronal
269 AChE. Knowing that there is low percentage of similarity between mammal (human and
270 mouse), insect and fish AChE amino-acid sequences (Wiesner et al., 2007), one cannot
271 exclude that the affinity of nPoly-3-APS for mouse AChE is very low compared to that for
272 mouse muscle-type AChRs.

273 Table 1 near here

274 The partial block of nerve-evoked muscle twitch produced by nPoly-3-APS was completely
275 reversed by the potassium channel blocker 3,4-diaminopyridine, known to prolong the
276 duration of action potential, to increase both Ca^{2+} entry into nerve terminals and ACh release
277 from motor terminals (Molgò et al., 1980; Hong and Chang, 1990). Antagonist effects of 3,4-
278 diaminopyridine on mouse hemidiaphragm muscle, partially paralyzed by the action of nPoly-
279 3-APS, further suggest postsynaptic effects. In the range of the concentrations studied, nPoly-
280 3-APS also showed a concentration-dependent effect on muscle excitability and excitation-
281 contraction coupling processes, as determined by the observed small blockade of contractile
282 responses by the compound (18.1-54.4 μ M) upon single and tetanic direct muscle stimulation.
283 nPoly-3-APS has been previously reported to be hemolytic and cytotoxic against different
284 mammalian cell lines (Malovrh et al., 1999) and to produce concentration-dependent
285 reversible/irreversible plasma membrane depolarization in neuronal cells (McClelland et al.,
286 2003). Persistent depolarization may affect muscle excitability and directly-elicited muscle
287 contractility (for review see Ulbricht, 2005). However, our results clearly show that the
288 compound-induced small decrease of directly muscle-elicited twitch and tetanic contraction
289 cannot be explained by a reduction of rV_m .

290 In conclusion, our results reveal, for the first time, that micromolar concentrations of nPoly-3-

291 APS completely inhibit nerve-evoked twitch and tetanic contraction as well as, although
292 partly, directly muscle-elicited twitch and tetanic contraction. No evidence was obtained in
293 the present study for an eventual anticholinesterase effect of nPoly-3-APS. The *in vivo*
294 toxicity of the natural compound is thus likely to occur through a preferential inhibition of
295 neuromuscular transmission. The most likely mechanistic hypothesis to explain such an
296 inhibition is a slowly reversible antagonist effect on muscle-type nAChRs since nPoly-3-APS
297 reduced the number of open nAChRs by altering neither muscle fiber passive membrane
298 properties nor neurotransmitter ACh release. This blocking nAChR activity remains however
299 to be confirmed by studying the natural compound effects on muscle-type nAChRs expressed
300 or incorporated in *Xenopus* oocytes.

301

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305

306 **Compliance with Ethical Standards**

307 All experiments on isolated tissue followed ethical standards and were approved by The
308 Administration of the Republic of Slovenia for food safety, veterinary and plant protection (34401-
309 20/2009/30). The manuscript does not contain clinical studies or patient data.

