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Adenosine receptors and muscarinic receptors cooperate in acetylcholine release modulation in the neuromuscular synapse

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Complete List of Authors:	Santafe, Manel; Universitat Rovira i Virgili, Unitat Histologia i Neurobiologia Priego, Mercedes; Universitat Rovira i Virgili, Unitat Histologia i Neurobiologia Obis, Teresa; Universitat Rovira i Virgili, Unitat Histologia i Neurobiologia Garcia, Neus; Univeritat Rovira i Virgili, Histologia i Neurobiologia; Universitat Rovira i Virgili, Unitat Histologia i Neurobiologia Tomas, Marta; Univeritat Rovira i Virgili, Histologia i Neurobiologia Lanuza, Angel; Universitat Rovira iVirgili, Histologia i Neurobiologia Tomas, Josep; Univeritat Rovira i Virgili, Histologia i Neurobiologia
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Prof. John Foxe
Co-Editor in Chief, EJN

Dear Dr. Foxe,

Thank you for giving us the opportunity to respond to the referees' comments on our manuscript EJN-2014-11-22278 entitled: **"Adenosine receptors and muscarinic receptors cooperate in acetylcholine release modulation in the neuromuscular synapse"**.

We agree with the comments of the referee 1 and, as suggested, we modify explanation of the considered questions in the section of Results and Discussion. The changes we have made are highlighted in yellow.

Thank you for your cooperation.

Yours sincerely,

Dr. Manel M. Santafe
Unitat d'Histologia i Neurobiologia
Facultat de Medicina.
Universitat Rovira i Virgili
c/ St Llorenç, num 21.
43201 Reus (Spain)
e-mail: msm@fmcs.urv.es / manuel.santafe@urv.cat
Tel 0034 977759300/ 977759351

Adenosine receptors and muscarinic receptors cooperate in acetylcholine release modulation in the neuromuscular synapse

M.M. Santafe,* M. Priego, T. Obis, N. Garcia,* M. Tomàs, M.A. Lanuza and J. Tomàs*

Unitat d'Histologia i Neurobiologia (*UHN*): Facultat de Medicina i Ciències de la Salut, Universitat Rovira i Virgili, carrer St Llorenç num 21, 43201-Reus, SPAIN.

Correspondence should be addressed to either of the following:
Josep Tomàs (jmtf@fmcs.urv.es)
Neus Garcia (ngs@fmcs.urv.es)
Manel M Santafe (manuel.santafe@urv.cat)

For editorial communications, please send correspondence to:
Dr. MM Santafe
Unitat d'Histologia i Neurobiologia (*UHN*)
Facultat de Medicina i Ciències de la Salut, Universitat Rovira i Virgili, Carrer Sant Llorenç, 21, 43201-Reus, SPAIN
Telephone number: (54 -11) 977 759351
Fax number: (54 -11) 977 759322
Email address: msm@fmcs.urv.es

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ABSTRACT

Adenosine receptors (AR) are present in the motor terminals at the mouse neuromuscular junction (NMJ). AR and the presynaptic muscarinic autoreceptors (mAChR) share the functional control of the NMJ. We analysed here their mutual interaction in transmitter release modulation. In electrophysiological experiments with unaltered synaptic transmission (muscles paralyzed by blocking the voltage-dependent sodium channel of the muscle cells with μ-CgTx-GIIIB), we found that: i) a collaborative action between different AR subtypes reduce synaptic depression at moderate activity level (40Hz); ii) at high activity levels (100Hz), endogenous adenosine (ADO) production in the synaptic cleft is sufficient to reduce depression through A_1R and $A_{2A}R$ receptors; iii) when the non metabolizable 2-chloroadenosine (CADO) agonist is used, both quantal content and depression are reduced; iv) the protective effect of CADO on depression is mediated by A_1R whereas $A_{2A}R$ receptors seem to modulate A_1R; v) AR and mAChR absolutely depend upon each other for the modulation of evoked and spontaneous ACh release in basal conditions and in experimental conditions with CADO stimulation; vi) the purinergic and muscarinic mechanisms cooperate in the control of depression by sharing a common pathway though the purinergic control is more powerful than the muscarinic one; vii) the imbalance of the AR created by using subtype selective and non-selective inhibitory and stimulatory agents uncouples protein kinase C (PKC) from evoked transmitter release. In summary, ARs (A_1R, $A_{2A}R$) and mAChRs (M_1, M_2) cooperate in the control of activity-dependent synaptic depression and may share a common PKC pathway.

INTRODUCTION

In the neuromuscular synapse, presynaptic muscarinic ACh autoreceptors (mAChR) directly couple ACh release to the regulation of the nerve ending release mechanism itself (Caulfield, 1993; Slutsky *et al.*, 1999; Minic *et al.*, 2002; Santafe *et al.*, 2003; 2004; Garcia *et al.*, 2005). Also, adenosine (ADO) secretion (co-released from cholinergic endings) and the presynaptic adenosine receptors (AR) modulate transmitter release (Correia-de-Sa *et al.*, 1991).

The physiological role of the AR in the adult NMJ was best observed when the evoked neuromuscular transmission was not directly altered (in experiments made by blocking the voltage-dependent sodium channel of the muscle cells with μ-CgTx-GIIIB to prevent contraction instead of using high Mg^{2+}, low Ca^{2+} or curare (Garcia *et al.*, 2013; Tomas *et al.*, 2014)). In this basal condition, A_1R can conserve neurotransmitter resources by limiting spontaneous quantal leak of ACh (in mouse adult NMJ, the spontaneous minis frequency at rest is about 1 per second). In addition, the coordinated involvement of A_1R and $A_{2A}R$ seems to protect synaptic function by reducing the magnitude of depression during repetitive activity (Garcia *et al.*, 2013). Adenosine deaminase (ADA) does not change the amplitude of evoked endplate potentials (EPP), although the MEPP frequency increased significantly (as it did with the non-selective AR blocker 8SPT and the selective A_1R blocker DPCPX). This supports the idea that endogenous ADO really contributes to limiting the spontaneous quantal leak of ACh through A_1R. However, ADA does not change the magnitude of the depression that normally occurs during high frequency stimulation (40Hz), which indicates that endogenous adenosine production does not change depression in these activity conditions (Garcia *et al.*, 2013).

However, we do not know if high levels of ADO in the synaptic cleft, similar to those obtained by applying exogenous ADO, are produced in higher activity conditions. The

purinergic mechanism that limits depression may work preferentially at very high synaptic activity. Thus, we analysed the effect of ADA and the agents ADO and 8SPT on depression when muscles were electrically stimulated at a higher rate (100Hz). 2-chloroadenosine (CADO) shows this issue from a different view. CADO (a non-specific agonist of all subtypes of adenosine receptor) has the same affinity as adenosine for AR but it is not metabolized, which allows a more stable and prolonged effect on receptors. We studied how CADO influences ACh release and synaptic depression and which AR subtypes are involved. Because AR and mAChR share some functional control of the NMJ – both sets of presynaptic autoreceptors promote the reduction of depression (Tomas *et al.*, 2014) – we analysed here their mutual interaction in transmitter release modulation. Finally, we investigated the functional relation in transmitter release between AR and the protein kinases A (PKA) and C (PKC).

The main results indicate that at high activity levels, endogenous ADO production in the synaptic cleft could be sufficient to interact mainly with A_1R receptors and protect against depression. Also, AR and mAChR are mutually dependent on modulating spontaneous, evoked and activity-dependent (depression) ACh release.

METHODS

Animals

Experiments were performed on the *Levator auris longus* (LAL) muscle of adult male Swiss mice (30–40 days postnatal; Criffa, Barcelona, Spain). The mice were cared for in accordance with the guidelines of the European Community's Council Directive of 24 November 1986 (86/609/EEC) for the humane treatment of laboratory animals. The animals were anesthetized with 2% tribromoethanol (0.15 ml /10 g body weight, I.P.). This study was approved by the Ethics Committee of the Rovira i Virgili University (Ref. number 233).

Electrophysiological recordings

The LAL muscle and its nerve supply were surgically removed and pinned in a Sylgard-lined 35-mm Petri dish containing normal Ringer solution (in mM) – NaCl 135, KCl 5, $CaCl_2$ 2.5, $MgSO_4$ 1, NaH_2PO_4 1,$NaHCO_3$ 15, glucose 11 – and bubbled continuously with 95% O_2, 5% CO_2, which flowed into the Petri dish to superfuse the muscle preparation. The overflow was evacuated by suction. The solution was not bubbled directly in the Petri dish in order to minimize vibration during electrophysiological recording. Temperature and humidity were set to 26°C and 50%, respectively. The bath temperature was monitored during the experiments (23.4 ºC ± 1.7, Digital Thermometer TMP 812, Letica, Barcelona, Spain). We also performed some experiments at a bath temperature of 31ºC but we found no significant change in the adenosine effect. Intracellular recordings (end plate potentials, EPPs, and miniature end plate potentials, MEPPs) were performed with conventional glass microelectrodes filled with 3M KCl (resistance: 20-40 MΩ). Recording electrodes were connected to an amplifier (Tecktronics, AMS02, Oregon, USA), and a distant Ag-AgCl electrode connected to the bath solution via an agar bridge (agar 3.5% in 137 mM NaCl) was used as reference. The signals were digitized (DIGIDATA 1322A Interface, Axon

Instruments Inc., Weatherford, TX, USA), stored and computer-analyzed. The software Axoscope 9.0 (Axon Instruments Inc.) was used for data acquisition and analysis.

To prevent muscle contraction during EPP recordings, we used μ-conotoxin GIIIB (μ-CgTx-GIIIB, 1.5 μM) with a recirculation system. After a muscle fiber had been impaled, the nerve was continuously stimulated (70 stimuli at 0.5Hz) using two platinum electrodes coupled to a pulse generator (CIBERTEC CS-20) and linked to a stimulus isolation unit. We recorded the last 50 EPPs. We selected fibers with membrane potentials of no less than -70mV (however, see below for the single-fiber experiments) and used only those results from preparations which did not deviate by more than 5 mV during the recording. The mean amplitude (mV) per fiber was calculated and corrected for non-linear summation (EPPs were usually more than 4mV) (McLachlan & Martin, 1981) assuming a membrane potential of – 80 mV. Quantal content (M) was estimated by the direct method, which consists of recording mEPPs and EPPs simultaneously and then calculating the ratio: M =Average Peak EPP/Average Peak mEPP. Incubation with the drugs took place for one hour. We studied a minimum of 15 fibers per muscle and usually a minimum of 5 muscles in each type of experiment. In the single-fiber experiments (time course of the effect of drugs on EPPs of the same permanently impaled fiber), the drugs were added to the bathing solution and EPPs were recorded as previously described every 15 minutes for a minimum of 60 minutes. During the 60-minute period of single-fiber experiments, the membrane potential sometimes fell below -70 mV. In these particular cases, the muscle fiber was discarded when the resting potential was less than -45 mV.

