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RESEARCH ARTICLE

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Effect of endogenous purines on electrically evoked ACh release at the mouse neuromuscular junction

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Abstract

At the mouse neuromuscular junction, adenosine triphosphate (ATP), which is co-released with the neurotransmitter acetylcholine (ACh), and its metabolite adenosine, modulate neurotransmitter release by activating presynaptic inhibitory $P2Y_{13}$ receptors (a subtype of ATP/adenosine diphosphate [ADP] receptor), inhibitory A_1 and A_3 adenosine receptors, and excitatory A_{2A} adenosine receptors. To study the effect of endogenous purines, when phrenic-diaphragm preparations are depolarized by different nerve stimulation patterns, we analyzed the effect of the antagonists for $P2Y_{13}$, A_1, A_3, and A_{2A} receptors (AR-C69931MX, 8-cyclopentyl-1,3-dipropylxanthine, MRS-1191, and SCH-58261, respectively) on the amplitude of the end-plate potentials of the trains, and contrasted these results with those obtained with the selective agonists of these receptors (2-methylthioadenosine 5′-diphosphate trisodium salt hydrate, 2-chloro-N^6-cyclopentyl-adenosine, inosine, and PSB-0777, respectively). During continuous 0.5-Hz stimulation, the amount of endogenous purines was not enough to activate purinergic receptors, while at continuous 5-Hz stimulation, an incipient action of endogenous purines on $P2Y_{13}$, A_1 and A3 receptors might be evident just at the end of the trains. During continuous 50-Hz stimulation, the concentration of endogenous ATP/ADP and adenosine exerted an inhibitory action on ACh release after of the initial phase of the train, but when the nerve was stimulated at intermittent 50 Hz (5 bursts), this behavior was not observed. Excitatory A_{2A} receptors were only activated when continuous 100-Hz stimulation was applied. In conclusion, when motor nerve terminals are depolarized by repetitive stimulation of the phrenic nerve, endogenous ATP/ADP and adenosine are able to fine-tune neurosecretion depending on the frequency and pattern of stimulation.

KEYWORDS
adenosine, ATP/ADP, electrical depolarization, purinergic receptors

Abbreviations: ACh, acetylcholine; AR-C69931MX, *N*-[2-(methylthio)ethyl]-2-[3,3,3-trifluoropropyl]thio-5′-adenylic acid, monoanhydride with (dichloromethylene)bis[phosphonic acid], tetrasodium salt; ADP, adenosine diphosphate; ATP, adenosine triphosphate; CCPA, 2-chloro-N^6-cyclopentyl-adenosine; CICUAL, Institutional Commission for the Care and Use of Laboratory Animals; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; EPP, end-plate potential; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MEPPs, miniature end-plate potentials; 2-MeSADP, 2-methylthioadenosine 5′-diphosphate trisodium salt hydrate; MRS-1191, (3-Ethyl-5-benzyl-2-methyl-4-phenylethynyl-6- phenyl-1, 4-(±)-dihydropyridine-3,5-, dicarboxylate; nAChR, nicotinic acetylcholine receptor; NBTI, S-(p-nitrobenzyl)-6-thioinosine; NMJ, neuromuscular junction; PSB-0777, 4-[2-[(6-Amino-9-b-D-ribofuranosyl-9*H*-purin-2-yl)thio]ethyl] benzenesulfonic acid ammonium salt; SCH-58261, 2-(2-Furanyl)-7-(2-phenylethyl)-7*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-c]pyrimidin-5-amine; SEM, standard error of mean; VDCCs, voltage-dependent calcium channels; Vm, resting membrane potential.

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1 | INTRODUCTION

Synaptic transmission is under the control of extracellular purines, which by activating their specific receptors (Rs), modulate the neural activity. At the neuromuscular junction (NMJ), adenosine triphosphate (ATP) is released together with acetylcholine (ACh) into the synaptic space, where it is degraded to adenosine via ectonucleotidases (Cunha & Sebastião, 1991; Magalhães-Cardoso et al., 2003; Redman & Silinsky, 1994). This means that the concentration of adenosine in the synaptic cleft depends on synaptic activity (Magalhães-Cardoso et al., 2003; Silinsky & Redman, 1996), although the nucleoside can be released as such via bi-directional nucleoside transporters (Barroso et al., 2007; Correia-de-Sá et al., 1996) present in all cell membranes (reviewed in Kong et al., 2004). Besides, it was described that purines can be released from activated muscle fibers (Cunha & Sebastião, 1993. Santos et al., 2003; Smith, 1991) and perisynaptic Schwann cells (PSC, Liu et al., 2005; Todd & Robitaille, 2006).

It is known that, at motor nerve terminals, ATP/adenosine diphosphate (ADP) as well as adenosine regulates neurotransmitter release when activating presynaptic inhibitory and facilitatory P2 and P1 Rs, respectively (Correia-de-Sá et al., 1996; De Lorenzo et al., 2004, 2006; Giniatullin & Sokolova, 1998; Salgado et al., 2000; Sebastião & Ribeiro, 2000; Sokolova et al., 2003; reviewed by Ziganshin et al., 2020). At the mouse NMJs, we have previously found that the cholinergic release is modulated by activation of inhibitory $P2Y_{13}$Rs (De Lorenzo et al., 2006; Guarracino et al., 2016; Veggetti et al., 2008), A_1Rs and A_3Rs (Cinalli et al., 2013; De Lorenzo et al., 2004), and excitatory A_{2A}Rs (Palma et al., 2011).

At mature mammalian NMJs, high K^+ concentration induces a sustained elevation of miniature end-plate potential (MEPP) frequency which depends on the membrane potential depolarization of the motor nerve terminals (Lliley, 1956). This enhancement in the asynchronic ACh release is attributed to the increase in the Ca^{2+}influx through the voltage-dependent calcium channels (VDCCs) type P/Q (Losavio & Muchnik, 1997; Protti & Uchitel, 1993), the same channels that are involved in the electrically evoked ACh release (Bowersox et al., 1995; Hong & Chang, 1995; Protti & Uchitel, 1993; Wright & Angus, 1996), although under certain stimulation pattern (Correia-de-Sá et al., 2000) or physiopathological conditions (García & Beam, 1996; Katz et al., 1996; Santafé et al., 2000), L-type VDCCs may contribute to such release. We have recently studied the role of the endogenous purines on cholinergic release when the nerve terminals were depolarized by increased K^+ concentrations (10–20 mM K^+; Guarracino et al., 2018). We found that at 10 mM K^+, ATP/ADP bound to $P2Y_{13}$Rs provoking the inhibition of ACh release, whereas adenosine levels in the synaptic cleft were not enough to activate A_1, A_{2A}, and A_3Rs possibly because the nucleoside was carried out to the interior of the cells by equilibrative transporters. In fact, inhibition of the adenosine uptake by NBTI provoked a reduction of 10 mM K^+-evoked ACh release which was prevented in the presence of the A_1R antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), suggesting that endogenous adenosine was being removed from the synaptic space by the transporters. At 15 and 20 mM K^+, endogenous ATP/ADP activated its Rs and endogenous adenosine, now was able to bind to inhibitory A_1 and A_3Rs. The activation of excitatory A_{2A}Rs could only be observed at 20 mM K^+. These findings suggest that, at high K^+ concentration, the activity of the equilibrative nucleoside transporters was not enough to decrease adenosine concentration in the synaptic cleft.

Taken into account these results, the aim of this investigation was to analyze the effect of endogenous nucleotides and nucleosides at the mice NMJs when phrenic-diaphragm preparations are depolarized by nerve stimulation. Although high K^+- and electrically evoked ACh release share some characteristics, the correlation between the sustained depolarization induced by increased K^+ concentrations and the phasic depolarization provoked by increased stimulation patterns (0.5–50 Hz) is not clear. Thus, we studied the effect of the antagonists for $P2Y_{13}$, A_1, A_3, and A_{2A}Rs on the end-plate potential (EPP) amplitude when the phrenic nerve was stimulated at different stimulation patterns. Then, the results were contrasted with those obtained in the presence of agonists of each of the purinergic Rs.

Significance

At mammalian neuromuscular junction, adenosine triphosphate is co-released with acetylcholine, and once in the synaptic cleft, it is hydrolyzed to adenosine. Both purines bind to specific presynaptic receptors. We found that, when motor nerve terminals are depolarized by electrical stimulation, endogenous purines are able to modulate neurotransmitter release depending on the frequency and pattern of stimulation. These findings contribute to understand in more detail the role of the purines as neuromodulators of the cholinergic transmission and raise the possibility to use selective ligands of the different purinergic receptors as a therapeutic tool to improve neurotransmission in the neuromuscular diseases.

2 | METHODS

2.1 | Preparations and solutions

All experimental studies were approved by the Institutional Animal Care and Use Committee (IACUC of the Instituto de Investigaciones Médicas Alfredo Lanari, Re. #058-17-059-17), and protocols were performed following the *National Institute of Health Guide for the Care and Use of Laboratory Animals* (NIH Publications no. 80-23), revised 1996. Mice were housed in cages of four animals containing physical enrichment and kept on 12-h light–dark cycles, at 20 ± 2°C and 60%–70% humidity. The cages were cleaned twice a week, and food and water were available ad libitum. A total of 157 CF1 mice (83 males and 74 females), aged 12–32 weeks (30–40 g), were anesthetized with ketamine 100 mg.kg^{-1} + xylazine 10 mg.kg^{-1}

intraperitoneally. The experiments were performed on left phrenic nerve-hemidiaphragm preparations superfused with a bathing solution containing (mM): NaCl 135, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, D-glucose 11, HEPES 5, pH 7.3–7.4, and bubbled with O_2.

2.2 | Electrophysiological recordings

Intracellular recordings were performed at the end-plate region from muscle fibers using borosilicate glass microelectrodes (WP Instruments, Sarasota, FL, USA) filled with 3 M KCl and a resistance of 5–10 MΩ. When resting membrane potential (Vm) was less negative than −60 mV or EPP had a rise time greater than 1 ms, the muscle fiber was excluded. We only accepted those fibers where their Vm did not deviate by more than 5 mV during the train. The nerve was stimulated through a suction electrode placed near its entrance to the muscle, with supramaximal stimuli (pulse width 0.1 ms) at different stimulation patterns: continuous at 0.5 Hz (50 pulses), continuous at 5 Hz (750 pulses), continuous at 50 Hz (750 pulses), intermittent at 50 Hz (5 bursts of 150 pulses, 20s interburst interval, Correia-de-Sá et al., 1996), and continuous at 100 Hz (750 pulses). Each hemidiaphragm underwent only a single stimulation paradigm in the presence of a single drug (agonist/antagonist for $P2Y_{13}$, A_1, A_3, or A_{2A}Rs). Typically, in each experiment, EPPs were measured at control solution (9–10 fibers) with the selected stimulation frequency; then, the preparation was washed and incubated with the antagonist/agonist of a purinergic receptor for 20–40 min., and finally the EPPs were recorded in the solution containing the drug with the stimulation pattern used in control saline (9–10 fibers). A recovery period of 10–15 min was allowed between trains. Pulses were delivered by a Grass S48 stimulator (Grass Instruments, Quincy, MA, USA) linked to a stimulus isolation unit (Grass SIU5). To prevent muscle contraction during EPP recordings, a submaximal concentration (0.8–1.6 μM) of *d*-tubocurarine was used. EPP amplitudes were normalized to −75 mV, assuming 0 mV as the reversal potential for ACh-induced current (Magleby & Stevens, 1972), using the formula $Vc = [Vo \times (-75)]/E$, where Vc is the corrected EPP amplitude, Vo is the observed EPP, and E the Vm. Signals were amplified with Axoclamp 2B (Axon Instruments, USA), digitized with Digidata 1200 (Axon Instruments), and analyzed via the software pClamp 8.2 (Axon Instruments). Experiments were performed at room temperature.