310

311 **Conflicts of interest**

312 The authors declare that there are no conflicts of interest.

313

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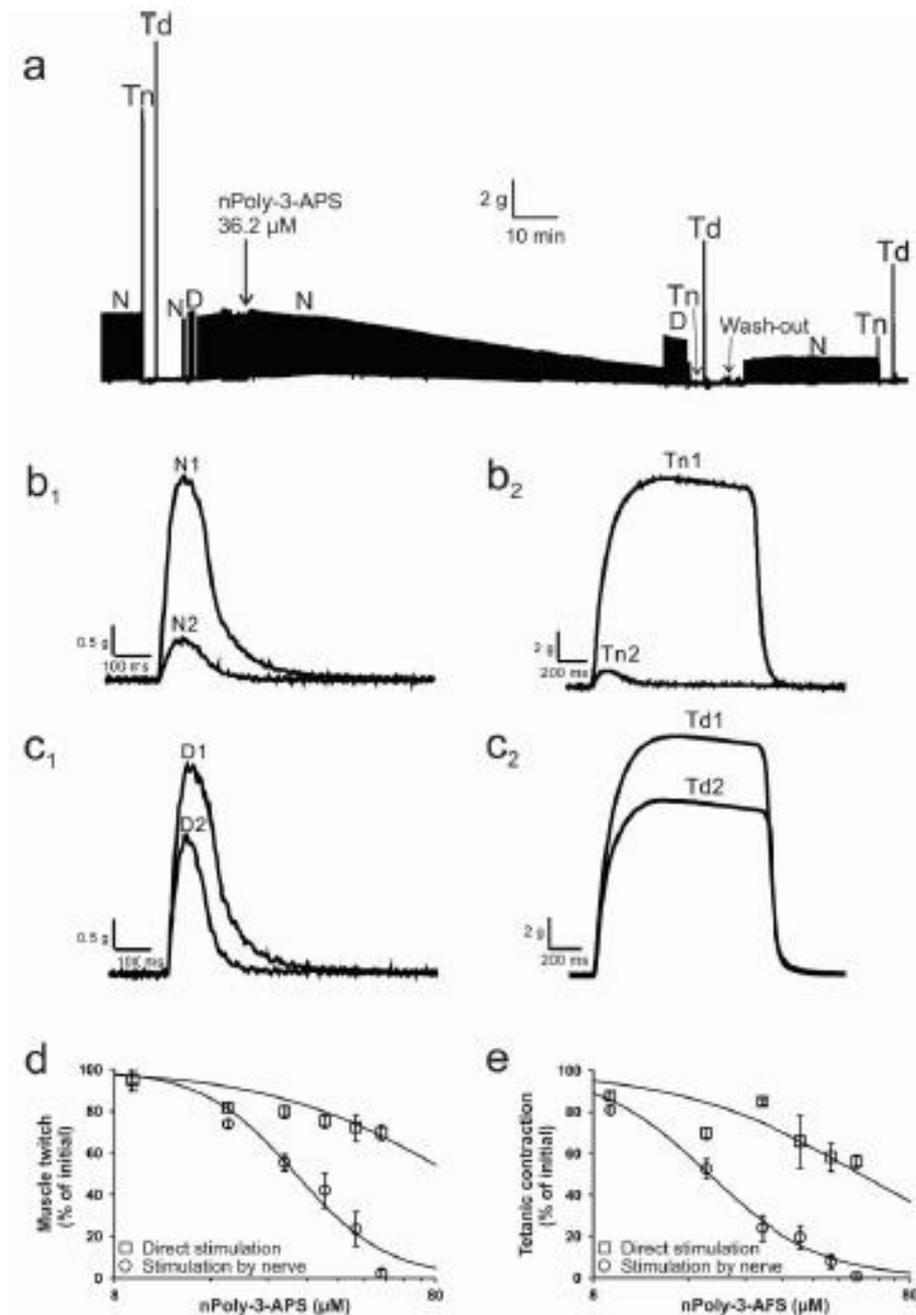
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420

421 **Table 1** Investigated parameters of mouse neuromuscular system in function of protocol stimulation,
 422 present results obtained from nPoly-3-APS effects on these parameters, and expected results assuming
 423 that the natural compound either inhibits AChE activity or blocks nAChR functioning.

Protocol stimulation	Investigated parameters ¹	Present results ¹	Expected results with nAChR blockage ¹	Expected results with AChE inhibition
Nerve	Twitch amplitude	↘ (important)	↘	↗
	Twitch decay phase	→	→	↗
	Tetanic contraction amplitude	↘ (important)	↘	↘
	Tetanic contraction decay phase	→	→	↗
	EPP amplitude	↘ (important)	↘	↗
	EPP decay phase	→	→	↗
Muscle	Twitch amplitude	↘ (small)	→	→
	Twitch decay phase	→	→	→
	Tetanic contraction amplitude	↘ (small)	→	→
	Tetanic contraction decay phase	→	→	→
None	MEPP amplitude	↘ (important)	↘	↗
	MEPP frequency	↘ (high concentrations)	↘	↗
	MEPP decay phase	→	→	↗
	Resting membrane potential	→	→	↘

424 ¹ Specific effects due to nAChR blockage are underline in grey.