We also applied repetitive stimulation (trains at 40 and 100 Hz for 2 minutes) to evaluate the effects of the different drugs on synaptic depression. There were 10-minute intervals between trains to allow for muscle recovery. We recorded 2 minutes of EPPS and used the first 15 and the last 15 EPPS to evaluate changes in depression. We evaluated the ratio between the mean size

of the last 15 EPPs of each train and the mean size of the first 15 EPPs. We also analyzed possible facilitation as the ratio between the sizes of the second EPP and the first EPP in each train of 40Hz. We also experimented with paired pulses at 100 Hz. We studied between 8 and 10 fibers per muscle and usually between 5 and 8 muscles in each type of experiment. In the single-fiber experiments, the drugs were added to the bathing solution and the EPPs were recorded every 15 minutes for 60 minutes.

Standard sharp-electrode intracellular recording techniques were used to show that miniature endplate potential (MEPP) amplitudes and postsynaptic resting membrane potentials were unaffected and, therefore, that all the compounds used act presynaptically in the present conditions. The MEPP frequency in each solution was recorded for 100 s from at least 15 different neuromuscular junctions and the values were averaged. ACh in the synaptic cleft can increase during trains of 40 Hz or 100 Hz. This may modify the sensitivity of the AChRs. Therefore, we evaluated the size of the MEPPs during the trains and did not notice any significant change with the drugs used. For example, the change in the amplitude of MEPPs during trains of 40Hz in the presence of 25μM adenosine was 14.90% ± 2.07 and for 8-SPT it was 2.41% ± 4.65 ($P>0.05$).

Consecutive incubations with two substances are used as a pharmacological tool to investigate the possible occlusive or additive crosstalk effects between them. We recorded and measured control EPPs, and then we incubated the muscle for one hour in the first compound. After recording the EPPs again, we incubated it for one hour in the second compound (in the presence of the first drug) and then recorded the EPPs.

Chemicals

All stock solutions were stored at -20ºC for less than four weeks. We chose drug concentrations that did not change the size of the MEPPs in the concentration-response curves

performed in previous experiments. The final DMSO concentration in control and drug-treated preparations was 0.1% (v/v). In control experiments, this concentration of DMSO did not affect any of the parameters studied (data not shown).

Several stock solutions were dissolved in deionized water: Adenosine (Sigma-Aldrich); 2-chloroadenosine (CADO; Sigma-Aldrich); 8-(p-Sulfophenyl) theophylline hydrate (8-SPT; Sigma-Aldrich); Pirenzepine dihydrochloride (Tocris); Methoctramine tetrahydrochloride (Sigma); Atropine (Sigma); Adenosine 3′,5′-cyclic Monophosphorothioate, 8-Bromo-, Rp-Isomer, Sodium Salt (Sp-8-BrcAMPs, Calbiochem); methyl-beta-cyclodextrin (MCD) 2% (Sigma).

Several stock solutions were dissolved in dimethylsulfoxide (DMSO, Tocris): 2-Chloro-N6-cyclopentyladenosine (CCPA; Sigma-Aldrich); 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX; Sigma-Aldrich); 2-p-(2-Carboxyethyl) phenethylamino-5′-N-ethylcarboxamidoadenosine hydrochloride hydrate (CGS-21680; Sigma-Aldrich); 2-(2-Furanyl)-7-(2-phenylethyl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine (SCH-58261; Tocris); N-(4-Acetylphenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)phenoxy]acetamide (MRS1706; Tocris); 1,4-Dihydro-2-methyl-6-phenyl-4-(phenylethynyl)-3,5-pyridinedicarboxylic acid 3-ethyl-5-[(3nitrophenyl)methyl] ester (MRS1334; Tocris); Phorbol 12-myristate 13-acetate (PMA, Sigma); Calphostin C (CaC, Sigma); N-[2-((p-Bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, 2HCl (H-89, Calbiochem).

Statistical procedure

The statistical software SPSS© v17.0 was used to analyze the results. Values are expressed as means ± SEM. The values are sometimes expressed as "Percentage of change". This is defined as: [final value / initial value] X 100. We used the two-tailed Welch's t-test for

unpaired values because our variances were not equal. We prefer this test because it is more conservative than the ordinary t-test. Differences were considered significant at $P < 0.05$.

RESULTS

When the voltage-dependent sodium channel of the muscle cells was blocked with μ-CgTx-GIIIB (to prevent contraction in electrophysiological experiments), some of the effects reported for AR in the modulation of ACh release at the adult NMJ are not clearly observed (Garcia *et al.*, 2013; Tomas *et al.*, 2014). By way of introducing the present results, the bar chart in figure 1A shows some representative data. No effect is observed on quantal content after AR unselective exogenous stimulation (initial quantal content: 43.00 ± 4.01, ADO: 42.69 ± 3.05, $t_{85} = 0.3167$, P = 0.7526, n = 5 muscles with 15 fibres per muscle), inhibition (initial quantal content: 41.03 ± 3.00, 8SPT: 42.15 ± 4.16, $t_{83} = 1.519$, P = 0.1235, n = 5 muscles with 15 fibres per muscle) or eliminating endogenous adenosine with adenosine deaminase (initial quantal content: 44.85 ± 2,74, ADA: 41.95 ± 3.12, $t_{93} = 0.2541$, P = 0.8000, n = 5 muscles with 15 fibres per muscle). In addition, the A_1R and $A_{2A}R$ selective antagonists DPCPX and SCH-58261 have no effect on quantal content (initial quantal content: 44.07 ± 3.38, DPCPX: 47.65 ± 4.33, $t_{82} = 0.3413$, P = 0.7337; initial quantal content: 44.51 ± 3.56, SCH-58261: 43.77 ± 4.20, $t_{86} = 1.072$, P = 0.2867; in both cases, n = 5 muscles with 15 fibres per muscle).

However, AR are involved in control of spontaneous release (MEPP), which seems to be mediated by A_1R [MEPP frequency is increased by 8SPT ($t_{87} = 2.225$, P = 0.0267) and DPCPX ($t_{73} = 3.514$, P = 0.0008), in both cases, n = 5 muscles with 15 fibres per muscle]. Because $A_{2B}R$ and A_3R have recently been found in the motor nerve terminals at the mouse neuromuscular junction (Garcia *et al.*, 2014), here we investigate the $A_{2B}R$ and A_3R selective antagonists MRS1706 and MRS1334. The raw data in figure 1A show no change in evoked and spontaneous release (the numerical data are: quantal content, % of change: MRS1706, 10.14 ± 29.60, $t_{82} = 0.2913$, P = 0.7715; MRS1334, 10.02 ± 9.06, $t_{98} = 0.4626$, P = 0.6447; in both cases, n = 5 muscles with 15 fibres per muscle; the MEPPs frequency, % of change:

MRS1706, 2.26 ± 2.15, t_{86} = 0.3834, P = 0.7023; MRS1334, 4.19 ± 2.56, t_{84} = 0.4957, P = 0.6114; in both cases, n = 5 muscles with 15 fibres per muscle).

One of the important roles of AR is that it controls activity-dependent synaptic depression. In a previous study (Garcia *et al.*, 2013; Tomas *et al.*, 2014), we found that stimulation with adenosine reduces the synaptic depression produced during trains of 2 minutes at 40Hz, whereas blocking AR with 8SPT considerably increases it. When endogenous adenosine is removed with ADA, no change in depression is observed. In addition, the selective A_1R and $A_{2A}R$ inhibitors DPCPX and SCH-58261 have no effect on synaptic depression. Some of these data at 40 Hz, are reproduced in figure 1B so that they can be better compared with the present data obtained at 100Hz). Here we found (raw data in figure 1B) that the $A_{2B}R$ and A_3R selective antagonists MRS1706 and MRS1334 do not change depression (synaptic depression, % of change: MRS1706, 2.83 ± 7.38, t_{95} = 0.1137, P = 0.9097; MRS1334, 9.48 ± 8.51, t_{83} = 0.5730, P = 0.5652; in both cases, n = 5 muscles with 8-10 fibres per muscle). Thus, judging by the effect on depression of the exogenous nonselective agents ADO and 8SPT, some collaboration between different AR subtypes may influence depression. However, the experiments with ADA indicate that the endogenous production of ADO cannot effectively reduce depression and this raises doubts about the physiological significance of the exogenously added ADO and 8SPT observations. The level of endogenous ADO in the synaptic cleft during trains at 40Hz may be unsufficient. Thus, in the present study we analysed the effect of ADA and the agents ADO and 8SPT on the control of depression during the electrical stimulation of muscles at 100Hz (trains of 2 minutes at 100Hz). At this high frequency of evoked synaptic activity, depression soon becomes 75-80 % of the initial EPP size (mean size of the first 15 EPPs: 30.42mV ± 4.30, mean size of the last 15 EPPs: 6.24 mV± 1.97; n= 22 animals, 8-10 fibers per muscle) although the trains can be reproduced for at least 2-3 hours. Transmission failures (stimuli

that do not produce EPP) are frecuently observed whereas at 40 Hz failures are scarce. The data in the bar chart of figure 1B show that neither ADO nor 8SPT can change the depression of synaptic potentials in these stimulation conditions (ADO: t_{96} = 0.2832, P = 0.7776; 8SPT: t_{96} = 0.8213, P = 0.9347; n= 5 animals, 8-10 fibers per muscle). However, in the presence of ADA the depression produced at 100 Hz increases considerably (t_{83} = 4.618, P = <0.0001, n= 5 animals, 8-10 fibers per muscle). This seems to indicate that at high activity levels, the amount of endogenous ADO in the synaptic cleft could be sufficient to interact with AR and might protect against additional depression. However, whereas the selective A_1R inhibitor DPCPX does not affect depression at 100 Hz (t_{96} = 0.5988, P = 0.5844, n= 5 animals, 8-10 fibers per muscle), the $A_{2A}R$ inhibitor SCH-58261 considerably reduces depression (t_{57} = 2.210, P = 0.0311, n= 5 animals, 8-10 fibers per muscle). Although several interpretations are possible, on the whole, these data indicate that there is some collaboration between A_1R and $A_{2A}R$ receptor subtypes in the control of depression. The collaboration between A_1R and $A_{2A}R$ receptors is additionally suggested in experiments with DPCPX in the presence of SCH-58261 and reciprocally. The results indicate that neither DPCPX in the presence of SCH-58261 at 100 Hz (% of change: 6.72 ± 5.41, t_{65} = 0.05909, P = 0.9531, n= 3 animals, 8-10 fibers per muscle) nor SCH-58261 in presence of DPCPX at 100 Hz have any effect (% of change: 87.86 ± 14.18, t_{58} = 2.317, P = 0.0241, n= 3 animals, 8-10 fibers per muscle).