2.3 | Data analysis

Data are reported as mean ± SD and *n* expresses number of animals. Statistical analyses were performed in Graph Pad Prim 8 and Matlab 2017b. The sample size was calculated with G Power 3.1.9.7 using values found in literature. The distribution of the data in each experiment was tested for normality using Shapiro–Wilk test. No outliers were identified and no data were excluded from the analysis. When EPP amplitudes were measured in the same diaphragm, in the presence of control and antagonist/agonist solution, paired Student's *t* test was used. Repeated measures one-way analysis of variance (ANOVA) followed by Tukey test was used for the analysis of the five bursts at 50 Hz. Statistical comparisons among three or more groups were performed using one-way ANOVA followed by Tukey test (to compare all pairs of columns) or Dunnett test (to compare all columns to control column). Differences were considered to be significant when $p < .05$.

2.4 | Chemicals

2-chloro-N^6-cyclopentyl-adenosine (CCPA, 500 nM), DPCPX (0.1 μM), 3-ethyl-5-benzyl-2-methyl-4-phenylethynyl-6- phenyl-1, 4-(±)-dihydropyridine-3,5-dicarboxylate (MRS-1191, 5 μM), inosine (100 μM), and 2-methylthioadenosine 5′-diphosphate trisodium salt hydrate (2-MeSADP, 150 nM) were purchased from Sigma-Aldrich Corp., St. Luis, MO, USA; 2-(2-furanyl)-7-(2-phenylethyl)-7*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-5-amine (SCH-58261, 50 nM) and 4-[2-[(6-amino-9-b-D-ribofuranosyl-9*H*-purin-2-yl)thio] ethyl] benzenesulfonic acid ammonium salt (PSB-0777, 20 nM) were from Tocris Bioscience, Ellisville, MO, USA; and *N*-[2-(methylthio)ethyl]-2-[(3,3,3-trifluoropropyl) thio]-5′-adenylic acid, monoanhydride with (dichloromethylene)bis[phosphonic acid], tetrasodium salt (AR-C69931MX, 1 μM) was obtained from Johnson Matthey Pharma Services, Devens, MA, USA. All other reagents were of the highest purity available. CCPA, DPCPX, MRS-1191, PSB-0777, and SCH-58261 were made up in dimethyl sulfoxide, and inosine, 2-MeSADP, and AR-C69931MX were made up in pure water. All stock solutions were aliquoted and frozen at −20°C. Aqueous dilutions of these stock solutions were made daily, and appropriate solvent controls were done.

3 | RESULTS

3.1 | Activation of $P2Y_{13}$Rs by endogenous ATP/ADP when ACh release is evoked by different stimulation patterns

Nucleotide Rs can be divided into P2XRs, which are ligand-gated ion channels, and P2YRs that are G protein-coupled Rs (Fredholm et al., 1994; Ravelic & Burnstock, 1998). At NMJs, the presence of both types of Rs was demonstrated (Choi et al., 2001; Deuchars et al., 2001; Moores et al., 2005). So, it has been found that, β,γ-imido ATP facilitates [^{3}H]ACh release (37°C, 5 Hz) from rat hemidiaphragm preparations presumably by activation of P2XRs (Salgado et al., 2000) but conversely, it was shown that ATP decreased evoked neurosecretion by activating P2YRs at the frog NMJs (Giniatullin & Sokolova, 1998; Sokolova et al., 2003) and, Galkin et al. (2001) depicted that ATP reduced MEPP frequency and non-quantal ACh release at mammalian NMJs due to a direct action on presynaptic metabotropic P2Rs. In previous reports, we have demonstrated that ATP and β,γ-imido ATP activate P2YRs and decrease spontaneous

release by a mechanism that involves the reduction of Ca^{2+} entry through the calcium channels related to spontaneous release (De Lorenzo et al., 2006), as well as through an effect on a Ca^{2+}-independent step in the cascade of the exocytotic process (Veggetti et al., 2008) and later on, we showed that the P2YR involved in the modulation of ACh release was of the subtype $P2Y_{13}$ (Guarracino et al., 2016). The apparent discrepancy of our findings with those by Salgado et al. (2000) might be due to differences in target species, in the recording systems, or in the experimental temperature (22°C vs. 37°C).

Our first goal was to study the effect of endogenous nucleotides upon evoked ACh release by activating $P2Y_{13}$Rs when the phrenic nerve was stimulated at 0.5, 5, and 50 Hz (continuous and intermittent stimulation). So, we analyzed the effect of the $P2Y_{13}$R antagonist AR-C6993MX (1 μM, Fumagalli et al., 2004; Jacobson et al., 2009; Marteau et al., 2003; Takasaki et al., 2001; Wirkner et al., 2004) and the $P2Y_{13}$R agonist 2-MeSADP (150 nM, Communi et al., 2001; Guarracino et al., 2016; Kubista et al., 2003) on the amplitude of the first EPP of the train, the mean amplitude of all the EPPs of the train, the last 20 EPPs of the train and the relation of the last 20 EPPs with respect to the first EPP during different stimulation frequencies.

When at control conditions the nerve is stimulated at 0.5 Hz, EPP amplitude decreases along the train following a single exponential (data not shown), likely due to the decrease in the number of synaptic vesicles of the pool of release-ready transmitter vesicles (de Freitas Lima et al., 2010; Perissinotti & Uchitel, 2010; Zefirov et al., 2009). At this nerve stimulation frequency, AR-C69931MX did not modify any of the parameters of EPP amplitude ($n = 4$, Table 1, Figure 1a,c). On the contrary, incubation of the preparations with the $P2Y_{13}$R agonist 2-MeSADP ($n = 4$) reduced the amplitude of the first EPP to ~53% of control values as well as the amplitude of the rest of the EPPs (Table 1, Figure 1b,c).

During 5-Hz stimulation (750 pulses), it is observed that at control solution, EPP amplitude declines along the train (~72% of the initial EPP) with a temporal course that fitted with a bi-exponential function: a fast phase that may reflect the rapid depletion of the rapidly releasable pool, and a slow phase that indicates a steady state level of release that may correspond to the rate of refilling of the rapidly releasable pool with vesicles from the recycling pool (de Freitas Lima et al., 2010; Perissinotti & Uchitel, 2010; Zefirov et al., 2009). Figure 1d,f, and Table 1 depict that AR-C69931MX ($n = 4$) did not significantly alter any of the EPP amplitude parameters compared to control values. Conversely, the $P2Y_{13}$R agonist 2-MeSADP ($n = 4$) significantly reduced the amplitude of the first EPP and the average of all EPPs of the train, but as it is observed in Table 1 and Figure 1e,f, the mean amplitude of the last 20 EPPs of the train was not significantly different from control solution. In fact, the ratio between the mean amplitude of the last 20 EPPs of the train and the amplitude of the first EPP in the preparations exposed to 2-MeSADP was greater than that in control solution, suggesting that the inhibitory effect of 2-MeSADP during the last EPPs was lower than the effect upon the first EPP (24% vs. 50%).

As it was observed by other authors (Bazzy, 1994; Fournier et al., 1991; Hubbard & Wilson, 1973; Krnjevic & Miledi, 1958; Moyer & Van Lunteren, 1999), continuous stimulation of the phrenic nerve at 50 Hz (750 pulses) in control solution, also evidenced an initial fall of the EPP amplitude followed by a slow declination along the train. The magnitude of the rundown at high frequency was ~88% with respect to the initial EPP. At his stimulation rate, the $P2Y_{13}$R antagonist AR-C69931MX ($n = 4$), as expected, did not alter first EPP amplitude, but significantly increased the mean amplitude of all EPPs of the train, especially at the expense of the stationary phase (See Figure 1g). So, AR-C69931MX significantly increased the mean amplitude of EPPs, the amplitude of the last 20 EPPs as well as the relation between the last 20 EPPs and the first EPP (Table 1, Figure 1g,i). On the other hand, 2-MeSADP ($n = 5$) reduced the amplitude of the initial EPP and the average of all EPPs of the train but not the amplitude of the last 20 EPPs (Table 1, Figure 1h,i). Therefore, the relation of the last 20 EPPs with respect to the first EPP was significantly increased by 2-MeSADP when compared to control solution.

Considering that respiratory muscles are activated intermittently, with a duty cycle as short as 0.25–0.35 (Kong & Berge, 1986; St John & Bartlett Jr., 1979), we studied the effect of endogenous purines when the phrenic nerve was stimulated at 50 Hz (750 pulses), but through a series of 5 bursts of 150 pulses applied with a 20-s interburst interval (Correia-de-Sá et al., 1996; Oliveira et al., 2004). In control solution, it is observed the typical rundown of the EPP amplitude in each burst which adjusted to a mono-exponential curve and a recovery of the first EPP amplitude at the beginning of the subsequent burst. We observed that the percentage of rundown of the amplitude of the last 20 EPPs in relation to the first EPP in each burst was lower than that observed with continuous 50-Hz stimulation (~70% vs. ~88%). However, the percentage of declination of the EPP amplitude during intermittent 50-Hz stimulation in each burst was similar to that obtained during the first 150 pulses of continuous 50-Hz stimulation. With this stimulation pattern, AR-C69931MX ($n = 6$) did not modify the amplitude of the first EPP of each burst, the mean amplitude of all EPPs, nor the mean amplitude of the last 20 EPPs when compared to control solution. Nevertheless, when the relation of the last 20 EPPs with respect to the first EPP was analyzed in each burst, it was significantly higher than the control (Table 1, Figure 1j,l). Conversely, 2-MeSADP ($n = 5$) in each burst reduced the amplitude of the first EPP, the mean amplitude of all EPPs and the mean size of the last 20 EPPs, although the relation last 20 EPPs/1° EPP was significantly enhanced in the last 2 bursts (Table 1, Figure 1k,l). We did not observed differences in the amplitude parameters inter-trains (among the 5 bursts of each train, repeated measures ANOVA) studied in the presence of AR-C69931MX or 2-MeSADP. In summary, data obtained during 50-Hz intermittent stimulation in the presence of AR-C69931MX only depicted an increase in the relation of the last 20 EPPs with respect to the first EPP, although this value (~31% in the 5 bursts) is lower than that observed during the 50-Hz continuous stimulation (~68%), suggesting that, at high frequency, more than 150 pulses are required to make the effect of endogenous nucleotides really obvious.