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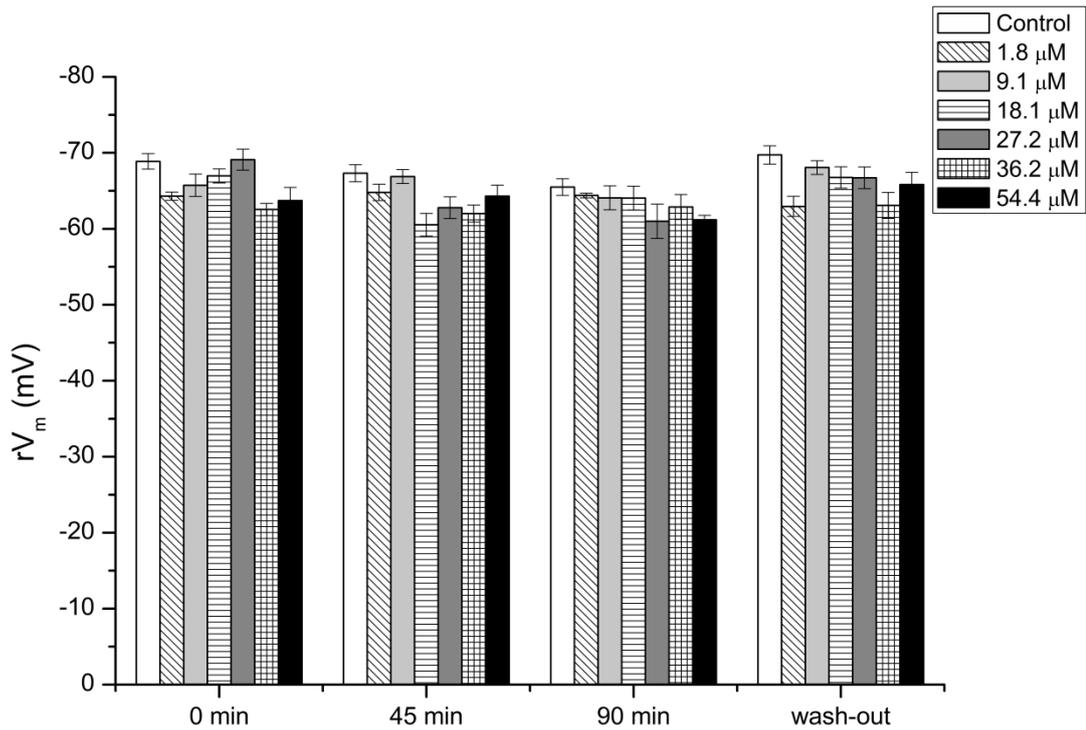
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428 **Fig 1** Effects of nPoly-3-APS on nerve-evoked and directly muscle-elicited twitch and tetanic
 429 contraction in isolated mouse hemidiaphragm neuromuscular preparations. (a) Representative
 430 time-course of nPoly-3-APS (36.2 μM) effects on muscle contraction showing the partial and
 431 reversible block of nerve-evoked and directly muscle-elicited twitch and tetanic contraction
 432 induced by the compound. N denotes nerve-evoked muscle twitch; D denotes directly muscle-
 433 elicited twitch; Tn denotes nerve-evoked tetanic contraction; Td denotes directly muscle-
 434 elicited tetanic contraction. (b1) Traces of nerve-evoked twitch recorded before (N1) and 90
 435 min after (N2) the application of 36.2 μM nPoly-3-APS. (b2) Traces of tetanic contraction
 436 recorded before (Tn1) and 90 min after (Tn2) the application of 36.2 μM nPoly-3-APS. (c1)
 437 Representative recordings of twitch elicited by direct muscle stimulation, before (D1) and 90
 438 min after (D2) the application of 36.2 μM nPoly-3-APS. (c2) Representative recordings of