Effect of 2-chloroadenosine (CADO) on evoked ACh release in the presence of μ-CgTx-GIIIB

Because differences in the frequency of synaptic activation reveal complementary aspects of AR coupling to depression control, another point of view may be obtained by the induction of a more stable and prolonged effect on receptors. CADO has the same affinity as adenosine for the ARs but is not metabolized, which leads to a prolonged effect. We studied

how the unselective agonist CADO influences ACh release in μ-CgTx-GIIIB treated muscles. Figure 2A (see also the summary diagram in figure 6A) shows that CADO 10μM reduces evoked quantal release by about 35% (% of inhibition: 35.54 ± 3.57, n = 5 muscles with 15 fibres per muscle, t_{106} = 2.046, P = 0.0433). This may reveal that, in these circumstances, ARs play a negative role in transmitter release, as decribed elsewhere (Di Angelantonio *et al.*, 2011; Pousinha *et al.*, 2012). However, CADO applied at 300 nM does not affect quantal content (data not show in the figure 2A; the numerical data are: by itself: % of change 2.97 ± 9.03, t_{115} = 0.2422, P = 0.8091; after preincubation with DPCPX: % of change 18.32 ± 8.38, t_{147} = 0.5377, P = 0.5916; after SCH-58261: % of change 8.02 ± 6.65, t_{137} = 0.3077, P = 0.7588; after 8SPT: % of change 4.94 ± 4.11, t_{147} = 0.1554, P = 0.8767; in all cases, n = 5 muscles with 15 fibres per muscle).

Which AR subtypes are involved in the CADO effect at 10μM? We analysed how CADO changes evoked transmitter release after the selective block of A_1R and $A_{2A}R$ receptors. Figure 2A shows that after A_1R block with DPCPX or $A_{2A}R$ inhibition with SCH-58261, CADO was able to still reduce quantal content [% of inhibition: 35.78 ± 4.18 (t_{108} = 4.070 , P = <0.0001) and 27.72 ± 0.05 (t_{86} = 2.182, P = 0.0318) respectively; in both cases, n = 5 mucles with and 15 fibres per muscle]. Interestingly, after the non-selective block (8SPT) of the AR subtypes CADO had no effect (t_{137} = 0.6227, P = 0.5345, n = 5 muscles with and 15 fibres per muscle). One interpretation of these results is that the effect of CADO on ACh release (quantal content reduction at 10μM) again depends on collaborative work between A_1R and $A_{2A}R$. At least one AR subtype is necessary for the CADO effect.

With respect to spontaneous ACh release (figure 2B), CADO (10μM) did not change MEPP frequency. Neither did it change MEPP frequency after preincubation with DPCPX, SCH-58261 or 8SPT [CADO by itself: % of change 7.66 ± 5.99 (t_{128} = 0.4236, P = 0.6726); after preincubation with DPCPX, SCH-58261 or 8SPT: % of change 10.32 ± 9.39 (t_{147} =

1.564, P = 0.1199), 10.54 ± 8.09 (t_{133} = 1.003, P = 0.3177) and 3.69 ± 3.97 (t_{112} = 0.6014, P = 0.5488) respectively; in all cases, n = 5 muscles with 15 fibres per muscle]. Surprisingly, however, CADO incubation at 300 nM increased MEPP frequency by a significant 42%, although not when CADO was added after preincubation with DPCPX, SCH-58261 or 8SPT [CADO by itself: % of increase 41.72 ± 2.74 (t_{148} = 7.044, P = <0.0001); this increase is prevented preincubating with DPCPX, SCH-58261 or 8SPT: % of change 14.08 ± 3.00 (t_{143} = 1.668, P = 0.0974), 3.64 ± 2.53 (t_{104} = 0.7047, P = 0.4820) and 1.08 ± 4.10 (t_{134} = 0.1315, P = 0.8956) respectively; in all cases, n = 5 muscles with 15 fibres per muscle]. Thus, some colaborative work between the receptors seems to be required if CADO is to have an effect on MEPP at low concentrations. Both A_1R and $A_{2A}R$ receptors are necessary in this case.

We also studied how the prolonged effect of CADO on adenosine receptors influenced activity-dependent synaptic depression and which AR subtypes were involved (figure 2C and the recapitulation diagram in figure 6B). We found that CADO (300 nM) had no effect on depression at 40Hz (data not show). However, a non-significant 17% of depression reduction (the same tendency as ADO) was observed at 10μM (t_{80} = 1.068, P = 0.2891, n = 5–8 muscles, with 8–10 fibres per muscle). After A_1R block with DPCPX the small non-significant CADO effect was reversed (depression underwent a non-significant increase of 19%; t_{69} = 1.299, P = 0.1981, n = 5–8 muscles, with 8–10 fibres per muscle). Although they are clearly a long way from being significant, these results suggest that A_1R tends to have a small protective effect on depression. After $A_{2A}R$ receptors had been blocked with SCH-58261 a strong protective effect of CADO was found (similar in magnitude to the effect of ADO in the same conditions, see figure 1B; t_{71} = 2.991, P = 0.0038, n = 5–8 muscles, with 8–10 fibres per muscle). Thus the protective effect of CADO seems to be mediated by A_1R because it increased after $A_{2A}R$ block and was prevented when both receptors were blocked (8SPT: t_{96} = 0.6130, P = 0.5313, n = 5–8 muscles, with 8–10 fibres per muscle) or in

the simultaneous presence of SCH-58261 and DPCPX (percentage of change 3.96 ± 3.76, t_{47} = 0.2358, P = 0.8146; n = 5-8 muscles with 8–10 fibres per muscle). These data also suggest that $A_{2A}R$ receptors have a negative modulatory role on A_1R in these CADO conditions.

Interaction between ARs and mAChRs in transmitter release modulation

In the adult, M_1 (inhibited by pirenzepine, PIR) and M_2 (inhibited by methoctramine, MET) muscarinic ACh receptors (mAChRs) subtypes modulate evoked transmitter release by positive and negative feedbacks, respectively (Santafe *et al.*, 2003; Santafe *et al.*, 2006). Both M_1- and M_2-mediated mechanisms operate in parallel and their simultaneous unspecific stimulation with oxotremorine M (OXO-M) decreases release whereas the unspecific block of all mAChRs by atropine (AT) increases transmitter output probably because of the predominance of the M_2 subtype. This suggests a tonic inhibition of ACh release in the basal situation when the M_1-M_2 couple is directly operated by endogenous ACh, thus saving the synapse function by decreasing the extent of evoked release. In addition, mAChRs can stabilize the conditions of the spontaneous quantal output of ACh because any imbalance results in decreased MEPP frequency [(Tomas *et al.*, 2014); some of these published data are in figure 3 A,B and C].

Figure 3A shows that AT cannot modify evoked and spontaneous ACh release when ARs are previously stimulated (ADO, t_{82} = 0.04634, P = 0.9631, n = 5 muscles with 15 fibres per muscle) or inhibited (8SPT, t_{120} = 0.05831, P = 0.9536, n = 5 muscles with 15 fibres per muscle). The insets in this figure show raw data of the effect of AT on evoked and spontaneous release (figure 3, inset Aa) and the lack of AT effect in the presence of 8SPT (figure 3, inset Ab). Although 8SPT did not change quantal content by itself, it can prevent AT from affecting evoked release. In reciprocal experiments, blocking mAChRs with AT did not allow AR to be coupled to evoked release [ADO (t_{124} = 0.9575, P = 0.3402) and 8SPT

(t_{124} = 1.306, P = 0.1940) cannot change quantal content as normally occurs without AT preincubation; in both cases, n = 5 muscles with 15 fibres per muscle]. However, AT preincubation effectively suppressed the effect that ADO (t_{141} = 0.5911, P = 0.5554, n = 5 muscles with 15 fibres per muscle) and 8SPT (t_{128} = 0.2925, P = 0.7703, n = 5 muscles with 15 fibres per muscle) had on decreasing and increasing MEPP frequency, respectively (see also figure 1A). Because CADO (10μM) reduces evoked quantal release by about 35%, which suggests that a prolonged agonist effect on ARs may help to regulate ACh release, we assayed how preincubation with AT modifies the CADO effect and performed the reciprocal experiment. We found that neither AT nor CADO can affect quantal content or MEPP frequency after the muscle is preincubated with the other compound (quantal content, CADO *vs* AT: t_{121} = 0.2572, P = 0.7974; quantal content, AT *vs* CADO: t_{150} = 0.7314, P = 0.4657; MEPP frequency, CADO *vs* AT: t_{147} = 0.5297 , P = 0.5971; MEPP frequency, AT *vs* CADO: t_{130} = 0.2811, P = 0.7790; in all cases, n = 5 muscles with 15 fibres per muscle). In addition, in figures 3B and 3C we analysed the effect of several selective inhibitors of the various AR and mAChR subtypes. The data show that neither PIR nor MET can modify evoked and spontaneous ACh release when ARs are previously stimulated (ADO) or inhibited (8SPT). Nor do they work after A_1R inhibition with DPCPX or $A_{2A}R$ inhibition with SCH-58261.

The first conclusion to be drawn is that if mAChRs are to operate normally, the set of ARs needs to operate normally and the second is that inhibition of the mAChRs does not allow the ARs to couple to evoked release. Moreover, if ARs are to couple to spontaneous release the mAChRs need to operate normally and vice versa. Thus, there is an absolute interdependence of ARs (A_1R, $A_{2A}R$) and mAChRs (M_1, M_2) in order to modulate evoked and spontaneous ACh release in basal conditions.

Because of the mutual dependence of AR and mAChR on the control of evoked and spontaneous ACh release, we have investigated the possible involvement of lipid raft

microdomains in controlling the interaction of the two receptor systems. We found that after preincubation with MCD (2%) (Xu *et al.*, 2006), incubation with AT (2 μM) or CADO (10 μM) leads, respectively, to the normal increase (% of variation: 206.56 ± 53.64, n = 3 muscles with 15 fibres per muscle, $t_{85} = 6.65$, $P < 0.0001$) and decrease (% of variation: 57.26 ± 4.01, n = 3 muscles with 15 fibres per muscle, $t_{66} = 4.819$, $P < 0.0001$) of the quantal content, and also the normal expected changes in the MEPP frequency. Neither can AT modify quantal content (% of variation: 5.20 ± 6.65, n = 3 muscles with 15 fibres per muscle, $t_{84} = 0.1551$, $P = 0.8871$) or MEPP frequency (% of variation: 93.75 ± 6.54, n = 3 muscles with 15 fibres per muscle, $t_{60} = 0.4831$, $P = 0.6308$) after preincubation with 8SPT in the presence of MCD as occurs without the lipid raft disruptor. Altogether, these data suggest that lipid rafts do not control AR and mAChR binding and signaling relations.

Interaction between ARs and mAChRs in the control of synaptic depression

In previous studies, we observed that blocking M_1-M_2 receptors with AT results in more depression during activity (inset in figure 4; $t_{137} = 2.563$, $P = 0.0114$) though M_1-M_2 stimulation with OXO-M do not change depression. This suggest that mAChRs are involved in tonically protect from depression (Tomas *et al.*, 2014). Thus, adenosine and muscarinic receptors seem to share some functional control of synaptic depression [blocking them with 8SPT ($t_{787} = 4.369$, $P = <0.0001$) and AT, respectively, increases depression]. We have analysed here the possible occlusive or additive crosstalk effects between the two receptor groups in the control of depression to gain insight into the respective mechanisms and possible shared features. Thus, we have investigated (in depression conditions induced at 40Hz), the effect of AT after incubation with 8SPT and vice versa. We have also investigated the possible effect on depression of AT after ADO and vice versa (figure 4). We found that ADO cannot reduce depression after AT preincubation ($t_{144} = 0.2787$, $P = 0.7809$) and,

reciprocally, AT cannot increase depression after ADO preincubation (t_{147} = 0.9342, P = 0.9342). However, whereas AT does not increase depression after 8SPT (t_{145} = 1.028, P = 0.3054), 8SPT increase depression after AT (t_{127} = 2.037, P = 0.0437) to the same extent as when it acts by itself. In conclusion, these data indicate that the purinergic and muscarinic mechanisms cooperate in the control of depression and may share a common pathway. The fact that AT does not increase depression after 8SPT may be due to a strong saturation effect of the purinergic inhibitor.