TABLE 1 Effect of AR-C69931MX and 2-MeSADP on end-plate potential (EPP) amplitudes during different nerve stimulation patterns

Solution	First EPP	EPPs[a]	Last 20 EPPs	Last 20 EPPs/first EPP	Stimulation rate
AR-C69931MX *n* = 4	98.54 ± 20.38 *t* 0.1435 *p* .8950	95.10 ± 21.86 *t* 0.4485 *p* .6842	97.56 ± 23.47 *t* 0.2077 *p* .8487	96.74 ± 12.54 *t* 0.5204 *p* .6387	Continuous 0.5 Hz (50 pulses)
2-MeSADP *n* = 4	53.14 ± 2.72*** *t* 34.49 <.0001	60.16 ± 2.91*** *t* 27.43 *p* <.0001	62.48 ± 4.26*** *t* 17.63 *p* .0004	116.80 ± 13.16 *t* 2.552 *p* .0838	
AR-C69931MX *n* = 4	117.30 ± 25.42 *t* 1.358 *p* .2677	115.10 ± 17.17 *t* 1.753 *p* .1778	110.40 ± 20.12 *t* 1.031 *p* .3783	97.15 ± 6.26 *t* 0.9104 *p* .4297	Continuous 5 Hz (750 pulses)
2-MeSADP *n* = 4	49.81 ± 9.10 ** *t* 11.03 *p* .0016	69.63 ± 17.63 * *t* 3.444 *p* .0411	76.06 ± 17.41 *t* 2.751 *p* .0707	151.00 ± 19.69 * *t* 5.186 *p* .0139	
AR-C69931MX *n* = 4	88.65 ± 9.68 *t* 2.345 *p* .1008	120.50 ± 4.27 ** *t* 9.565 *p* .0024	139.80 ± 13.90 * *t* 5.724 *p* .0106	168.10 ± 34.29 * *t* 3.971 *p* .0285	Continuous 50 Hz (750 pulses)
2-MeSADP *n* = 5	44.52 ± 7.43*** *t* 16.71 *p* < .0001	67.47 ± 13.69** *t* 5.314 *p* .0060	80.57 ± 17.52 *t* 2.480 *p* .0682	191.60 ± 51.15 * *t* 4.006 *p* .0161	
AR-C69931MX					
1	92.38 ± 27.04	112.50 ± 27.12	115.20 ± 26.33	133.40 ± 24.05***	Intermittent 50 Hz (5 bursts of 150 pulses)
2	87.32 ± 19.89	111.90 ± 20.73	113.40 ± 20.94	138.50 ± 18.53***	
3	91.13 ± 24.44	111.80 ± 18.79	113.00 ± 17.66	126.80 ± 17.78***	
4	92.13 ± 23.63	111.90 ± 19.74	116.40 ± 20.46	129.10 ± 20.37**	
5	95.97 ± 30.15	114.40 ± 19.86	121.90 ± 23.82	128.60 ± 21.54**	
n = 6	*F* 0.7062	*F* 1.905	*F* 2.964	*F* 11.98	
2-MeSADP					
1	57.38 ± 11.89***	64.05 ± 5.49***	69.47 ± 12.26***	122.00 ± 30.39	
2	56.77 ± 3.74***	66.93 ± 8.43***	72.51 ± 12.67***	123.70 ± 15.63	
3	57.07 ± 5.23***	69.57 ± 8.64***	75.27 ± 22.37**	127.60 ± 32.40	
4	55.98 ± 4.21***	71.58 ± 7.29***	76.33 ± 15.43**	136.60 ± 26.20*	
5	57.39 ± 5.65***	71.88 ± 6.36***	79.81 ± 13.69*	140.60 ± 28.27*	
n = 5	*F* 114.3	*F* 81.90	*F* 11.85	*F* 5.278	

Note: Values are expressed as % of control values (media ± SD).

[a]Mean amplitude of all EPPs evoked during the train. Paired Student's *t* test at continuous 0.5, 5, and 50 Hz. Repeated measures one-way analysis of variance followed by Tukey test at intermittent 50 Hz.

***$p < .001$; **$p < .01$; *$p < .05$.

3.2 | Activation of inhibitory A_1 and A_3Rs, and excitatory A_{2A}Rs by endogenous adenosine when ACh release is evoked by different stimulation patterns

Adenosine is a key modulator of neuromuscular transmission that regulates ACh release by acting on inhibitory (A_1, A_3) and excitatory (A_{2A}) adenosine Rs. At mammalian neuromuscular preparations, Correia-de-Sá and Ribeiro (1996) found that the inhibitory effect induced by activation of A_1Rs prevails when the nerve is stimulated at low frequency, whereas higher synaptic adenosine levels are required to activate A_{2A}Rs. Moreover, we have demonstrated, by pharmacological and immunohistochemical studies, that A_3Rs are present at the motor nerve terminals and that inosine binds selectively to these Rs (Cinalli et al., 2013). So, the next step was to elucidate how the adenosine generated during 0.5-Hz, 5-Hz and continuous and intermittent 50-Hz stimulation affects ACh release by activating the different adenosine Rs.

When analyzing the effect of endogenous adenosine on inhibitory A_1 and A_3Rs we found that, at 0.5 Hz (50 pulses), the A_1R antagonist DPCPX 0.1 μM (Lohse et al., 1987) did not modify the first EPP amplitude, the mean amplitude of all EPPs, the mean size of the last 20 EPPs, and the ratio last 20 EPP/1° EPP (Table 2, Figure 2a,c, *n* = 5). These data are coherent with the results obtained in the presence of the A_1R agonist CCPA (500 nM, *n* = 4, De Lorenzo et al., 2004). In this condition, CCPA induced its typical inhibitory effect upon the amplitude of all EPPs of the train (Table 2, Figure 2b,c). Similar results were observed when experiments were performed at 0.5 Hz in the presence of the A_3R antagonist, MRS-1191 (5 μM, Jacobson et al., 1997; Jiang et al., 1996; Table 3, Figure 3a,c, *n* = 4), or the A_3R agonist inosine 100 μM (Cinalli et al., 2013; Table 3, Figure 3b,c, *n* = 6).

During 5 Hz-stimulation (750 pulses), DPCPX (*n* = 6) did not affect first EPP amplitude, mean amplitude of all EPPs, last 20 EPPs amplitude, and last 20 EPP/1° EPP ratio (Table 2, Figure 2d,f). Conversely, when the effect of CCPA (*n* = 5) was studied upon EPP amplitude parameters, it was observed that the A_1R agonist significantly reduced the amplitude of the first EPP and the rest of the EPPs of the train, although the relation between last 20 EPPs versus 1° EPP amplitude was higher than in control solution (Table 2, Figure 2e,f). When analyzing the action of endogenous adenosine on A_3Rs we found that, at 5 Hz, MRS-1191 (*n* = 4) and inosine (*n* = 4) induced similar results to that obtained with the A_1Rs (Table 3, Figure 3d–f).

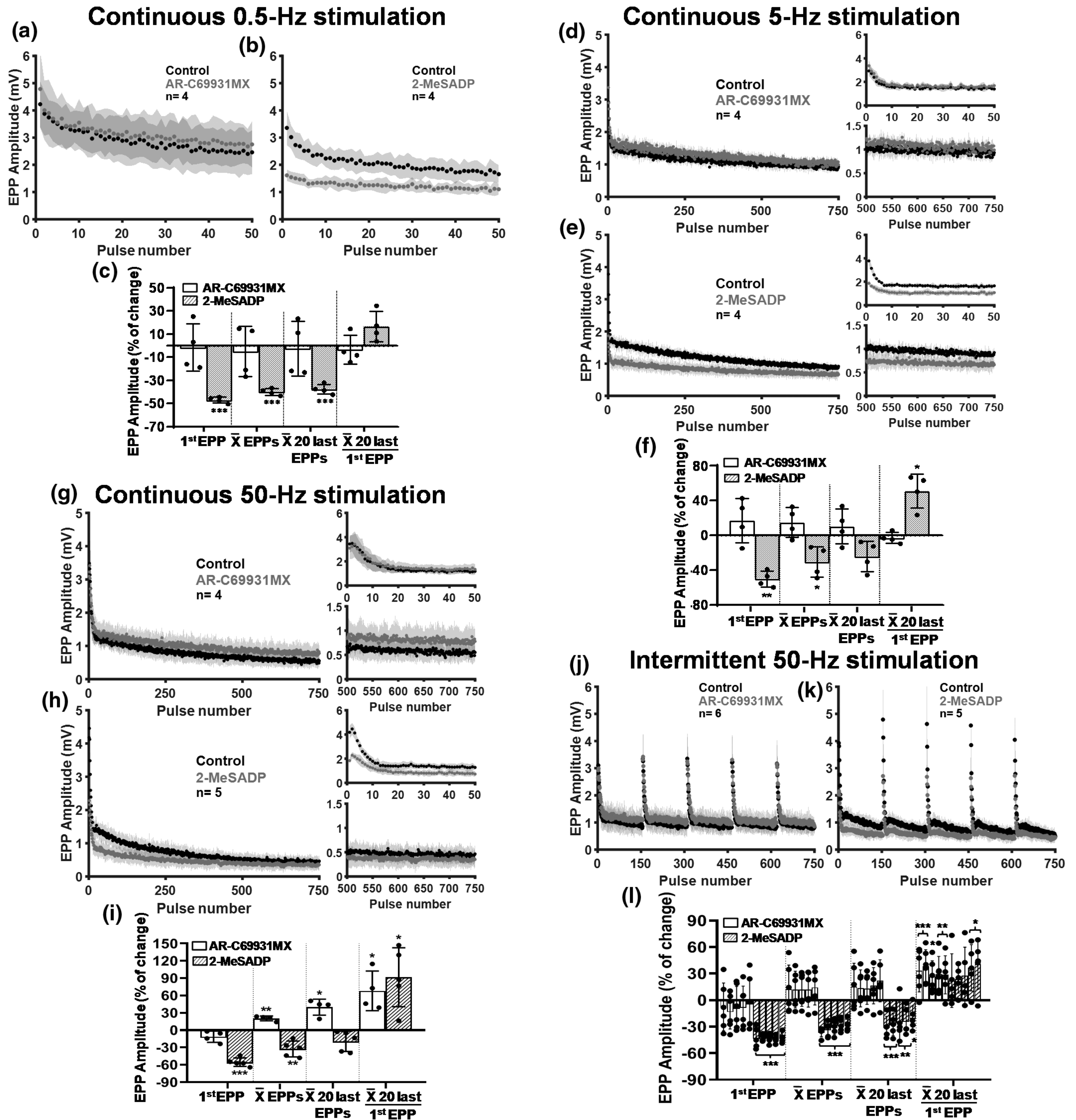

FIGURE 1 Effect of the P2Y$_{13}$R antagonist, AR-C69931MX, and the P2Y$_{13}$R agonist, 2-MeSADP, upon the end-plate potential (EPP) amplitudes during continuous 0.5-Hz (50 pulses), continuous 5-Hz (750 pulses), continuous 50-Hz (750 pulses), and intermittent 50-Hz stimulation (5 bursts of 150 pulses, 20 s interburst interval). a, b, d, and e (left panel), g and h (left panel), j, and k show EPP amplitudes along the trains/bursts in the presence of AR-C69931MX and 2-MeSADP, respectively, and expanded (d, e, g, h), during the first 50 pulses (upper right panel) and last 250 pulses (lower right panel). Symbols and shades represent media ± SD. (c, f, i, l) Summary graph (scatter plot with bar) shows the action of AR-C69931MX and 2-MeSADP on the amplitude of the first EPP, the mean value of the amplitudes of all EPPs, the mean value of the last 20 EPPs, and the ratio between the last 20 EPPs and the first EPP. Each point (individual experiment) and bar (media ± SD) represent the effect of the drug, expressed as % of change, with respect to its control. Continuous 0.5-, 5-, and 50-Hz stimulation: ***$p < .001$, **$p < .01$, *$p < .05$, paired *t*-test. Intermittent 50-Hz stimulation ***$p < .001$, **$p < .01$, *$p < .05$, repeated measures one-way analysis of variance followed by Tukey test.