439 tetanic contraction elicited by direct muscle stimulation, before (Td1) and 90 min after (Td2)
440 the application of 36.2 μM nPoly-3-APS. (d) Concentration-response curves for the inhibition
441 of directly muscle-elicited and nerve-evoked twitch by nPoly-3-APS. Muscle twitch was
442 expressed as percentage of its maximal initial value. Each value represents the mean \pm S.E.
443 obtained from five different muscles. IC_{50} values, determined by fitting the relationships with
444 the four-parameter nonlinear regression model, were 29.4 and 90 μM for nerve-evoked and
445 directly muscle-elicited twitches, respectively ($r^2 \geq 0.993$). (e) Concentration-response curves
446 for the inhibition of directly muscle-elicited and nerve-evoked tetanic contraction by nPoly-3-
447 APS. Tetanic contraction was expressed as percentage of its maximal initial value. Each value
448 represents the mean \pm S.E. obtained from five different muscles. IC_{50} values, determined by
449 fitting the relationships with the four-parameter nonlinear regression model, were 18.5 and 56
450 μM for nerve-evoked and directly muscle-elicited tetanic contractions, respectively ($r^2 \geq$
451 0.978)

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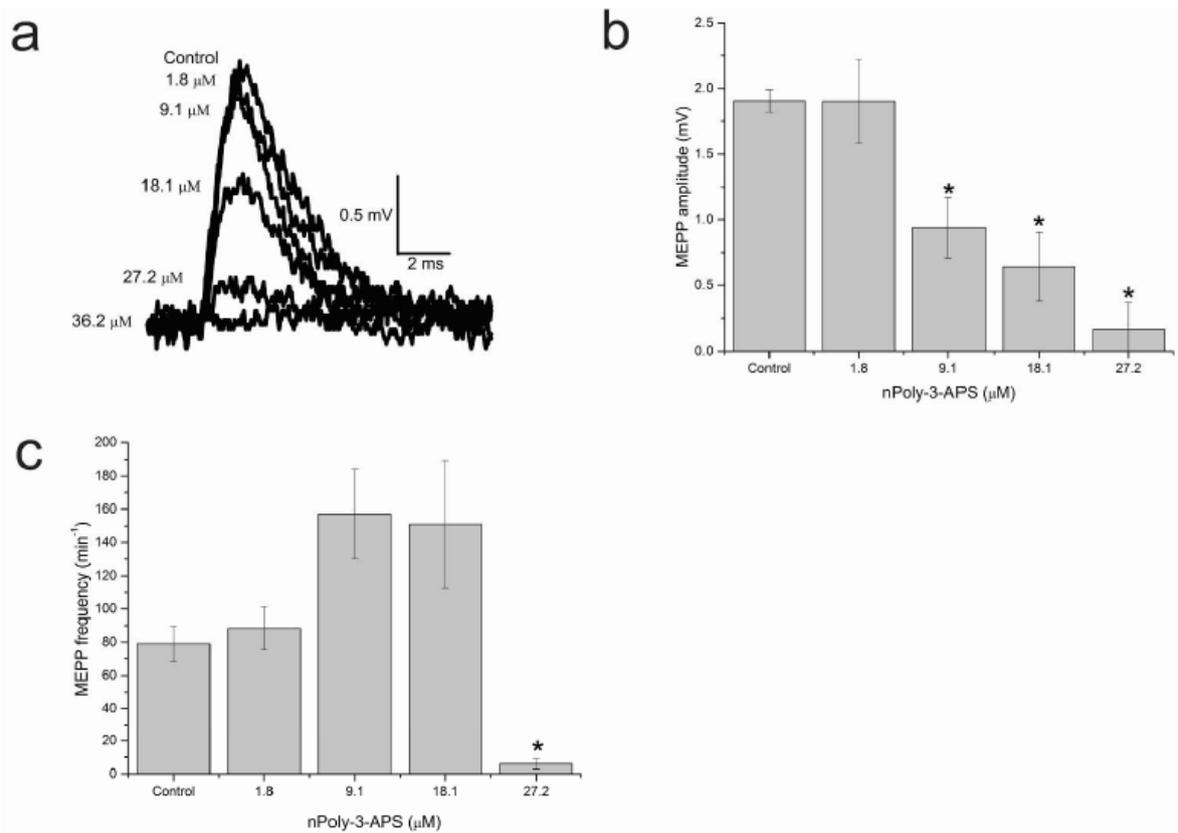