Interaction between ARs and serine kinases PKC and PKA

In basal conditions, ACh release can be reduced and increased by the PKA modulators, the inhibitor H-89 and the stimulator Sp8Br. Figure 5A shows that after inhibiting or stimulating AR (with 8SPT or ADO), PKA works (can be inhibited with H-89; H-89 *vs* 8SPT: t_{105} = 2.199, P = 0.0300; H-89 *vs* ADO: t_{144} = 2.151, P = 0.0331) but cannot be additionally stimulated (with Sp8Br; Sp8Br *vs* 8SPT: t_{148} = 0.09428, P = 0.9250 ; Sp8Br *vs* ADO: t_{82} = 0.01888, P = 0.9850). The MEPP frequency in H-89 (no change; t_{140} = 1.785, P = 0.0764) and in Sp8Br (increases over 150%; t_{126} = 6.865, P = <0.0001) is the same as the frequency observed when these substances are applied by themselves and independently of a previous incubation with ADO (Sp8Br *vs* ADO: t_{104} = 6.240, P = <0.0001; H-89 *vs* ADO: t_{110} = 1.943, P = 0.0545) or 8SPT (Sp8Br *vs* 8SPT: t_{115} = 14.675, P = <0.0001; H-89 *vs* 8SPT: t_{137} = 1.761, P = 0.0805). These data indicate that the tonic coupling of the PKA to promote ACh release (which can be inhibited with H-89) was maintained after stimulating (ADO) or inhibiting (8SPT) adenosine receptors. However, in both these conditions, the influence of PKA on ACh release cannot be increased with Sp8Br. As far as spontaneous release is concerned, the state of AR does not affect the coupling of PKA. Interestingly, there is a difference with mAChRs because when fully inhibited in the presence of AT, the two PKA

modulators (H-89 and Sp8Br) respectively decrease and increase ACh release (Santafe *et al.*, 2006).

In basal conditions, PKC did not seem to be tonically involved in transmitter release because the blocker calphostin C (CaC) did not reduce release [data not shown; (Santafe *et al.*, 2007b)]. However, increasing Ca^{2+} in the physiological solution (to 5 mM) or maintaining NMJ activity by continuous electrical stimulation (1 Hz), couples PKC to ACh release, which can now be inhibited by CaC (by about 40%) Figures 5B (quantal content) and 5C [MEPP frequency; see also (Santafe *et al.*, 2007b; Besalduch *et al.*, 2010)]. We observed here that previously blocking or stimulating AR with 8SPT or ADO, respectively, in both high external Ca^{2+} (CaC *vs* 8SPT: $t_{93} = 0.3056$, $P = 0.7606$; CaC *vs* ADO: $t_{87} = 0.008471$, $P = 0.9972$) or during continuous trains at 1 Hz (CaC *vs* 8SPT: $t_{148} = 0.02593$, $P = 0.9794$; CaC v*s* ADO: $t_{148} = 0.7124$, $P = 0.4773$), completely prevents the effect of CaC on quantal content. We conclude that the activity coupling of PKC to ACh release needs normally balanced adenosine receptors.

Phorbol esters like PMA (potent PKC stimulators) increase quantal content by about 100% and MEPP frequency by about 200% (Santafe *et al.*, 2007a). However, previous incubation with most AR agents (ADO and 8SPT in figures 5B and 5C) completely abolishes the effect of PMA on evoked ACh release (PKC canot be stimulated with PMA; PMA *vs* ADO: $t_{87} = 1.074$, $P = 0.2856$; PMA $_{vs}$ 8SPT: $t_{98} = 1.303$, $P = 0.1956$) although it does not change the PMA-dependent MEPP frequency increase (PMA *vs* ADO: $t_{87} = 8.045$, $P < 0.0001$; PMA *vs* 8SPT: $t_{98} = 8.797$, $P < 0.0001$). It seems that the imbalance of the AR prevents PKC from coupling to evoked transmitter release. When the AR are fully inhibited in the presence of 8SPT or stimulated with ADO, the effect of CaC (in high Ca^{2+} and stimulation conditions at 1Hz) and the effect of PMA on quantal content cannot be demonstrated.

DISCUSSION

~~The main results show that~~ Adenosine reduced the number of spontaneous quantal events through A_1R receptors and ~~that~~ at high activity levels there may be enough endogenous ADO in the synaptic cleft to interact mainly with A_1R receptors and protect against depression. When non metabolizable CADO is used, both quantal content and depression are reduced when A_1R and $A_{2A}R$ receptors are involved. AR and mAChR are mutually dependent on modulating spontaneous, evoked and activity-dependent (depression) ACh release although purinergic control seems to be more efficient than muscarinic control at controlling depression.

A_1R, $A_{2A}R$, $A_{2B}R$ and A_3R molecules are present in the pre- and postsynaptic sites at the NMJ and A_1R receptors are also present in the Schwann cells (Garcia *et al.*, 2013; Garcia *et al.*, 2014). Robitaille's group has clearly shown that glia are actively involved in synaptic stability and plasticity (post-tetanic potentiation or depression depending on the activity pattern of the synapses). Schwann cells decode the pattern of synaptic activity through AR and subsequently provide bidirectional feedback to synapses (Todd & Robitaille, 2006). Also, mAChRs are present in Schwann cells (Garcia *et al.*, 2005; Wright *et al.*, 2009) and may regulate glial cell activation (in response to low synaptic activity) that induces and guides compensatory axonal growth. These data indicate that the receptors are located in different compartments and suggest a complex multicellular signalling that has not been evaluated here. We focused on the direct role of AR in ACh release modulation.

Effect on evoked and spontaneous ACh release of adenosine receptors in the presence of μ-CgTx-GIIIB

Experiments were made with μ-CgTx-GIIIB. Here we have confirmed that in these basal conditions A_1R reduces spontaneous quantal leak of ACh, although in test stimulations

at 1 Hz any receptor subtype can be involved in the modulation of evoked release. However, in the same conditions, the non metabolizable 2-chloroadenosine (CADO) agonist can be used to forcibly reduce quantal content. In recording conditions (to prevent contraction) that directly interfere with neurotransmission – for example, high Mg^{2+} (ACh release reduction) or d-tubocurarine (reduction of the postsynaptic response) – added adenosine reduces EPP quantal content and/or MEPP frequency in both frog (Bennett *et al.*, 1991; Karunanithi *et al.*, 1992; Searl & Silinsky, 2005; Shakirzyanova *et al.*, 2006; Adamek *et al.*, 2010) and rodent NMJs (De Lorenzo *et al.*, 2006; Pousinha *et al.*, 2010). Also, in these recording conditions (with high Mg^{2+} or d-tubocurarine), the findings suggest that at low stimulation frequencies there is a predominant tonic inhibition mediated by A_1R. At higher frequencies, however, adenosine facilitates ACh release through the phasic activation of adenosine $A_{2A}R$.

Thus, when the sodium channel of the muscle cells was blocked with μ-CgTx-GIIIB, some of the effects previously reported for AR in ACh release are not observed (Garcia *et al.*, 2013; Tomas *et al.*, 2014). Several synaptic molecules change their normal functional expression if neurotransmission is altered. There are many examples. When ACh release is low (high external Mg^{2+} or low Ca^{2+}) or when there is less ACh in the cleft (when acetylcholinesterase is added), or even in the postnatal low-release nerve endings, the whole response of M1 and M2 mAChRs to endogenously released ACh (which is normally to reduce ACh secretion in the adult; (Santafe *et al.*, 2003; Santafe *et al.*, 2006) shifts to increase release (Santafe *et al.*, 2007b). Also, in the presence of curare, the selective mAChR blockers no longer change ACh release (Santafe *et al.*, 2006), and in high Mg^{2+} conditions, protein kinases C and A lose their normal coupling to ACh release (Santafe *et al.*, 2007b).

Though interference with neurotransmission reveals several features of how AR works, we believe that the use of μ-CTX GIIIB in electrophysiological experiments creates a more physiological scenario. This scenario differs from the high Mg^{2+} condition and from

curare-paralyzed muscles in the normal postsynaptic membrane activation that occurs in the presence of μ-CgTx-GIIIB. Our control studies show that the toxin by itself does not affect quantal content, MEPP frequency and size, time course of the EPPs, and no EPP responses are observed close to saturation (because too much transmitter is released in μ-CTX GIIIB-treated muscles) (Garcia *et al.*, 2010).

Thus, at this point, the conclusion can be drawn (see diagram in figure 6A) that in the absence of any interference with neurotransmission endogenous adenosine (ADO) tonically influences A_1R adenosine receptors so that spontaneous release remains low in basal conditions but does not affect evoked release. Interestingly, however, the prolonged effect of the non metabolizable agonist CADO leads to a different situation (Di Angelantonio *et al.*, 2011; Pousinha *et al.*, 2012). At submicromolar concentrations of CADO, MEPP frequency increases as a result of colaborative work between A_1R and $A_{2A}R$. Also, quantal content can be reduced by using CADO at micromolar concentrations depending on both A_1R and $A_{2A}R$. Increased spontaneous release (CADO at 300 nM) may alter the availability of the readily releasable pool for exocytosis resulting in the CADO-induced evoked release reduction. However, this seems unlikely because at the concentration that CADO reduces evoked release (10 μM), MEPP frequency is not affected.

On the whole, the results reveal a complex involvement of both $A_{2A}R$ and A_1R in spontaneous and evoked release when they are strongly stimulated (CADO) beyond the limits of the basal condition.

AR and depression control

The present results confirm that ARs are involved in the control of depression. Again, some collaborative work between AR subtypes may reduce synaptic depression. However, adenosine deaminase (ADA) cannot reduce depression at 40 Hz, which suggests that the

endogenous production of ADO may be insufficient. This raises doubts about the physiological relevance of the observations when exogenously added ADO and 8SPT are used. Whereas the full block of ARs (8SPT) results in large depression, endogenous ADO elimination with ADA does not. Two explanations are: i) the tonic effect of ARs when they are blocked with 8SPT may be partly independent of stimulation with ADO. A transactivation of the AR similar to that of the receptor tyrosine kinases in response to G-protein-coupled receptor signaling may intervene (Daub *et al.*, 1996; Lee & Chao, 2001; Fischer *et al.*, 2003; Wiese *et al.*, 2007; Oliveira *et al.*, 2009; Garcia *et al.*, 2010); ii) a fraction of endogenous ADO may avoid breaking by ADA (perhaps because produced very close to the AR).