TABLE 2 Effect of DPCPX and CCPA on end-plate potential (EPP) amplitudes during different nerve stimulation patterns

Solution	First EPP	EPPs[a]	Last 20 EPPs	Last 20 EPPs/first EPP	Stimulation rate
DPCPX $n = 5$	93.53 ± 6.98 t 2.072 p .1070	94.97 ± 15.47 t 0.7272 p .5074	94.72 ± 18.09 t 0.6520 p .5499	102.70 ± 14.78 t 0.4146 p .6997	Continuous 0.5 Hz (50 pulses)
CCPA $n = 4$	49.02 ± 13.80** t 7.391 p .0051	53.40 ± 16.89 * t 5.519 p .0117	53.44 ± 17.76 * t 5.242 p .0135	109.90 ± 11.83 t 1.679 p .1917	
DPCPX $n = 6$	98.98 ± 22.88 t 0.1095 p .9171	90.13 ± 12.72 t 1.901 p .1157	90.72 ± 12.26 t 1.855 p .1228	92.83 ± 18.40 t 0.954 p .3835	Continuous 5 Hz (750 pulses)
CCPA $n = 5$	46.15 ± 12.16*** t 9.898 p .0006	60.59 ± 13.30** t 6.624 p .0027	64.22 ± 16.89** t 4.737 p .0091	138.80 ± 26.65* t 3.253 p .0313	
DPCPX $n = 6$	99.56 ± 17.89 t 0.06025 p .9543	121.90 ± 13.76* t 3.900 p .0114	128.60 ± 15.38* t 4.564 p .0060	127.60 ± 21.20* t 3.194 p .0242	Continuous 50 Hz (750 pulses)
CCPA $n = 4$	46.05 ± 11.04** t 9.772 p .0023	68.90 ± 10.13** t 6.142 p .0087	79.31 ± 13.88 t 2.980 p .0586	178.10 ± 25.25** t 6.182 p .0085	
DPCPX					
1	94.87 ± 12.03	95.73 ± 26.09	92.13 ± 20.23	96.54 ± 14.49	Intermittent 50 Hz (5 bursts of 150 pulses)
2	97.01 ± 21.66	92.70 ± 20.75	90.44 ± 22.22	94.16 ± 17.13	
3	92.82 ± 18.90	85.36 ± 21.27	87.53 ± 22.58	94.04 ± 10.42	
4	89.68 ± 16.44	88.80 ± 25.39	86.26 ± 26.90	95.34 ± 21.69	
5	85.86 ± 15.85	86.41 ± 25.58	83.25 ± 28.92	94.04 ± 18.05	
$n = 6$	F 1.636	F 1.288	F 1.562	F 0.4961	
CCPA					
1	43.88 ± 4.11 ***	57.40 ± 6.41 ***	64.36 ± 9.23 ***	148.40 ± 23.03 *	
2	42.97 ± 5.14 ***	56.72 ± 4.49 ***	62.08 ± 5.49 ***	144.30 ± 15.68 *	
3	44.51 ± 7.10 ***	58.38 ± 5.56 ***	63.58 ± 6.45 ***	145.70 ± 30.42 *	
4	40.67 ± 7.43 ***	58.45 ± 5.04 ***	63.41 ± 5.48 ***	161.10 ± 33.39 **	
5	43.70 ± 4.44 ***	57.80 ± 4.06 ***	63.57 ± 7.17 ***	145.30 ± 18.79 *	
$n = 4$	F 317.2	F 211.8	F 86.78	F 8.67	

Note: Values are expressed as % of control values (media ± SD).

[a]Mean amplitude of all EPPs evoked during the train. Paired Student's t test at continuous 0.5, 5, and 50 Hz. Repeated measures one-way analysis of variance followed by Tukey test at intermittent 50 Hz.

***$p < .001$; **$p < .01$; *$p < .05$.

When the stimulation frequency was increased to 50 Hz (750 pulses), DPCPX ($n = 6$) did not change the amplitude of the 1° EPP, but significantly enhanced the mean amplitude of all EPPs of the train, the last 20 EPPs and the relation between the last 20 EPP/1° EPP when compared to control values (Table 2, Figure 2g,i). As counterpart, CCPA ($n = 4$) significantly decreased the amplitude of the 1° EPP and the mean amplitude of the 750 EPPs of the train, whereas the mean amplitude of the last 20 EPPs remained unchanged. Moreover, CCPA increased last 20 EPPs/1° EPP ratio (Table 2, Figure 2h,i). As expected, at 50 Hz-continuous stimulation, endogenous adenosine also activated A_3Rs. So, MRS-1191 ($n = 4$) did not modify the amplitude of the first EPP but significantly increased the average of the amplitudes of all EPPs, the last 20 EPPs and the relation between the last 20 EPPs and 1° EPP (Table 3, Figure 3g,i) and inosine ($n = 5$) reduced the amplitude of the 1° EPP and the mean amplitude of all EPPs of the train, although the average of the last 20 EPPs was not significant different from controls, then increasing the ratio last 20 EPPs/1° EPP (Table 3, Figure 3h,i).

The modulatory effect of the endogenous adenosine depicted during continuous 50 Hz-stimulation could not be observed when the phrenic nerve was stimulated intermittently at the same frequency (5 bursts of 150 pulses each one). So, DPCPX ($n = 6$) did not change any of the EPP amplitude parameters compared with control values, nor there were variations inter-bursts (Table 2, Figure 2j,l). On the other hand, incubation of the preparations with CCPA ($n = 4$) reduced the amplitude of the 1° EPP, the mean of the 150 EPPs and the last 20 EPPs amplitude of each burst, but significantly increased the last 20 EPPs/1° EPP ratio (Table 2, Figure 2k,l). We did not observe differences in the amplitude parameters among the five bursts with the two drugs. Similar behavior was observed when the effects of MRS-1191 ($n = 5$) and inosine ($n = 5$) were investigated at intermittent 50-Hz stimulation (Table 3, Figure 3j–l).

The last step was to investigate the action of endogenous adenosine on excitatory A_{2A}Rs when the phrenic nerve was stimulated at 0.5 Hz, 5 Hz, and continuous or intermittent 50 Hz, in the presence of the selective A_{2A}R antagonist (SCH-58261 50 nM, Zocchi et al., 1996) or the A_{2A}R agonist PSB-0777 (20 nM, El-Tayeb & Gollos, 2013). To state the adequate concentration for PSB-0777 at the NMJ, we first analyzed the effect of the drug on MEPP frequency in a concentration–response curve. As it is observed in Figure 4a, the selected concentration was 20 nM (EC50 5.76 nM). PSB-0777 did not modify the MEPP amplitude, but increased its frequency to 155.7 ± 4.17% of control values ($n = 4$, $p < .0001$),

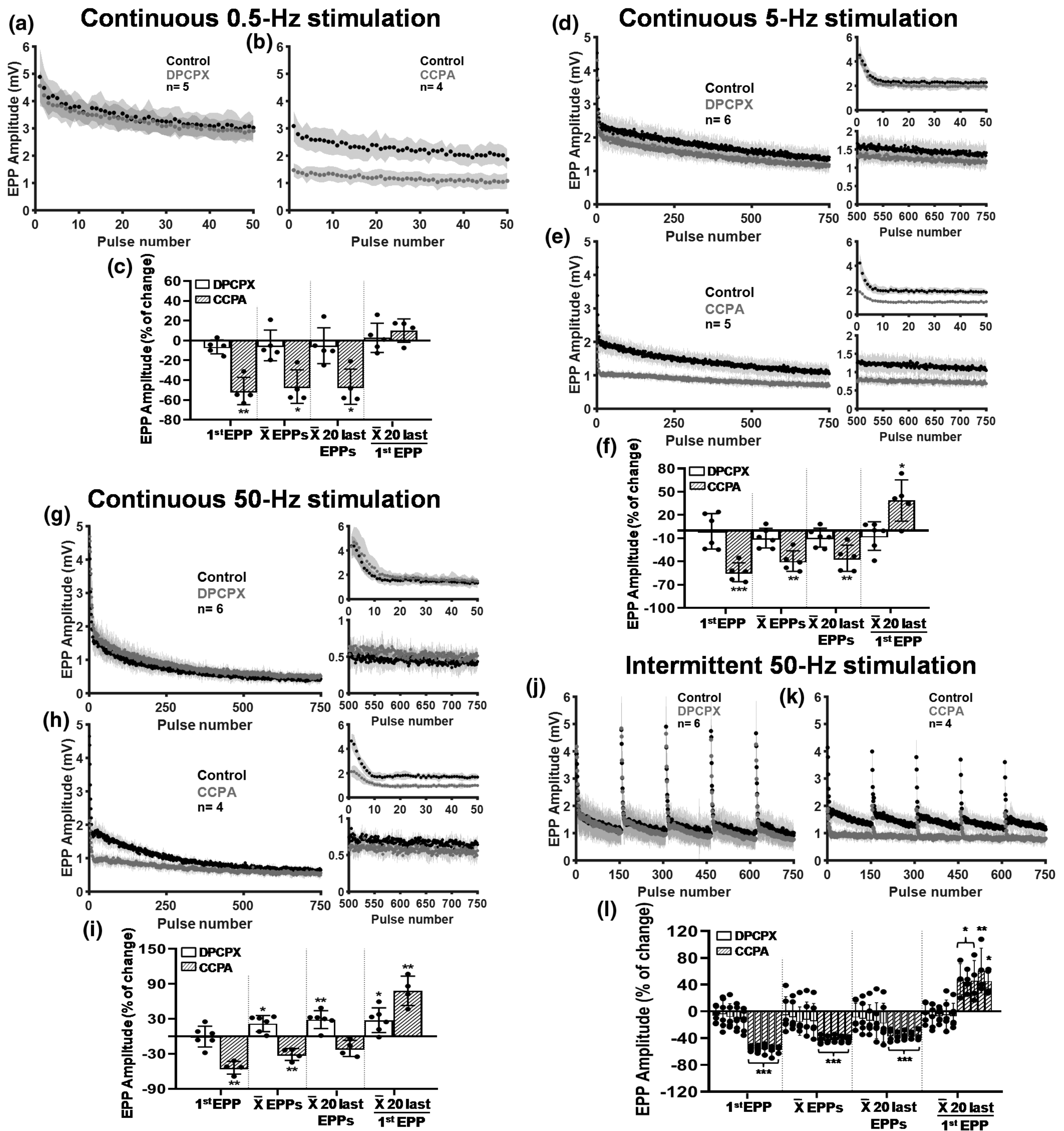

FIGURE 2 Effect of the A_1R antagonist, DPCPX, and the A_1R agonist, CCPA, upon the EPP amplitudes during continuous 0.5-Hz (50 pulses), continuous 5-Hz (750 pulses), continuous 50-Hz (750 pulses) and intermittent 50-Hz stimulation (5 bursts of 150 pulses, 20 s interburst interval). a, b, d, and e (left panel), g and h (left panel), j, and k, show end-plate potential (EPP) amplitudes along the trains/bursts in the presence of DPCPX and CCPA, respectively, and expanded (d, e, g, h), during the first 50 pulses (upper right panel) and last 250 pulses (lower right panel). Symbols and shades represent media ± SD. (c, f, i, l) Summary graph (scatter plot with bar) shows the action of DPCPX and CCPA on the amplitude of the first EPP, the mean value of the amplitudes of all EPPs, the mean value of the last 20 EPPs, and the ratio between the last 20 EPPs and the first EPP. Each point (individual experiment) and bar (media ± SD) represent the effect of the drug, expressed as % of change, with respect to its control. Continuous 0.5-, 5-, and 50-Hz stimulation: ***$p < .001$, **$p < .01$, *$p < .05$, paired t-test. Intermittent 50-Hz stimulation ***$p < .001$, **$p < .01$, *$p < .05$, repeated measures one-way analysis of variance followed by Tukey test.