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455 **Fig 2** Concentration- and time-dependent effect of nPoly-3-APS on the muscle fiber resting
 456 membrane potential (rV_m) of the mouse hemidiaphragm. Neuromuscular preparations were
 457 exposed to 1.8-54.4 μM nPoly-3-APS. Measurements were performed before (time 0), 45 and
 458 90 min after nPoly-3-APS application, and after about 30 min of thorough washing of the
 459 preparation with standard physiological solution. Each value represents the mean \pm S.E. of
 460 rV_m recorded from 3 different hemidiaphragms ($n = 7-10$ fibers from each)

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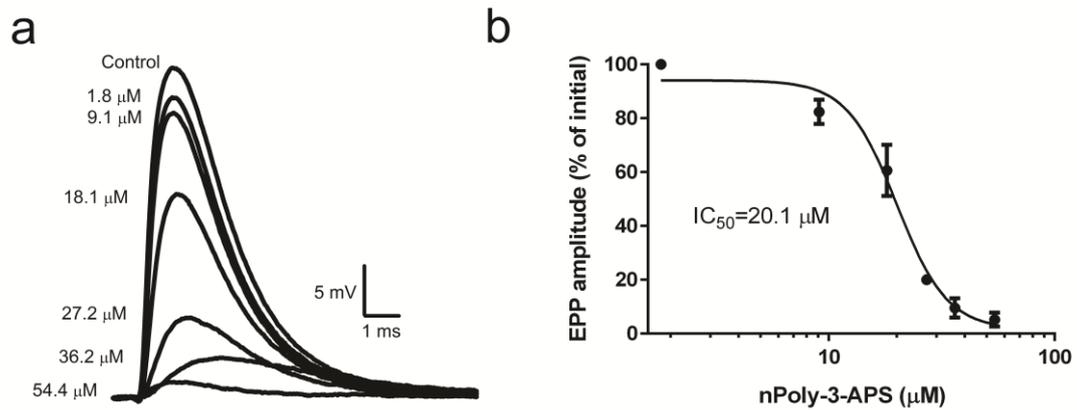


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464 **Fig 3** Effects of nPoly-3-APS on the MEPP amplitude and frequency in the muscle fibers of
 465 isolated mouse hemidiaphragm. (a) MEPP traces recorded under control conditions and after
 466 the indicated concentrations of nPoly-3-APS applied for 90 min. At 36.2 μM, the compound
 467 completely blocked MEPPs. (b) MEPP amplitude and (c) MEPP frequency in muscle fibers
 468 exposed to different nPoly-3-APS concentrations. Each bar represents the mean value ± S.E.
 469 from 3 different hemidiaphragms ($n = 7-10$ fibers from each). * indicates statistically
 470 significant difference ($P \leq 0.05$)

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474 **Fig 4** Effects of nPoly-3-APS on EPP amplitude in mouse hemidiaphragm skeletal muscle
 475 fibers. The compound blocked EPPs in a concentration-dependent manner. (a) EPP traces
 476 recorded under control conditions and 90 min after exposure of muscle fibers to various
 477 nPoly-3-APS concentrations. Note that the decrease of EPP amplitudes occurred without
 478 significant change in rV_m . (b) Concentration-response curve for the inhibition of EPP
 479 amplitude, expressed as a percentage of its maximal initial value, by nPoly-3-APS3. Each
 480 value represents the mean \pm S.E. of EPPs recorded from at least 3 different hemidiaphragms
 481 ($n = 7-10$ muscle fibers from each). The IC_{50} value, determined by fitting the relationship with
 482 the four-parameter nonlinear regression model, was $20.1 \mu M$ ($r^2 = 0.985$)