Therefore, we analysed depression while muscles were being electrically stimulated at 100Hz so that endogenous ADO production would increase. We also forced the AR mechanism by using stimulation with CADO (summary diagram in figure 6B). At high activity levels (100Hz), ADA greatly increases depression, which indicates that endogenous ADO production in the synaptic cleft could be sufficient to reduce depression in this case. However, in these stimulation conditions, neither added ADO nor 8SPT can change the depression of synaptic potentials. Additional exogenous ADO may find AR occupied or desensitized. The high level of endogenous adenosine may prevent any additional effect of exogenously added adenosine. Surprisingly, however, the fact that 8SPT at 100 Hz has no effect on depression indicates that when these receptors are not operative, depression does not increase. Another set of modulators (ACh itself) and receptors (mAChR) may substitute AR in the control of depression (see below and Tomas et al., 2014 (Tomas *et al.*, 2014)). Alternatively, endogenous ADO can displace 8SPT from the receptors to have a protective effect on depression. In the absence of endogenous ADO (preincubation with ADA) there is much more depression than in the control. However, depression is not greater than in the control after A_1R receptor block (preincubation with DPCPX), which suggests a small

facilitatory tonus of endogenous ADO though $A_{2A}R$ receptors. When $A_{2A}R$ receptors are blocked (SCH-58261), endogenous ADO has a potent facilitatory effect through A_1R receptors. This may indicate that at high activity rates endogenous ADO reduces depression through A_1R and $A_{2A}R$ receptors.

In most systems, ADO was a full agonist at all four adenosine receptors (in functional assay using changes in cAMP formation and EC50 determination). Potency was highest at the A_1R receptor and second highest at the $A_{2A}R$ receptor. Thus, the ADO present under physiological conditions mainly activates A_1R, whereas the $A_{2A}R$ receptors are expected to be less activated by physiological levels of adenosine (Fredholm *et al.*, 2001).

Interestingly, results show that CADO has some ability to reduce depression (by a non-significant 17%). This protective effect can be mediated by A_1R and $A_{2A}R$ can have some modulatory effect because protection was significantly increased after $A_{2A}R$ block and was prevented when both receptors were blocked.

In summary (see diagram in figure 6B), it seems that endogenous ADO can protect for depression through A_1R stimulation mainly during intense repetitive activity whereas $A_{2A}R$ seems to modulate the A_1R response. It is not clear if reduced depression could be a simple consequence of a reduced release probability mediated by the AR. In fact, adenosine reduced the number of spontaneous quantal events (MEPP frequency) but not the quantal content of EPP. And stimulation with CADO (10μM) reduces quantal content but does not affect MEPP frequency.

Interaction between AR and mAChRs in transmitter release modulation

The overall outcome of the mAChR is to stabilize the conditions of the spontaneous quantal output of ACh (an M_1 and M_2 role), and save the synapse function by decreasing the extent of evoked release (mainly an M_2 action) and reducing depression (Tomas *et al.*, 2014).

Therefore, AR and mAChRs intervene differently in spontaneous and evoked ACh release modulation but seem to share some functional control over synaptic depression. In low release conditions (high Mg^{2+}), it has also been reported that adenosine outflow from active motor nerve terminals may (through A_1R) be coupled to reduce M2 function and adenylyl cyclase may be the common intracellular link (Oliveira *et al.*, 2009). We observed here an absolute mutual dependence of AR and mAChR on the modulation of evoked and spontaneous ACh release in basal conditions and in experimental conditions with CADO stimulation (figure 3 and diagram in figure 6C). The well established effects of mAChRs antagonists on evoked release do not come about after AR are blocked or stimulated. In addition, the effect of CADO was prevented by preincubation with AT. This suggests that the muscarinic involvement depends on some sort of purinergic permission, and vice versa, and both mechanisms seem to share the same intracellular routing. In addition, the purinergic and muscarinic mechanisms cooperate in the control of depression (diagram in figure 6D) although the purinergic control seems to be more powerful than the muscarinic one because depression can also be increased with 8SPT after an initial increase in depression by muscarinic block with AT. The neuromodulation exerted by the purinergic system may do more than simply control the release of neurotransmitter and must behave as a modulatory system playing a permissive role for the action of other signaling molecules and pathways (Ribeiro *et al.*, 1996; Sebastiao & Ribeiro, 2000; Fredholm *et al.*, 2005).

The plasma membranes contain combinations of glycosphingolipids and protein receptors organized in lipid rafts. These specialized membrane microdomains compartmentalize cellular processes by serving as organizing centers for the assembly of signaling molecules. Several G protein-coupled receptors function within lipid raft plasma membrane microdomains, including purinergic receptors (Giniatullin *et al.*, 2015), which may be important for regulating signal transduction, receptor trafficking and neurotransmission.

The mutual dependence of AR and mAChR on ACh release and their cooperation in the control of depression may be related with their belonging to a lipid raft. The data show, however, that the disruption of lipid rafts does not change the normal coupling and mutual relations of AR and mAChRs.

Interaction between AR and serine kinases

Membrane receptors are coupled to intracellular pathways that converge on a limited repertoire of effector kinases involved in transmitter exocytosis (West *et al.*, 1991; Numann *et al.*, 1994; Tanaka & Nishizuka, 1994; Byrne & Kandel, 1996; Catterall, 1999; Santafe *et al.*, 2005; 2006; Santafe *et al.*, 2007b). How are the kinases PKC and PKA related to the purinergic and muscarinic mechanisms? It is known that AR modulate PKA pathway activity (Schulte & Fredholm, 2002; Fredholm *et al.*, 2003). The tonic coupling of the PKA to promote ACh release (which can be inhibited with H-89) was maintained after AR were stimulated or inhibited. However, in neither of these conditions can the influence of PKA on ACh release be increased with Sp8Br, which suggests that it is working close to its maximum. In the presence of exogenous ADO three ACh release conditions coincide: i) the last EPPs in a train are larger than in the control (less depression), ii) PKA is coupled tonically to potentiate ACh release (release is reduced by H-89), iii) PKA cannot be additionally stimulated (and their involvement in potentiating release increased) with Sp8Br, however. Thus, PKA may work close to their upper activity limit to modulate the reduction of depression. In the presence of 8SPT, the last EPPs in the trains are smaller than in the control (more depression) and the tonic PKA coupling to evoked release (modulation of the quantal content) cannot be stimulated in this case either, which suggests partial PKA inhibition in the absence of the AR function.

However, the coupling of PKC to release (which can be shown by inhibition with CaC) in several regulated conditions in which this coupling can be induced (as in high Ca^{2+}, continuous activity at 1 Hz and PMA stimulation) is fully prevented by previously stimulating or inhibiting AR (see diagram in figure 6E). We conclude that the activity coupling of PKC to ACh release needs normally balanced adenosine receptors. Although the relations described are very complex and there may be several alternative explanations, one main conclusion may be that blocking AR uncouples PKC from the modulation of ACh release. This uncoupling may increase depression in these circumstances because when PKC is coupled ACh release is potentiated (Santafe *et al.*, 2005; 2006; Santafe *et al.*, 2007b; Santafe *et al.*, 2009). On the other hand, PKC coupling may be necessary for mAChR operation (Santafe *et al.*, 2006; Santafe *et al.*, 2007b; Santafe *et al.*, 2009) and after AR block and the resulting PKC uncoupling, mAChR does not operate, so AT does not act. Likewise, blocking mAChR couples PKC to release and this may increase the size of the EPP with the result that the quantal resources destined to increasing the last EPPs in the trains will be quickly consumed. Therefore, AT increases depression.

The complementary function in spontaneous, evoked and activity-dependent ACh release of these presynaptic receptors and kinases can modulate the voltage-dependent calcium channels (VDCC) and the ready releasable pool of synaptic vesicles, which are the instruments of neurotransmission (Takamori, 2012). The influx of external Ca^{2+} required for Ca^{2+}-triggered exocytosis seems to be promoted by the activation of presynaptic mAChR and trkB receptors (Santafe *et al.*, 2006; Amaral & Pozzo-Miller, 2012). The appropiate function of the mAChR subtypes is modulated by adenosine coreleased with ACh at the NMJ (Oliveira *et al.*, 2009) and trkB (Garcia *et al.*, 2010). Both mAChR and trkB pathways share a link mediated by phospholipase C (PLC), diacylglycerol (DAG) and PKC, which leads to the

modulation of P/Q-type VDCC. A reduction in calcium entry would reduce transmitter release and thus reduce depression.

In summary, the results show that when synaptic activity is high, endogenous adenosine production in the synaptic cleft reduces depression with the involvement of A_1R and $A_{2A}R$ receptors. AR and mAChR depend on each other for the modulation of evoked and spontaneous ACh release and cooperate in the control of depression by making specific changes to PKA and PKC activity.

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Abbreviations

ADO, adenosine; ADA, adenosine deaminase; AR, adenosine receptors A1R, A1-type receptors; A2AR, A2A-type receptors; A2BR, A2B-type receptors; A3R, A3-type receptors; AT, atropine; CADO, 2-chloroadenosine; EPP, endplate potential; LAL, Levator auris longus muscle; mAChR, muscarinic acetylcholine receptor; MCD, methyl-beta-cyclodextrin; nAChR, nicotinic acetylcholine receptor; MEPPs, miniature endplate potentials; NMJ, neuromuscular junction; PKA, protein kinase A; PKC, protein kinase C.

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LEGENDS TO THE FIGURES

Figure 1. Effects of purinergic agents on short-term depression and spontaneous and evoked ACh release. (A), in μ-CgTx-GIIIB paralyzed muscles, no effect is observed on quantal content as a result of AR unselective stimulation (adenosine, ADO) or inhibition (8SPT), selectively blocking the AR subtypes (A_1R, $A_{2A}R$, $A_{2B}R$ and A_3R with DPCPX, SCH-58261, MRS1706 and MRS1334 respectively) or eliminating endogenous adenosine with adenosine deaminase (ADA) (ADO, t_{85} = 0.3167, P = 0.7526; 8SPT, t_{83} =1.519, P = 0.1235; ADA, t_{93} = 0.2541, P = 0.8000; DPCPX, t_{82} = 0.3413, P = 0.7337; SCH-58261, t_{86} = 1.072, P = 0.2867). However, MEPP frequency decreases after non-selective stimulation with ADO but increases after non-selective block with 8SPT, by preventing the action of endogenous adenosine with exogenous adenosine deaminase (ADA) or by blocking A_1Rs by incubation with DPCPX (ADO, t_{79} = 2.503, P = 0.0144; 8SPT, t87 = 2.225, P = 0.0267; ADA, t_{143} = 12.173, P = <0.0001; DPCPX, t73 = 3.514, P = 0.0008; SCH-58261, t_{85} = 0.3449, P = 0.7310). For each column, n = 5 muscles, with a minimum of 15 fibres per muscle. Values are expressed as means ± SEM. The raw data show the lack of effect of MRS1706 and MRS1334 on quantal content and MEPP frequency (EPP scale bars: horizontal, 5ms and vertical, 10mV. MEPP scale bars: horizontal, 25ms and vertical, 1mV). **(B)** Shows the effect of purinergic agents on synaptic depression. We produced nerve-delivered stimulation (40 or 100Hz, 2 min) and analysed the effect of several drugs on modulating changes in synaptic depression (increase or decrease of the last EPPs in a train). Depression at 40Hz is reduced by ADO (t_{70} = 4.518, P <0.0001) and increased by 8SPT t_{85} = 4.369, P <0.0001) whereas ADA (t_{95} = 0.3070, P = 0.7595), DPCPX (t_{86} = 0.3435, P = 0.7221) and SCH-58261 have no effect (t_{82} = 0. 3749, P = 0.7087). At 100Hz, ADA greatly increases depression (t_{83} = 4.618, P = <0.0001) whereas the $A_{2A}R$ inhibitor SCH-58261 greatly reduces it (t_{57} = 2.210, P = 0.0311; ADO: t_{96} =

0.2832, P = 0.7776; 8SPT: t_{96} = 0.8213, P = 0.9347; DPCPX: t_{96} = 0.5988, P = 0.5844). For each column, n = 5–8 muscles, with 8–10 fibres per muscle. The raw data show the lack of effect of MRS1706 and MRS1334 on synaptic depression at 40 Hz. One record has been selected out of every 100. The stimuli artifacts have been modified for clarity. Bars: horizontal, 20ms and vertical, 5mV. Values are expressed as mean ± SEM. *P < 0.05 with respect to control.