TABLE 3 Effect of MRS-1191 and inosine on end-plate potential (EPP) amplitudes during different nerve stimulation patterns

Solution	First EPP	EPPs[a]	Last 20 EPPs	Last 20 EPPs/first EPP	Stimulation rate
MRS-1191 *n* = 4	101.20 ± 16.74 *t* 0.1421 *p* .8960	104.40 ± 13.64 *t* 0.6523 *p* .5607	105.70 ± 13.55 *t* 0.8417 *p* .4618	107.10 ± 11.74 *t* 1.214 *p* .3115	Continuous 0.5 Hz (50 pulses)
Inosine *n* = 6	66.46 ± 14.98** *t* 5.484 *p* .0027	68.33 ± 10.44*** *t* 7.431 *p* .0007	69.59 ± 10.10*** *t* 7.377 *p* .0007	109.80 ± 13.28 *t* 1.810 *p* .1301	
MRS-1191 *n* = 4	86.01 ± 17.21 *t* 1.626 *p* .2024	92.38 ± 18.26 *t* 0.8343 *p* .4653	88.60 ± 15.97 *t* 1.0427 *p* .2488	100.5 ± 5.58 *t* 0.1754 *p* .8720	Continuous 5 Hz (750 pulses)
Inosine *n* = 4	59.70 ± 7.08** *t* 11.38 *p* .0015	66.72 ± 8.43** *t* 7.892 *p* .0042	71.7 ± 9.13** *t* 6.337 *p* .0079	119.30 ± 6.69* *t* 5.760 *p* .0104	
MRS-1191 *n* = 4	110.00 ± 15.88 *t* 1.262 *p* .2962	136.50 ± 22.33* *t* 3.281 *p* .0464	148.10 ± 17.38* *t* 5.536 *p* .0116	123.60 ± 11.60* *t* 4.076 *p* .0267	Continuous 50 Hz (750 pulses)
Inosine *n* = 5	70.20 ± 15.44* *t* 4.315 *p* .0125	71.78 ± 17.58* *t* 3.590 *p* .0230	79.20 ± 16.77 *t* 2.774 *p* .0501	114.70 ± 6.22 ** *t* 5.301 *p* .0061	
MRS-1191					
1	102.00 ± 22.06	111.70 ± 20.51	125.20 ± 27.86	120.90 ± 33.40	Intermittent 50 Hz (5 bursts of 150 pulses)
2	103.90 ± 27.56	127.20 ± 34.20	117.90 ± 22.35	114.40 ± 24.73	
3	99.82 ± 24.49	115.80 ± 18.96	125.80 ± 25.90	128.80 ± 36.92	
4	104.70 ± 27.45	117.10 ± 20.85	124.20 ± 24.34	118.00 ± 24.22	
5	106.50 ± 33.30	120.60 ± 20.85	127.30 ± 21.35	128.30 ± 37.54	
n = 5	*F* 0.1383	*F* 3.029	*F* 4.570	*F* 2.371	
Inosine					
1	60.67 ± 9.38***	76.29 ± 8.54***	82.40 ± 8.84***	134.90 ± 21.60*	
2	61.51 ± 10.52***	77.91 ± 7.31***	82.49 ± 10.81***	136.50 ± 28.18*	
3	62.68 ± 10.03***	77.69 ± 6.40***	81.77 ± 8.97 ***	133.60 ± 23.39 *	
4	60.14 ± 12.54***	77.58 ± 8.21***	84.25 ± 6.74***	146.80 ± 33.65***	
5	61.23 ± 6.21***	79.14 ± 6.94***	84.51 ± 10.86**	137.70 ± 21.67**	
n = 5	*F* 477.3	*F* 41.42	*F* 15.17	*F* 9.703	

Note: Values are expressed as % of control values (media ± SD).

[a]Mean amplitude of all EPPs evoked during the train. Paired Student's *t* test at continuous 0.5, 5, and 50 Hz. Repeated measures one-way analysis of variance followed by Tukey test at intermittent 50 Hz.

***$p < .001$; **$p < .01$; *$p < .05$.

indicating that the sensitivity of end-plates to ACh remained unchanged, whereas the drug has a typically presynaptic effect. The facilitatory effect of 20 nM PSB-0777 on MEPP frequency became evident after 20 min incubation with the drug (n = 4). Moreover, to verify the effect of PSB-0777 on A_{2A}Rs in our model, we studied its action in the presence of SCH-58261. The antagonist precluded the facilitatory effect of PSB-0777 on spontaneous ACh release (SCH-58261 95.72 ± 10.78% of control values, SCH-58261 + PSB-0777 92.60 ± 6.08%, n = 4, Figure 4b). These data indicate that PSB-0777, at a concentration of 20 nM, activates A_{2A}Rs and induces a facilitatory effect on ACh release.

When the nerve was stimulated at 0.5 Hz, SCH-58261 (n = 4) did not provoke any change in the EPP amplitude along the train (Table 4, Figure 5a,c). On the other hand, PSB-0777 (n = 4) significantly increased the amplitude of the first EPP, the mean amplitude of all the EPPs of the train and the last 20 EPPs, being the relation last 20 EPPs/1° PP amplitude not different from control values (Table 4, Figure 5b,c). Similar results were obtained when the effects of the A_{2A}R antagonist and the A_{2A}R agonist were investigated on EPP amplitudes at continuous 5-Hz and 50-Hz stimulation. As it is shown in Table 4, Figure 5d,f,g,i, SCH-58261 did not modify the amplitude of the first EPP, the mean amplitude of all EPPs of the trains, the last 20 EPPs, and the last 20 EPP/1° EPP ratio when the phrenic nerve was stimulated at 5 Hz (n = 4) or at 50 Hz (n = 5). Then, as it was expected, PSB-0777 could induce its facilitatory effect on ACh release by activating free A_{2A}Rs at continuous 5 Hz (n = 4) and 50 Hz (n = 4), since the drug significantly enhanced the amplitude of the EPPs along the trains, without affecting the last 20 EPP/1° EPP relation when compared to control values (Table 4, Figure 5e,f,h,i). The experiments performed with intermittent 50 Hz stimulation in the presence of SCH-58261 or PSB-0777 depicted similar results to those obtained with the other stimulation patterns. SCH-58261 (n = 4) did not produce modifications in any of the amplitude EPP parameters with respect to control values and PSB-0777 (n = 5) significantly increased EPP amplitude along each burst (Table 4, Figure 5j–l). Moreover, there were no differences in any of the studied parameters among the five bursts.

These results suggest that at 0.5, 5, and continuous or intermittent 50 Hz, the endogenous adenosine generated from ATP is not enough to activate A_{2A}Rs allowing then the action of the exogenous agonist. So, we decided in this case, to increase the stimulation frequency to 100 Hz to obtain the concentration of adenosine that

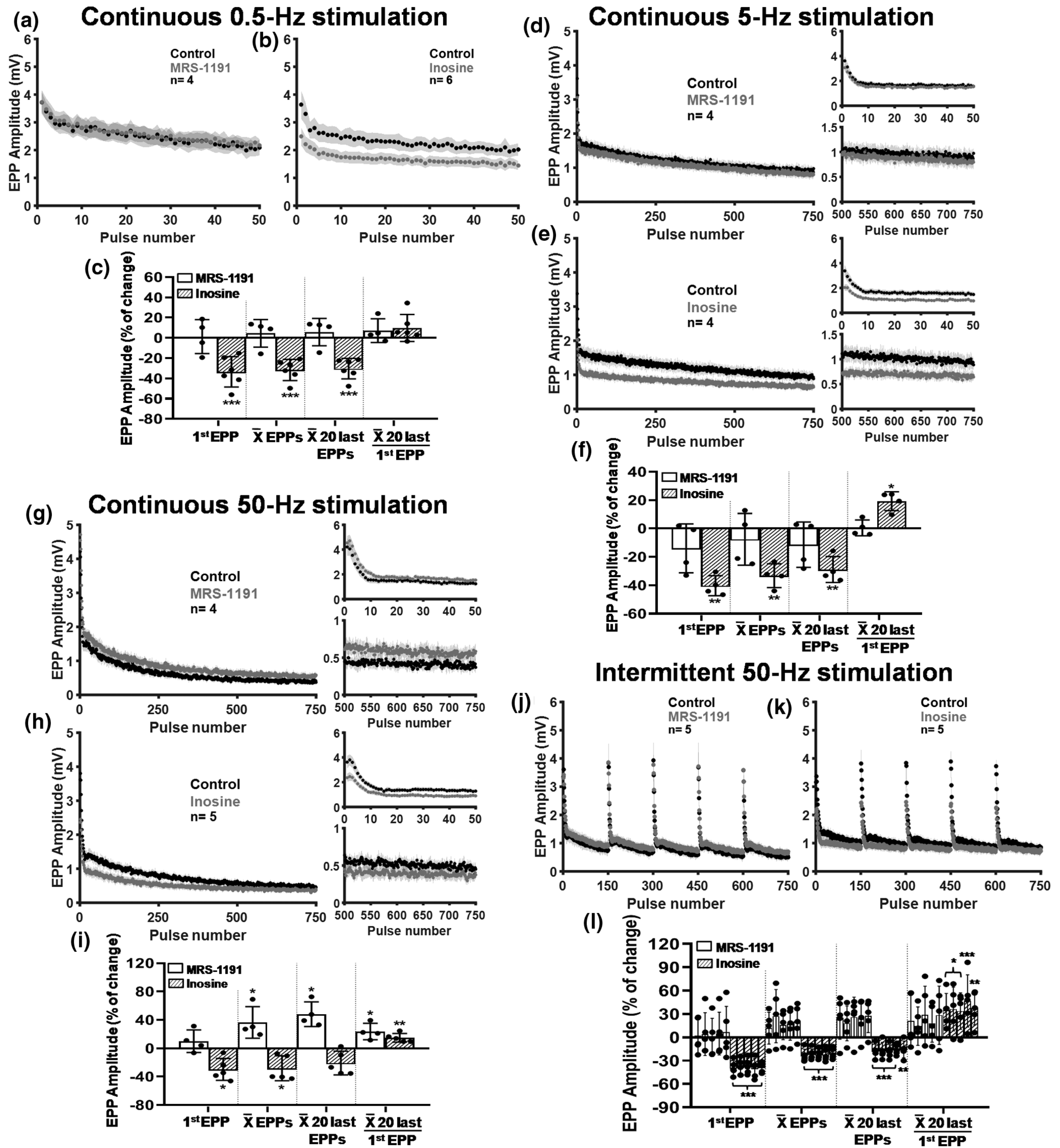

FIGURE 3 Effect of the A_3R antagonist, MRS-1191, and the A_3R agonist, inosine, upon the end-plate potential (EPP) amplitudes during continuous 0.5-Hz (50 pulses), continuous 5-Hz (750 pulses), continuous 50-Hz (750 pulses), and intermittent 50-Hz stimulation (5 bursts of 150 pulses, 20 s interburst interval). a, b, d, and e (left panel), g and h (left panel), j, and k, show EPP amplitudes along the trains/bursts in the presence of MRS-1191 and inosine, respectively, and expanded (d, e, g, h), during the first 50 pulses (upper right panel) and last 250 pulses (lower right panel). Symbols and shades represent media ± SD. (c, f, i, l) Summary graph (scatter plot with bar) shows the action of MRS-1191 and inosine on the amplitude of the first EPP, the mean value of the amplitudes of all EPPs, the mean value of the last 20 EPPs, and the ratio between the last 20 EPPs and the first EPP. Each point (individual experiment) and bar (media ± SD) represent the effect of the drug, expressed as % of change, with respect to its control. Continuous 0.5-, 5-, and 50-Hz stimulation: ***$p < .001$, **$p < .01$, *$p < .05$, paired t-test. Intermittent 50-Hz stimulation ***$p < .001$, **$p < .01$, *$p < .05$, repeated measures one-way analysis of variance followed by Tukey test.

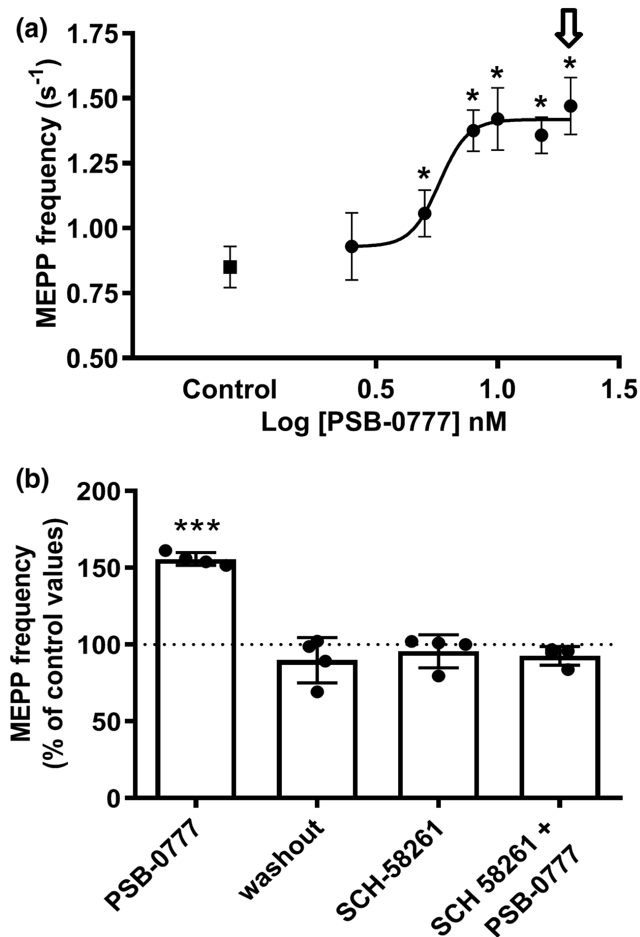

FIGURE 4 (a) Effect of PSB-0777 on miniature end-plate potential (MEPP) frequency (s^{-1}) as a function of its concentration. Each point represents mean ± SD (Control: 0.85 ± 0.08, n = 10; 2.5 nM 0.93 ± 0.13, n = 4; 5 nM 1.06 ± 0.09, n = 6; 8 nM 1.38 ± 0.08, n = 4; 10 nM 1.42 ± 0.12, n = 8; 15 nM 1.36 ± 0.07, n = 5; 20 nM 1.47 ± 0.11, n = 5. ***p < .001 versus control, analysis of variance (ANOVA) followed by Dunnett's test; F 37.19, EC_{50}: 5.76 nM. Arrow indicates the selected concentration (20 nM). (b) Effect of the $A_{2A}R$ agonist, PSB-0777 and the $A_{2A}R$ antagonist, SCH-58261 on MEPP frequency (n = 4). SCH-58261 abolished the facilitatory effect of PSB-0777. Each point (individual experiment) and bar (mean ± SD) are expressed as percentage of control values, ***p < .0001, F 41.16, ANOVA followed by Tukey test.

allows activating $A_{2A}Rs$. As it is shown in Table 4 and Figure 5m,o, SCH-58261 (n = 6) did not change first EPP amplitude, but significantly decreased mean EPP amplitude, 20 last EPP amplitude and last 20 EPP/1° EPP ratio, whereas PSB-0777 (n = 4) increased the amplitude of the first EPP, but did not significantly modify mean EPP amplitude and last 20 EPP amplitude, being last 20 EPP/1° EPP ratio lower than in control recordings (Table 4, Figure 5n,o).

4 | DISCUSSION

At mammalian NMJ, ATP is synchronously released with ACh into the synaptic cleft, in a frequency-dependent manner (Magalhães-Cardoso et al., 2003) and thus, the adenosine generated from it through ectonucleotidases, also depends on that frequency. Parallel to this, the nucleoside can be released as such from nerve terminals, muscle fibers, and PSCs (Cunha & Sebastião, 1993; Santos et al., 2003; Smith, 1991; Todd & Robitaille, 2006). In that sense, at rat NMJ, it was shown that phrenic nerve stimulation increased ATP accumulation in the bath effluent ~60%, which was dependent on extracellular Ca^{2+} and on neuronal activity (0 Ca^{2+} and TTX, respectively, prevented ATP outflow). In addition, electrical nerve stimulation also increased adenosine accumulation by ~50%, but paralysis of the muscle with μ-conotoxin GIIIB, decreased nerve-evoked ATP and adenosine outflow by 15% and 94%, respectively (Barroso et al., 2007; Magalhães-Cardoso et al., 2003; Noronha-Matos et al., 2011). On the other hand, it was recently suggested that activation of α7nAChRs, localized on the PSCs (Petrov et al., 2014), controls tetanic-induced ACh spillover from the NMJ by promoting adenosine release outflow from PSCs through equilibrative adenosine transporters and retrograde activation of presynaptic A_1Rs (Noronha-Matos et al., 2020; Petrov et al., 2014). In our experimental model, performed in curarized preparations, it is likely that purines come only from nerve terminals and not from muscle fibers and/or PSCs, since *d*-tubocurarine blocks both, $(\alpha1)_2\beta1\delta\varepsilon$nAChRs and α7nAChRs, expressed on skeletal muscle fibers and PSCs, respectively (Chavez-Noriega et al., 1997; Petrov et al., 2014).

The present data provide evidence that, at the mouse NMJs, depolarization of motor nerve terminals during repetitive stimulation of the phrenic nerve generates endogenous purines able to modulate ACh release depending on the stimulation pattern. During 0.5-Hz stimulation, neither the antagonists for inhibitory receptors: AR-C69931MX for $P2Y_{13}Rs$, DPCPX and MRS-1191, for A_1 and A_3Rs respectively, nor the antagonist for the excitatory $A_{2A}Rs$, SCH-58261, induced changes in the amplitude parameters studied (first EPP amplitude, the mean amplitude of all the EPPs of the train, the last 20 EPPs of the train and the relation of the last 20 EPPs with respect to the first EPP). On the other hand, the agonists of the purinergic receptors—2-MeSADP, CCPA, inosine, and PSB-0777, for $P2Y_{13}$, A_1, A_3 and $A_{2A}Rs$, respectively—provoked their typical modulatory effect on the evoked neurotransmitter release, being these results similar to those found by others authors in neuromuscular preparations (Bennett et al., 1991; Cinalli et al., 2013; Correia-de-Sá et al., 1991; Giniatullin & Sokolova, 1998; Ginsborg & Hirst, 1972; Guarracino et al., 2016; Sokolova et al., 2003). In fact, 2-MeSADP, CCPA, and inosine reduced the amplitude of all the EPPs of the train, whereas PSB-0777 increased it. These results suggest that at low stimulation rate (0.5 Hz) the concentration of endogenous purines in the synaptic cleft is not enough to activate purinergic Rs and to generate a modulatory response on neurosecretion, thus allowing the action of the exogenous agonists. It is likely that at low stimulation frequency, the ATP co-released with ACh has sufficient time to be degraded to adenosine, being the nucleoside carried into the cells by equilibrative nucleoside transporters following its concentration gradient (Correia-de-Sá & Ribeiro, 1996; Kong et al., 2004; Sebastião & Ribeiro, 1988).

TABLE 4 Effect of SCH-58261 and PSB-0777 on end-plate potential (EPP) amplitudes during different nerve stimulation patterns

Solution	First EPP	EPPs[a]	Last 20 EPPs	Last 20 EPPs/first EPP	Stimulation rate
SCH-58261 *n* = 4	104.90 ± 21.63 *t* 0.4517 *p* .6821	103.60 ± 8.70 *t* 0.8336 *p* .4657	103.63 ± 7.81 *t* 0.9236 *p* .4238	97.61 ± 12.67 *t* 0.3777 *p* .7308	Continuous 0.5 Hz (50 pulses)
PSB-0777 *n* = 4	140.02 ± 11.56** *t* 6.950 *p* .0061	136.50 ± 9.21** *t* 7.923 *p* .0042	137.50 ± 15.48* *t* 4.852 *p* .0167	97.80 ± 10.70 *t* 0.4110 *p* .7087	
SCH-58261 *n* = 4	105.70 ± 6.48 *t* 1.754 *p* .1778	106.40 ± 11.31 *t* 1.138 *p* .3376	108.40 ± 13.41 *t* 1.260 *p* .2969	100.40 ± 15.44 *t* 0.04723 *p* .9653	Continuous 5 Hz (750 pulses)
PSB-0777 *n* = 4	152.20 ± 18.48* *t* 5.652 *p* .0110	159.80 ± 19.45** *t* 6.154 *p* .0086	167.20 ± 25.65* *t* 5.244 *p* .0135	110.00 ± 22.39 *t* 0.8967 *p* .4360	
SCH-58261 *n* = 5	93.21 ± 20.46 *t* 0.7418 *p* .4994	96.87 ± 11.72 *t* 0.5967 *p* .5828	91.70 ± 18.75 *t* 0.9897 *p* .3783	99.13 ± 27.72 *t* 0.0703 *p* .9473	Continuous 50 Hz (750 pulses)
PSB-0777 *n* = 4	142.70 ± 16.22* *t* 5.269 *p* .0133	143.30 ± 12.32** *t* 7.026 *p* .0059	159.20 ± 15.60** *t* 7.596 *p* .0047	109.40 ± 11.97 *t* 1.567 *p* .2151	
SCH-58261					
1	98.04 ± 15.13	101.80 ± 9.08	100.90 ± 10.41	102.90 ± 13.27	Intermittent
2	105.40 ± 17.27	105.10 ± 5.73	108.50 ± 9.56	104.90 ± 7.40	50 Hz (5 bursts of 150 pulses)
3	108.90 ± 15.99	105.90 ± 4.11	107.10 ± 5.90	99.12 ± 8.48	
4	111.10 ± 13.46	107.00 ± 7.11	107.50 ± 13.00	96.00 ± 14.69	
5	110.40 ± 11.44	110.10 ± 6.31	107.00 ± 12.76	97.64 ± 14.31	
n = 4	*F* 1.515	*F* 2.848	*F* 1.298	*F* 0.4675	
PSB-0777					
1	143.40 ± 17.34**	151.70 ± 26.16***	149.00 ± 25.78***	104.70 ± 6.46	
2	139.00 ± 25.74*	147.00 ± 21.12***	147.10 ± 21.12***	110.70 ± 20.67	
3	143.10 ± 22.44**	140.80 ± 18.98***	147.90 ± 24.05***	114.90 ± 30.38	
4	149.80 ± 30.21***	144.90 ± 19.50***	141.50 ± 20.51***	100.40 ± 20.50	
5	148.90 ± 36.38***	148.00 ± 19.85***	144.00 ± 16.68***	104.30 ± 21.61	
n = 5	*F* 11.30	*F* 21.49	*F* 20.12	*F* 0.9202	
SCH-58261 *n* = 6	98.82 ± 13.56 *t* 0.2123 *p* .8403	82.75 ± 12.99 * *t* 3.254 *p* .0226	82.22 ± 11.78 * *t* 3.698 *p* .0140	87.85 ± 10.92 * *t* 2.726 *p* .0415	Continuous 100 Hz (750 pulses)
PSB-0777 *n* = 4	157.77 ± 19.46** *t* 5.938 *p* .0095	108.00 ± 9.65 *t* 1.652 *p* .1972	110.50 ± 13.46 *t* 1.559 *p* .2168	69.84 ± 8.77** *t* 6.875 *p* .0063	

Note: Values are expressed as % of control values (media ± SD).