Figure 2. Effects on evoked, spontaneous and activity-dependent ACh release of 2-chloroadenosine (CADO). (A) CADO at 10μM reduces evoked quantal release. In addition, after A_1R block with DPCPX or A_{2A}R inhibition with SCH-58261, CADO can still reduce quantal content as it can in physiological saline. However, 8SPT fully prevents the effect of CADO on quantal content. Scale bars: horizontal, 5ms and vertical, 5mV. **(B)** As far as spontaneous ACh release is concerned, CADO at 10μM did not change MEPP frequency (not show). At 300 nM CADO increases MEPP frequency although this increase is prevented by preincubation with DPCPX, SCH-58261 or 8SPT. Scale bars: horizontal, 25ms and vertical, 1mV. **(C)** We found that CADO has no effect on activity-dependent synaptic depression at 40Hz (t_{80} = 1.068, P = 0.2891). Interestingly, after A_{2A}R receptors are blocked with SCH-58261, CADO is found to have a protective effect [(t_{71} = 2.991, P = 0.0038) similar in magnitude to the effect of ADO in the same condition, see figure 1B]. CADO *vs* DPCPX: t_{69} = 1.299, P = 0.1981. CADO *vs* 8SPT: t_{96} = 0.6130, P = 0.5313. CADO *vs* the simultaneous presence of SCH-58261 and DPCPX: t_{47} = 0.2358, P = 0.8146. For each column, n = 5–8 muscles, with 8–10 fibres per muscle. Values are expressed as mean ± SEM. *P < 0.05 with respect to control values.

Figure 3. Interaction between ARs and mAChRs in transmitter release. The first two bars on the left of the histograms show the previously published effects of the main muscarinic selective and unspecific agents on NMJ transmission. M_1 antagonist pirenzepine (PIR), M_2 antagonist methoctramine (MET) and the unspecific blocker atropine (AT). **(A)** The histogram shows the action of unspecific agents for AR and mAChR. AT cannot change evoked and spontaneous ACh release when ARs are previously stimulated (AT *vs* ADO, evoked: $t_{82} = 0.04634$, $P = 0.9631$; spontaneous: $t_{141} = 0.5911$, $P = 0.5554$) or inhibited (AT *vs* 8SPT, evoked: $t_{121} = 0.05831$, $P = 0.9536$; spontaneous: $t_{128} = 0.2925$, $P = 0.7703$). In reciprocal experiments, blocking mAChRs with AT does not allow AR to influence evoked release (lack of action of ADO and 8SPT after AT preincubation), and normal AR uncoupling is maintained (ADO $_{vs}$ AT: $t_{124} = 0.9575$, $P = 0.3402$; 8SPT *vs* AT: $t_{120} = 1.306$, $P = 0.1940$). However, AT preincubation effectively suppresses the effect that ADO and 8SPT have on decreasing and increasing MEPP frequency (ADO $_{vs}$ AT: $t_{139} = 1847$, $P = 0.0668$; 8SPT *vs* AT: $t_{110} = 0.8050$, $P = 0.4225$), respectively (see this in figure 1A). Because CADO (10μM) reduces evoked quantal release (see figure 2A), we assayed how preincubation with AT modifies the CADO effect and reciprocally. Neither AT nor CADO can affect quantal content or MEPP frequency after preincubation with the other compound (quantal content, CADO *vs* AT: $t_{121} = 0.2572$, $P = 0.7974$; quantal content, AT *vs* CADO: $t_{147} = 0.7314$, $P = 0.4657$; MEPP frequency, CADO *vs* AT: $t_{150} = 0.5297$, $P = 0.5971$; MEPP frequency, AT *vs* CADO: $t_{130} = 0.2811$, $P = 0.7790$). Insets shown the raw data of AT action on evoked and spontaneous release (Aa) and the lack of AT effect in presence of 8SPT (Ab). Scale bars for EPP: horizontal 5ms and vertical 10ms. Scale bars for MEPPs: horizontal Aa, 25ms and Ab, 50ms; vertical, 1mv for both cases. **(B)** The M_1 antagonist pirenzepine (PIR) cannot modify evoked and spontaneous ACh release after preincubation with the purinergic agents ADO (evoked: $t_{121} = 0.3138$, $P = 0.7542$; spontaneous: $t_{123} = 0.4528$, $P = 0.6515$), 8SPT (evoked: $t_{82} =$

0.8616, P = 0.3914; spontaneous: t_{87} = 0.9646, P = 0.3376), DPCPX (evoked: t_{121} = 0.9552, P = 0.3414; spontaneous: t_{119} = 0.8266, P = 0.4101) or SCH-58261(evoked: t_{124} = 0.04233, P = 0.9663; spontaneous: t_{120} = 0.7132, P = 0.4770). **(C)** The M_2 antagonist methoctramine (MET) cannot modify evoked and spontaneous ACh release after preincubation with ADO (evoked: t_{121} = 0.08575, P = 0.9318; spontaneous: t_{100}= 1.449, P = 0.1505), 8SPT (evoked: t_{145} = 0.1878, P = 0.8513; spontaneous: t_{139} = 1.837, P = 0.0683), DPCPX (evoked: t_{102} = 1.558, P = 0.1223; spontaneous: t_{121} = 1.398, P = 0.1646) or SCH-58261(evoked: t_{120} = 0.3556, P = 0.7228; spontaneous: t_{124} = 0.5926, P = 0.5545). On the whole, the data indicate the absolute interdependence of AR and mAChR. For each column, n = 5 muscles, with a minimum of 15 fibres per muscle. Values are expressed as mean ± SEM. *P < 0.05 with respect to control values.

Figure 4. Interaction between ARs and mAChRs in synaptic depression. We stimulated the LAL nerve (40 Hz, 2 min) and analysed the effect of several drugs on modulating synaptic depression changes. The histogram shows the mean size of the first 15 EPPs of each train (gray columns) and the mean size of the last 15 EPPs (white columns). Previously published data are represented here in the inset on the left of the figure so that they can best be compared with the new data. Because AR and mAChR share functional control over synaptic depression [blocking them with 8SPT (t_{787} = 4.369, P = <0.0001) and AT (t_{137} = 2.563, P = 0.0114), respectively, increases depression induced at 40Hz], we analysed here the possible occlusive or additive crosstalk effects between the two receptor groups. We found that ADO cannot reduce depression after AT preincubation (t_{144} = 0.2787, P = 0.7809) and, reciprocally, that AT cannot increase depression after ADO preincubation (t_{147} = 0.9342, P = 0.9342). However, whereas AT does not increase depression after 8SPT (t_{145} = 1.028, P = 0.3054), 8SPT increase depression after AT (t_{127} = 2.037, P = 0.0437) to a similar extent as when

acting by itself. Thus, the purinergic and muscarinic mechanisms cooperate in the control of depression by sharing a common pathway. For each column, n = 5–8 muscles, with 8–10 fibres per muscle. Values are expressed as mean ± SEM. *P < 0.05 with respect to control values.

Figure 5. Interaction between ARs and serine kinases PKA and PKC. (A) In basal conditions, ACh release can be reduced and increased by the PKA inhibitor H-89 (t_{145} = 5.108, P = < 0.0001) and the stimulator Sp8Br (t_{111} = 4.365, P = < 0.0001), respectively. The histogram shows that after the inhibition or stimulation of the AR (with 8SPT or ADO), PKA can be inhibited with H-89 (H-89 *vs* 8SPT: t_{105} = 2.199, P = 0.0300; H-89 *vs* ADO: t_{144} = 2.151, P = 0.0331) but cannot be additionally stimulated with Sp8Br (Sp8Br *vs* 8SPT: t_{148} = 0.09428, P = 0.9250; Sp8Br *vs* ADO: t_{82} = 0.01888, P = 0.9850). The MEPP frequency in H-89 (no change; t_{140} = 1.785, P = 0.0764) and in Sp8Br (increases by about 150-200%; t_{126} = 6.865, P = <0.0001) is the same as the frequency observed when these substances are applied by themselves and independently of a previous incubation with ADO or 8SPT(Sp8Br *vs* ADO: t_{104} = 6.240, P = <0.0001; H-89 *vs* ADO: t_{110} = 1.943, P = 0.0545; Sp8Br *vs* 8SPT: t_{115} = 14.675, P = <0.0001; H-89 *vs* 8SPT: t_{137} = 1.761, P = 0.0805). (**B and C**) In basal conditions, PKC is not tonically involved in ACh release and the PKC paninhibitor CaC does not affect release (not shown here). However, with extracellular Ca^{2+} to 5 mM or electrical stimulation (1 Hz) couples PKC to release, which can now be inhibited by CaC. The interaction between ARs and PKC with respect to quantal content is shown in **B** and respect to MEPP frequency is shown in **C** [high external Ca^{2+} (CaC *vs* 8SPT: t_{147} = 1.847, P = 0.0668; CaC *vs* ADO: t_{144} = 0.7671, P = 0.4443); continuous trains at 1 Hz (CaC *vs* 8SPT: t_{96} = 6.168, P < 0.0001; CaC vs ADO: t_{106} = 4.490, P <0.0001)]. The previous block or stimulation of AR with 8SPT or ADO in both high external Ca^{2+} (CaC *vs* 8SPT: t_{93} = 0.3056, P = 0.7606; CaC

vs ADO: t_{87} = 0.008471, P = 0.9972) and continuous trains at 1 Hz (CaC *vs* 8SPT: t_{148} = 0.02593, P = 0.9794; CaC v*s* ADO: t_{148} = 0.7124, P = 0.4773) completely prevents the CaC effect on evoked release. PMA increases quantal content by about 100% (t_{146} = 18.638, P < 0.0001) and MEPP frequency by about 200% (t_{145}= 32.165, P < 0.0001). However, blocking or stimulating AR completely abolishes the PMA effect on evoked ACh release (PKC canot be stimulated with PMA; PMA *vs* ADO: t_{87} = 1.074, P = 0.2856; PMA $_{vs}$ 8SPT: t_{98} = 1.303, P = 0.1956) although it does not change the PMA-dependent MEPP frequency increase (PMA *vs* ADO: t_{87} = 8.045, P < 0.0001; PMA *vs* 8SPT: t_{98} = 8.797, P < 0.0001). For each column, n = 5 muscles, with a minimum of 15 fibres per muscle. Values are expressed as mean ± SEM. *P < 0.05 with respect to control values.