[a]Mean amplitude of all EPPs evoked during the train. Paired Student's *t* test at continuous 0.5, 5, 50, and 100 Hz. Repeated measures one-way analysis of variance followed by Tukey test at intermittent 50 Hz.

***$p < .001$; **$p < .01$; *$p < .05$.

During continuous 5-Hz stimulation (750 pulses), the results obtained with the antagonists and agonists for the $P2Y_{13}$, A_1, and A_3Rs were intriguing. Incubation of the preparations with AR-C69931MX, DPCPX, and MRS-1191 showed that, at 5-Hz stimulation, there were no significant changes in any of the parameters of amplitude recorded during the train. This behavior might be due to the rapid metabolism of ATP and adenosine and/or to the uptake of the nucleoside by equilibrative transporters. In this regard, Correia-de-Sá et al. (1996) found that, at 5 Hz, adenosine deaminase (ADA), the enzyme that deaminates adenosine into inosine, increased evoked [^{3}H]ACh release from rat phrenic nerve endings by about 30% and that the nucleoside uptake blocker NBTI increased endogenous adenosine concentration and provoked an inhibitory effect on evoked [^{3}H]ACh release that was enhanced as the number of pulses per train increased. The authors interpreted the increase in ACh release induced by ADA as endogenous adenosine exerting an inhibitory “tone” over neuromuscular transmission. If we only consider our results obtained with AR-C69931MX, DPCPX, and MRS-1191, we cannot reach that conclusion. This discrepancy might be due to differences between species, in the experimental model, or in the temperature of the bath (22°C vs. 37°C). In this sense, Masino et al. (2001) showed that increase in hippocampal slices temperature enhanced the adenosine concentration in the extracellular space. However, when experiments were performed (5 Hz) in the presence of the agonists for $P2Y_{13}$, A_1, and A_3Rs, we found that the typical inhibitory action on EPP amplitude seemed to be less evident by the end of the train (only statistical significant for $P2Y_{13}$Rs) (2-MeSADP ~50% of inhibition in the first EPP vs. ~24% in the last 20 EPPs; CCPA ~54% of inhibition in the first EPP vs. ~36% in the last 20 EPPs; inosine ~40% of inhibition in the first EPP vs. ~29% in the last 20 EPPs), which made that the ratios of the last 20 EPPs/first EPP were significant greater with 2-MeSADP, CCPA, and inosine compared to controls. One could speculate that, just at the end of the trains, the concentration of endogenous purines begins to increase in the synaptic cleft, thus activating some Rs and somewhat diminishing the

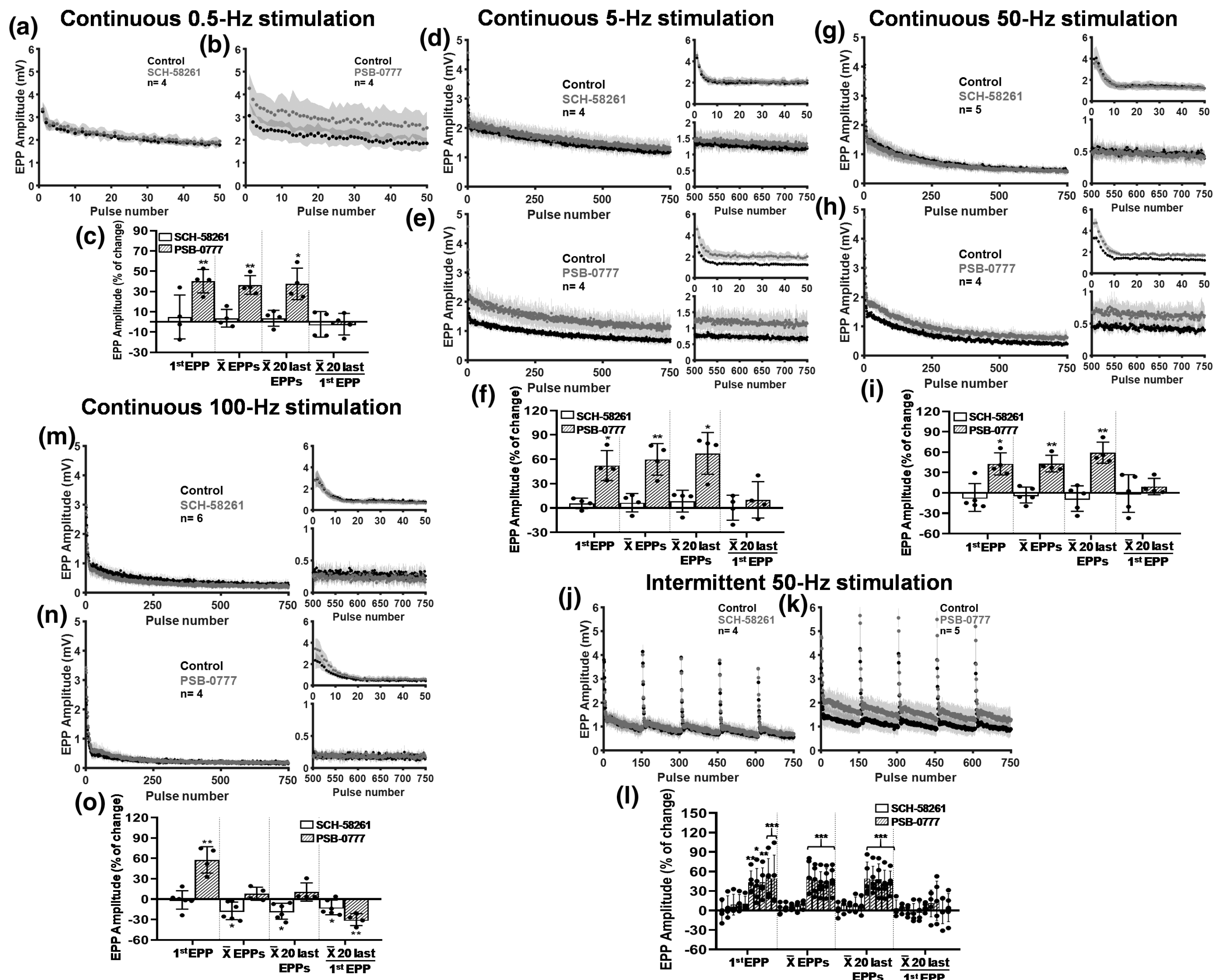

FIGURE 5 Effect of the $A_{2A}R$ antagonist, SCH-58261, and the $A_{2A}R$ agonist, PSB-0777, upon the end-plate potential (EPP) amplitudes during continuous 0.5-Hz (50 pulses), continuous 5-Hz (750 pulses), continuous 50-Hz (750 pulses), intermittent 50-Hz stimulation (5 bursts of 150 pulses, 20 s interburst interval), and continuous 100-Hz stimulation. a, b, d, and e (left panel), g and h (left panel), j, k, m, and n (left panel) show EPP amplitudes along the trains/bursts in the presence of SCH-58261 and PSB-0777, respectively, and expanded (d, e, g, h, m, n), during the first 50 pulses (upper right panel) and last 250 pulses (lower right panel). Symbols and shades represent media ± SD. (c, f, i, l, o) Summary graph (scatter plot with bar) shows the action of SCH-58261 and PSB-0777 on the amplitude of the first EPP, the mean value of the amplitudes of all EPPs, the mean value of the last 20 EPPs, and the ratio between the last 20 EPPs and the first EPP. Each point (individual experiment) and bar (media ± SD) represent the effect of the drug, expressed as % of change, with respect to its control. Continuous 0.5-, 5-, 50-, and 100-Hz stimulation: $^{**}p < .01$, $^{*}p < .05$, paired *t*-test. Intermittent 50-Hz stimulation $^{***}p < .001$, $^{**}p < .01$, $^{*}p < .05$, repeated measures one-way analysis of variance followed by Tukey test.

inhibitory action of agonists on cholinergic release. Eventually, increasing the frequency of stimulation or the number of applied pulses might allow endogenous purines to reach a concentration sufficient to permit observing effects of the antagonists.

On the other hand, at 5 Hz, endogenous purines were not able to generate an excitatory response by activation of $A_{2A}Rs$, since the antagonist of these Rs, SCH-58261, did not alter the EPP parameters and the selective $A_{2A}R$ agonist PSB-0777 depicted its facilitatory effect along the trains.

When the nerve was stimulated at continuous 50 Hz, exposition of the preparations to AR-C69931MX did not modify the amplitude of the first EPP, but significantly increased the mean amplitude of all EPPs of the train, that of the last 20 EPPs, as well as the ratio last 20 EPPs/1° EPP. When the experiments were performed in the presence of the agonist 2-MeSADP, it was observed a significant reduction of the amplitude of the first EPPs that was less evident along the train: the last 20 EPP amplitudes were not different from control values and the relation last 20 EPPs/1° EPP was ~92% greater than its control. These data suggest that the level of endogenous nucleotides in the synaptic space, along 750 pulses at 50 Hz, is able to activate to the $P2Y_{13}Rs$ generating an inhibitory tonus upon ACh release. The fact that AR-C69931MX did not provoke an increase in the amplitude of the first

EPP and that 2-MeSADP induced an inhibitory effect at the beginning of the train would indicate that basal ATP/ADP is not enough to induce a modulatory effect during the initial phase of the stimulation.

Similar results were observed when it was evaluated the effect of the endogenous adenosine upon A_1 and A_3Rs during continuous 50 Hz stimulation. DPCPX and MRS-1191 did not affect first EPP amplitude, but significantly enhanced the other EPP parameters, suggesting that as the train progresses, sufficient adenosine is being accumulated in the synaptic space and so, A_1 and A_3Rs are activated. These data are coherent with those obtained in the presence of the A_1 and A_3R agonists; CCPA and inosine decreased the first EPP amplitude but this modulation decreased during the rest of the train. One can speculate that, at this stimulation rate, equilibrative transporters might become saturated, causing an increase in the endogenous adenosine concentration and consequently activation of the inhibitory Rs, which would interfere with the action of the exogenous agonists. Similar results were obtained by other authors at this stimulation rate (Perissinotti & Uchitel, 2010). Although it was suggested that during continuous 50-Hz stimulation, the released ATP and/or ADP generated can feed-forward inhibit the ecto-5′-nucleotidase impairing adenosine generation (Correia-de-Sá et al., 1996); in our experimental model, this mechanism appears not to be the case, since the inhibition of A_1 or A_3Rs by DPCPX and MRS-1191 increased EPP amplitude after the beginning of the train and remained so until the end of the train, suggesting that during continuous 50-Hz stimulation enough endogenous adenosine is present to activate the inhibitory Rs.

Conversely, endogenous adenosine generated during continuous 50 Hz did not activate excitatory A_{2A}Rs since SCH-58261 did not alter any of the EPP parameters and PSB-0777 increased the EPP amplitude throughout the entire train. At rat NMJ, Correia-de-Sá and Ribeiro (1996), showed that adenosine concentrations between 10 and 100 μM inhibited the release of [^{3}H]-ACh by activation of A_1Rs while high concentrations (>100 μM) increased it when the nucleoside binds to A_{2A}Rs. In that sense, our experiments performed at 100 Hz (750 pulses) demonstrated that SCH-58261 reduced EPP amplitudes after the onset of the train, and PSB-0777 increased the amplitude of the first EPPs, but unlike 50 Hz, the agonist did not significantly modify the mean EPP amplitude and the last 20 EPP amplitude; so, the last 20 EPPs/1° EPP ratio was ~75% lower than the control recordings. These data suggest that, at 100 Hz, the adenosine concentration in the synaptic cleft reached a value able to activate A_{2A}Rs, thus impairing the action of the exogenous agonist after the initial phase.

When experiments were performed at intermittent 50-Hz stimulation (5 bursts of 150 pulses), we observed that, in all experimental situations, the degree of rundown of the EPP amplitude during intermittent stimulation (70%) is coincident with that obtained with continuous 5-Hz stimulation (~ 72%). In this sense, Correia-de-Sá et al. (1996), at rat NMJs, found that the quantity of [^{3}H]-ACh release in 5 Hz and 5 bursts at 50 Hz is not significantly different. With this stimulation pattern, incubation of the preparations with AR-C69931MX did not depict significant changes in any of the parameters of EPP amplitude, except in the last 20 EPP/first EPP ratio, which was higher than controls. On the contrary, the agonist 2-MeSADP decreased all EPP parameters in each burst, but increased the relation last 20 EPPs/first EPP, being significant in the last two bursts. These results indicate that at intermittent 50-Hz stimulation, unlike what happens with continuous 50-Hz stimulation, the concentration of endogenous nucleotides in the synaptic space in each burst does not induce a relevant modulatory response on neurotransmission. Similar results were observed with the antagonists and agonist of the inhibitory A_1 and A_3Rs; we only observed an increase in the relation last 20 EPPs/first EPP in the presence of CCPA and inosine compared with their controls. These data suggest that more than 150 pulses are needed to generate sufficient adenosine able to activate A_1 and A_3Rs. Furthermore, as it was expected, the excitatory A_{2A}Rs were not activated by the endogenous adenosine when the nerve was stimulated intermittently.

The differences observed with the results obtained at 50 Hz with continuous or intermittent stimulation indicate that the modulation of ACh release by endogenous purines not only depend on the stimulation frequency, but also on the pattern of stimulation, as it was previously suggested by Correia-de Sá et al. (1996) at rat NMJ.

The fact that A_1 and A_3Rs were activated by the endogenous adenosine accumulated extracellularly when the nerve was stimulated at 50 Hz, whereas the facilitation induced by A_{2A}Rs was only apparent when adenosine concentration was increased at 100 Hz stimulation, might suggest that inhibitory Rs have higher affinity for adenosine than the A_{2A}Rs (Correia-de-Sá & Ribeiro, 1996; Daly, 1982; Dunwiddie & Masino, 2001; Londos et al., 1980; Stockwell et al., 2017). Alternatively, the presence of crosstalk between A_1-A_{2A}R heteromers might provide a switch mechanism by which low and high concentrations of synaptic adenosine produce opposite effects on ACh release (Correia-de-Sá et al., 1996). In this regard, Ciruela et al. (2006) demonstrated in radioligand-binding experiments in co-transfected cells and rat striatum that heteromerization of A_1 and A_{2A}Rs allows adenosine to exert a fine-tuning modulation of glutamatergic neurotransmission since activation of A_{2A}Rs reduces the affinity of the A_1Rs for agonists. Likewise, there are several examples in different preparations of a functional cross-talk between P2 and P1 Rs (e.g., Dias et al., 2021; Tonazzini et al., 2007, reviewed by Agostinho et al., 2020). Besides, other factors such as the topographical arrangement of ecto-enzymes, transporters and other presynaptic Rs may also influence the effects mediated by A_1, A_3, or A_{2A}Rs.

In conclusion, when motor nerve terminals are depolarized by electrical stimulation of the phrenic nerve, there is a sequence of activation of purinergic receptors by endogenous ATP/ADP and adenosine that is able to fine-tune neurosecretion, depending on the frequency and pattern of stimulation. So, at continuous 0.5-Hz stimulation, endogenous ATP/ADP and adenosine are not enough to activate $P2Y_{13}$, and A_1, A_3, and A_{2A}Rs, respectively (Figure 6a). At moderate stimulation frequency (5 Hz), it is likely that the effect of endogenous purines begins to be evident just at the end of the train for the $P2Y_{13}$, A_1, and A_3Rs, being more relevant for the former (Figure 6b). At high stimulation frequency

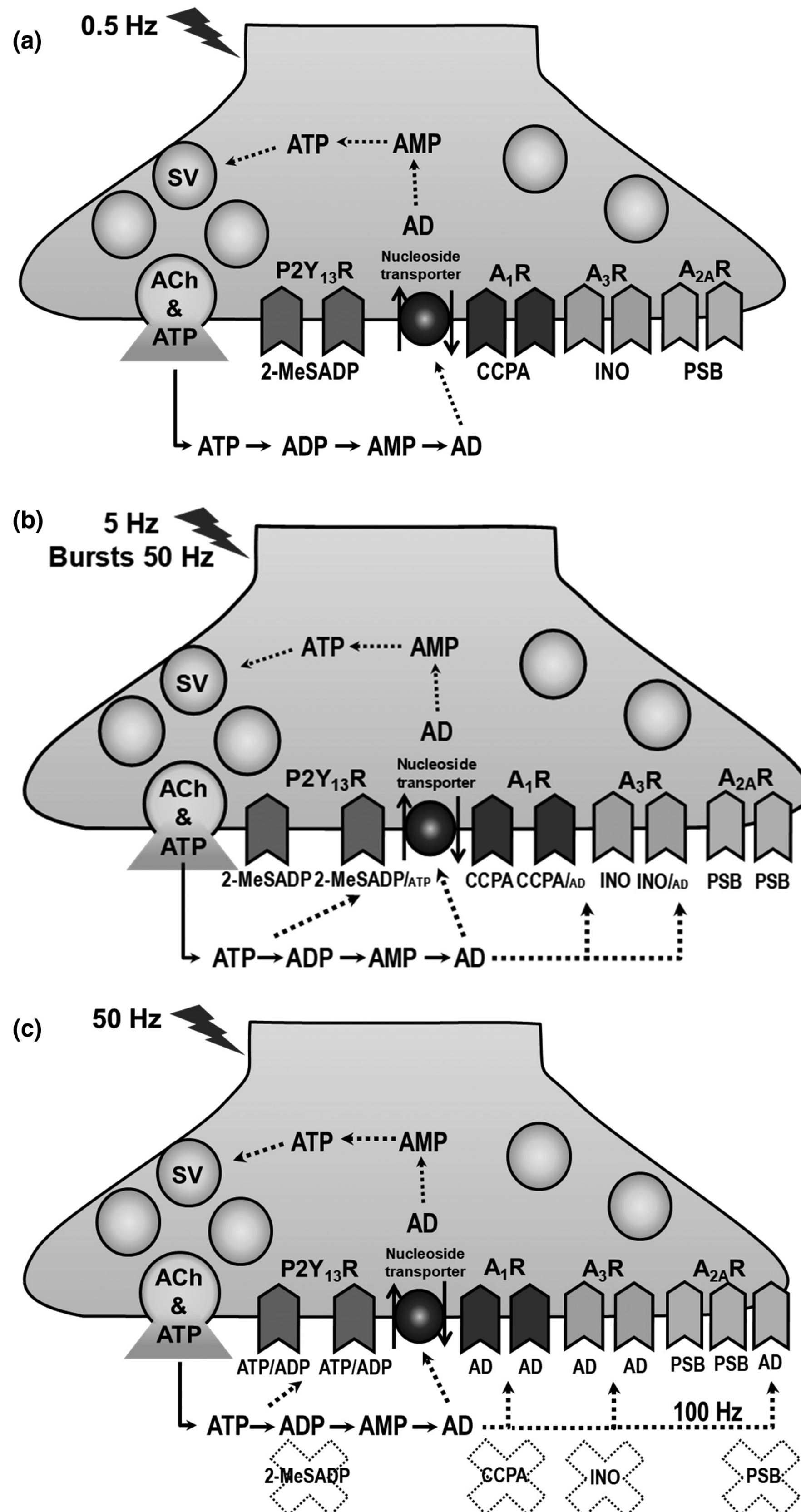

FIGURE 6 Effect of endogenous purines on electrically evoked acetylcholine release during continuous 0.5-Hz stimulation (a), continuous 5-Hz stimulation (b), continuous 50-Hz stimulation (c), and intermittent 50-Hz stimulation (b). See explanation in the text. Note that the effect of endogenous purines on A_{2A}Rs during continuous 100-Hz stimulation is also depicted in C. AD, adenosine; INO, inosine; PSB, PSB-0777; R, receptor; SV, synaptic vesicle.

(continuous at 50 Hz), the concentration of endogenous purines is sufficient to activate their inhibitory Rs, immediately after of the initial phase, provoking their typical inhibitory action on the evoked ACh release (Figure 6c). It is necessary more adenosine in the synaptic cleft (continuous at 100 Hz) to activate the excitatory A_{2A}Rs (Figure 6c). At high stimulation frequency (intermittent at 50 Hz), the putative effect of endogenous purines is similar to what happens at continuous 5-Hz stimulation (Figure 6b). These findings contribute to understand in more detail the role of the purines as neuromodulators of the cholinergic transmission at mammalian NMJs, in this case, during different stimulation frequencies. It is probable that the synthesis of potent and selective ligands for each of the purinergic Rs allows a better characterization of the physiological functions, in addition of representing a potential therapeutic tool for the diseases that affect such synapses. Through this investigation, we can hypothesize that, at least up to 50-Hz nerve stimulation, the activation of A_{2A}Rs by exogenous agonists could improve the neuromuscular transmission failure observed in neuromuscular diseases such as Myasthenia Gravis, Lambert-Eaton syndrome, or some congenical myasthenic syndromes, as it was suggested by Oliveira et al. (2015).

DECLARATION OF TRANSPARENCY

The authors, reviewers and editors affirm that in accordance to the policies set by the *Journal of Neuroscience Research*, this manuscript presents an accurate and transparent account of the study being reported and that all critical details describing the methods and results are present.

AUTHOR CONTRIBUTIONS

All authors had full access to all the data in the study and are responsible for the integrity of the data and the accuracy of the data analysis. *Conceptualization*, J.G.S., M.H.P., T.F., and A.L.; *Methodology*, J.G.S. and A.L.; *Investigation*, J.G.S., M.H.P., T.F., and A.L.; *Formal Analysis*, J.G.S., M.H.P., T.F., and A.L.; *Writing – Review & Editing*: J.G.S. and A.L.; *Supervision*, A.L.; *Funding Acquisition*: A.L.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request (idimneurofisio@gmail.com).

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