Figure 6. AR participation in ACh release. (A) in basal conditions, endogenous adenosine (ADOen) tonically reduces the efficacy of the unsynchronised ACh release mechanism through A_1R and spontaneous quantal events are reduced. Additional exogenous ADO (ADOex) potentiates this effect. Low concentrations of CADO increase MEPP frequency and higher concentrations of CADO (10μM) reduce EPP size in both cases with the involvement of both A_1R and $A_{2A}R$. $A_{2B}R$ and A_3R are not involved. **(B)** The analysis of the effect of endogenous and exogenous ADO shows that both A_1R and $A_{2A}R$ contribute to reducing depression. Interestingly, the analysis of the effect on depression of CADO at 40 Hz reveals a dual effect of A_1R and $A_{2A}R$ molecules. In summary, it seems that depression reduction may be mediated mainly by A1Rs. In **C**, the inhibition arrow between ARs and evoked release only occurs when ARs are stimulated with CADO. All data in the present study indicate that there is an absolute interdependency of AR and mAChR on the modulation of evoked and spontaneous ACh release in basal conditions. Any imbalance in one of the two receptor groups (when one or more subtypes are blocked) results in the other group being uncoupled

from release. In **D**, the purinergic and muscarinic mechanisms cooperate in the control of depression. However, if mAChRs are blocked (AT), ARs can continue protecting against depression (8SPT prevents this protection) almost at maximum capacity (additional ADO does not increase this protection). Reciprocally, if ARs are stimulated or inhibited, mAChRs are uncoupled from the depression control mechanism (their additional inhibition with AT does not change the size of the last EPPs in a train). Thus, purinergic control of depression seems to be more powerful than muscarinic control (in the conditions of the present experiments). In **E,** when ARs are blocked (8SPT), PKA is coupled although it cannot be additionally stimulated with Sp8Br (partial dependence), which suggests that it could be working close to capacity. However, the coupling to release of PKC in regulated conditions in which this coupling can be induced such as high Ca^{2+}, continuous activity at 1 Hz and PMA stimulation is fully prevented (full dependence). The arrow from PKA to PKC in this diagram represents some dependence of PKC on PKA activity in the fine control of the ACh release previously described (Santafe *et al.*, 2009). The arrow from PKA to ACh represents the tonic coupling of this kinase to release whereas the dashed arrow from PKC to ACh indicates that the coupling of PKC to ACh release occurs only in some regulated conditions.

FIGURE 1

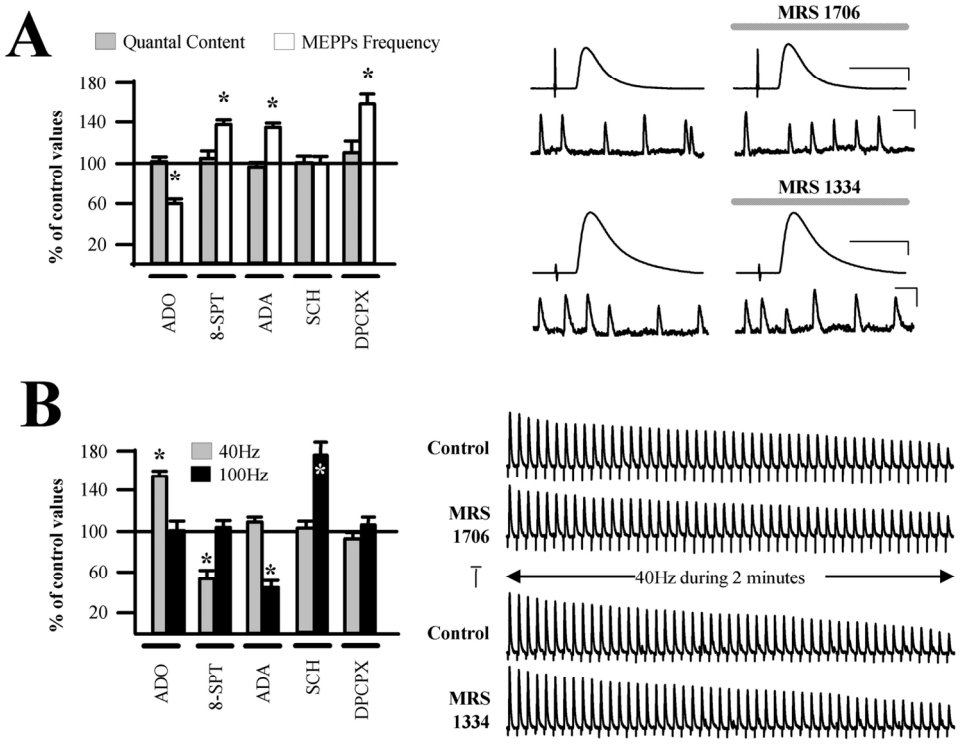

Figure 1. Effects of purinergic agents on short-term depression and spontaneous and evoked ACh release.
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FIGURE 2

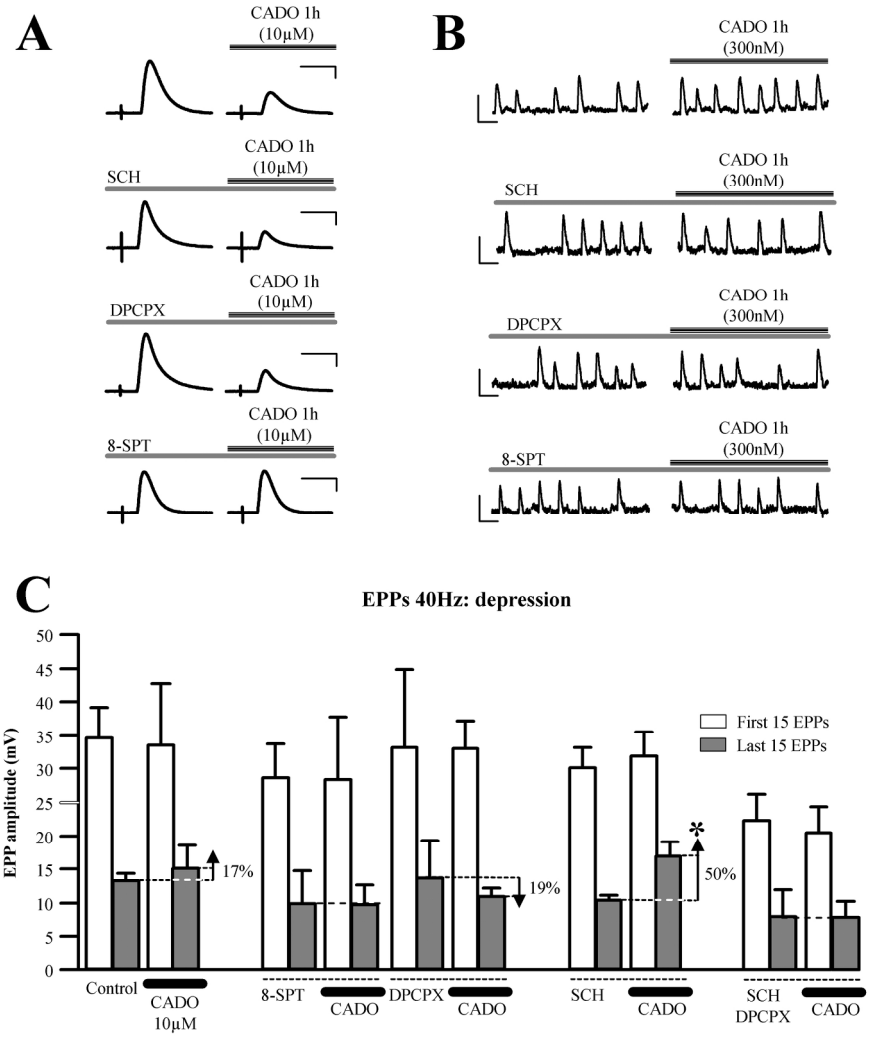

Figure 2. Effects on evoked, spontaneous and activity-dependent ACh release of 2-chloroadenosine (CADO).
194x253mm (300 x 300 DPI)

FIGURE 3

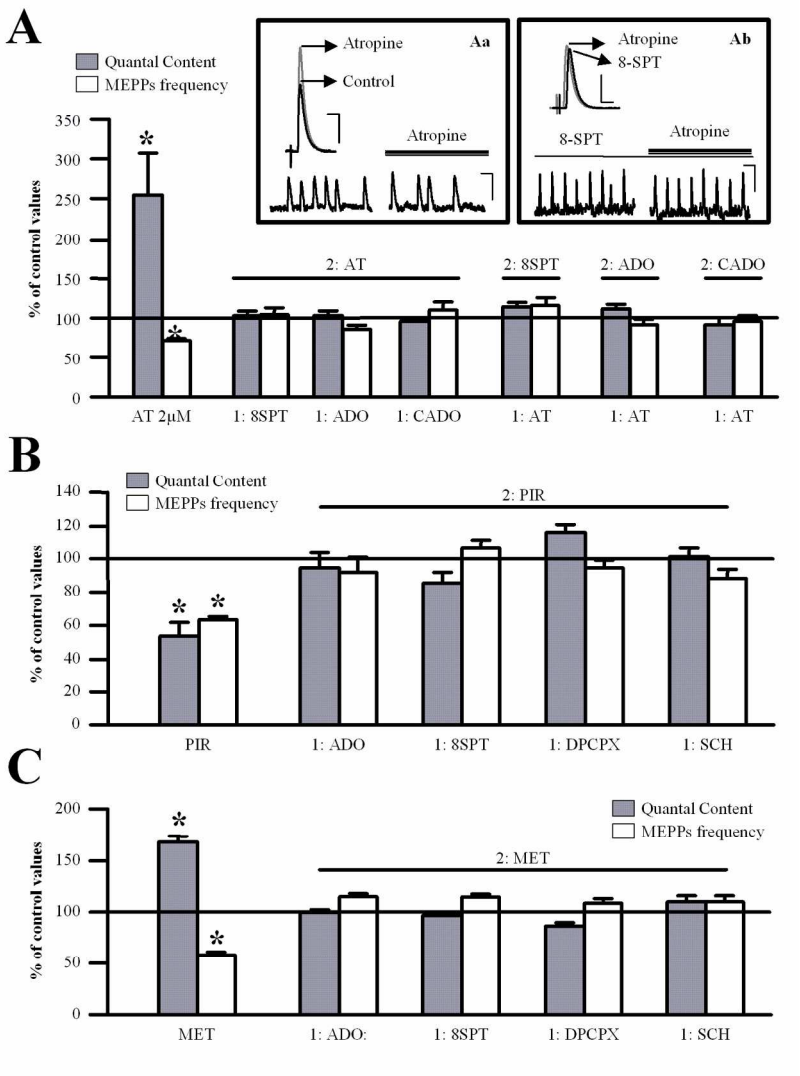

Figure 3. Interaction between ARs and mAChRs in transmitter release.
140x199mm (300 x 300 DPI)

FIGURE 4

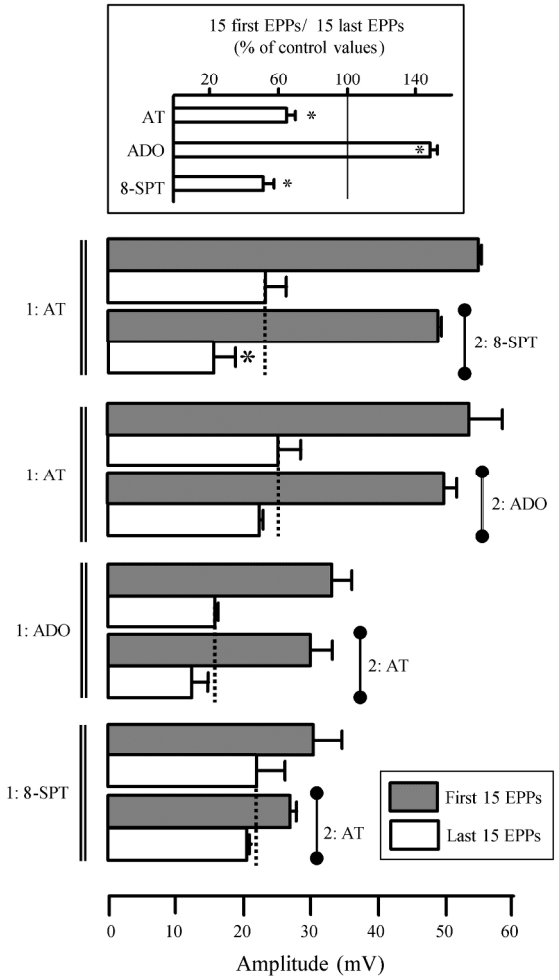

Figure 4. Interaction between ARs and mAChRs in synaptic depression.
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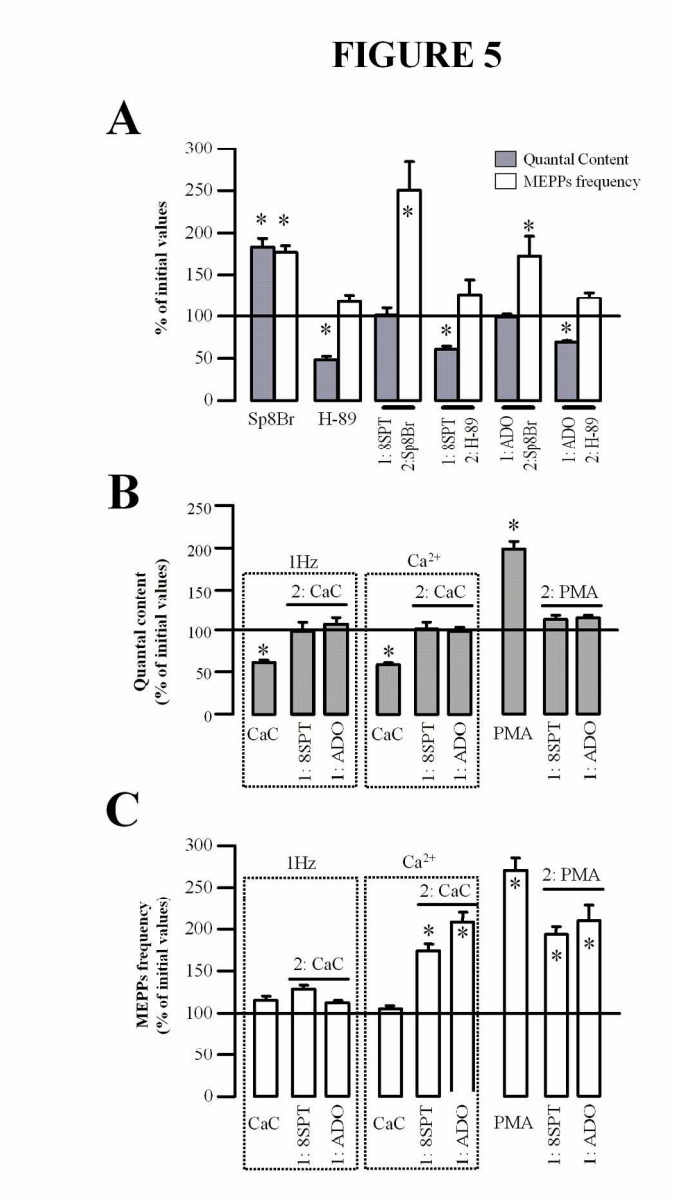


Figure 5. Interaction between ARs and serine kinases PKA and PKC.
114x203mm (300 x 300 DPI)

FIGURE 6

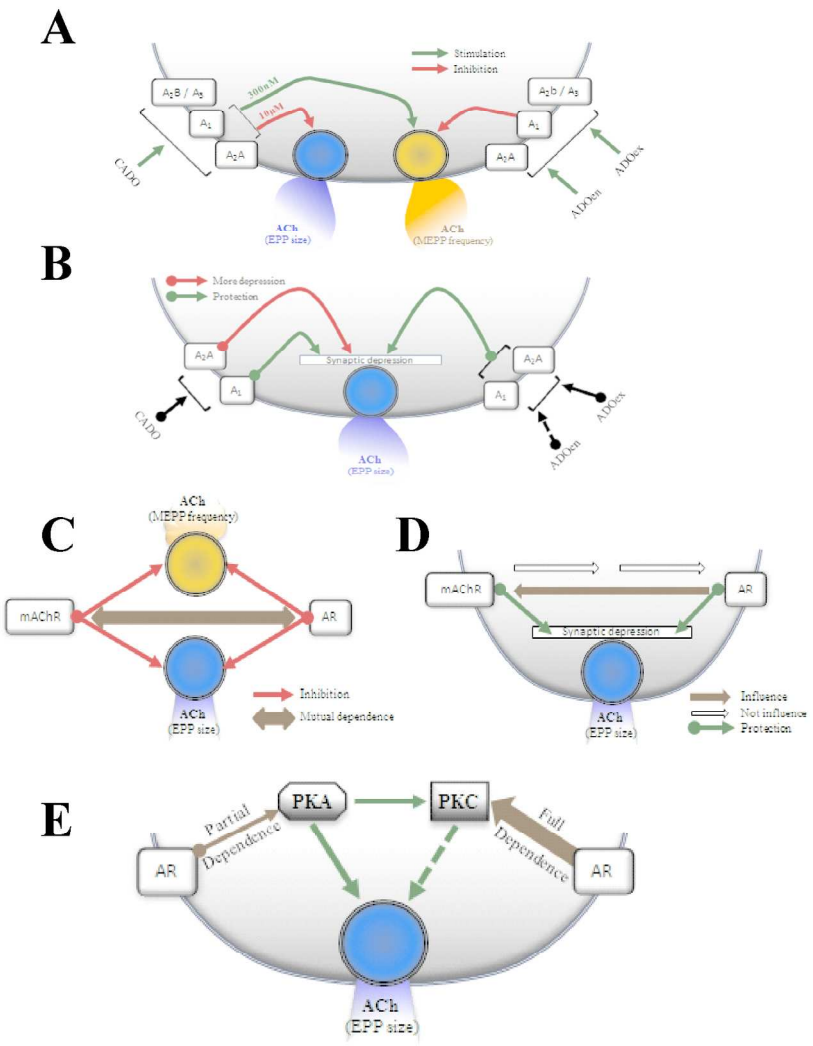
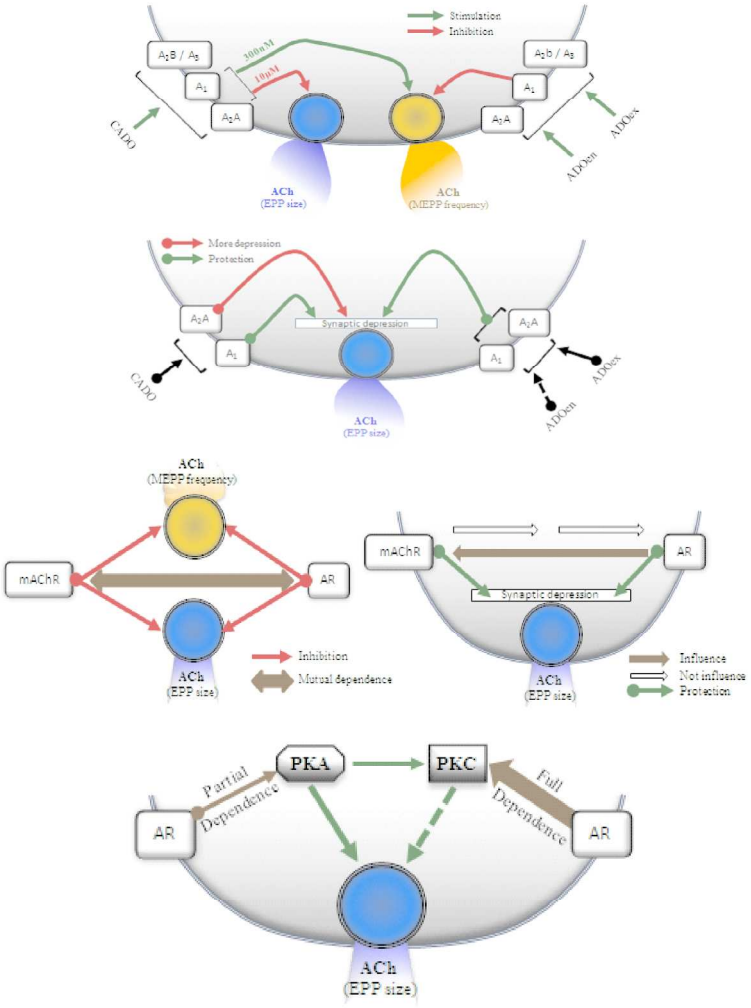


Figure 6. AR participation in ACh release
146x203mm (300 x 300 DPI)

GRAPHICAL ABSTRACT



135x203mm (300 x 300 DPI)

In basal conditions, adenosine tonically reduces the efficacy of the unsynchronised ACh release mechanism through A1R. Both A1R and A2AR contribute to reducing synaptic depression.

There is an absolute interdependency of AR and mAChR on the modulation of evoked and spontaneous ACh release and in the control of depression.

The imbalance of the AR created by using subtype selective and non-selective inhibitory and stimulatory agents uncouples protein kinase C (PKC) from evoked transmitter release.