Dual role of ATP on neuromuscular transmission in Experimental Autoimmune Myasthenia Gravis (EAMG)

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas .

O Presidente do Júri,

Porto, _____/_____/_____

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Brief-Note

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Dual role of ATP on neuromuscular transmission in Experimental Autoimmune *Myasthenia Gravis* (EAMG)

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Resumo

Miastenia gravis é uma doença autoimune mediada por anticorpos e dependente de células T que se caracteriza por uma falha na contração neuromuscular. Em indivíduos miasténicos, a falha na contração é rapidamente atingida aquando de uma estimulação muscular contínua que, no caso dos músculos respiratórios pode ser fatal para o indivíduo. Além do tratamento crónico com imunossupressores, o agravamento da situação clínica é normalmente controlado com inibidores das colinesterases, cujo uso acarreta efeitos laterais significativos (revisto em Conti-Fine et al., 2006). Assim sendo, outra estratégia terapêutica valiosa pode passar pela modulação dos sistemas capazes de influenciar a libertação de acetilcolina (ACh) na junção neuromuscular (JNM), nomeadamente o sistema purinérgico.

Recentemente, o nosso grupo demonstrou que nos terminais nervosos motores de ratazanas com miastenia gravis autoimune induzida experimentalmente (EAMG) ocorre uma diminuição da atividade tónica facilitatória mediada pelos recetores pré-sináticos da adenosina A_{2A} ($A_{2A}R$) devido a uma diminuição da acumulação de adenosina na fenda sináptica (Oliveira et al., 2015). O défice na acumulação extracelular de adenosina contrasta com a descoberta de que os animais EAMG libertam maiores quantidades de ATP após estimulação elétrica do nervo frénico (Neves, 2015, comunicação pessoal). Há muito se sabe que o ATP é co-libertado com a ACh a partir dos terminais nervosos motores (Silinsky, 1975) e que a sua ação pode ser direta, através da ativação de purinocetores P2 (Salgado et al., 2000), ou indireta, por intermédio de recetores $A_{2A}R$ após a sua conversão em adenosina na fenda sináptica por uma cascata de ecto-nucleotidases (Correia-de-Sá and Ribeiro, 1996). Assim, o objetivo deste trabalho consistiu em avaliar se existia ou não algum défice na atividade das ecto-nucleotidases que pudesse explicar o paradoxo aparente entre os níveis elevados de ATP e a deficiente acumulação de adenosina na JNM de animais EAMG. Tendo em consideração a acumulação de nucleótidos de adenina na JNM dos animais miasténicos, avaliou-se o papel neuromodulador dos recetores P2 sobre a libertação de ACh em ratazanas EAMG.

A indução de miastenia gravis autoimune foi realizada em ratazanas adultas de acordo com um protocolo previamente estabelecido no Laboratório de Farmacologia e Neurobiologia do ICBAS (Oliveira et al., 2015). Após o seu isolamento, as preparações de hemidiafragma inervado de ratazana foram usadas para experiências em que se avaliou a libertação de [^{3}H]-ACh induzida por estimulação elétrica do nervo frénico (750 pulsos de duração de 0.04 ms aplicados com a frequência de 5 Hz). Estas mesmas preparações foram usadas para estudos de cinética enzimática onde se avaliou o

catabolismo extracelular de ATP e ADP e a formação dos seus metabolitos por cromatografia líquida de alta eficiência com deteção UV (HPLC-UV).

Os resultados mostram que a deficiente acumulação de adenosina na JNM de ratazanas EAMG não pode ser atribuída a quaisquer alterações no catabolismo extracelular do ATP (30 μM) e do ADP (30 μM) verificada nos animais miasténicos comparativamente com o grupo controlo. O tempo de semivida do ATP não foi significativamente (P>0.05) diferente entre animais EAMG (10 ±1 min, *n*=4) e controlo (injetados apenas com CFA) (10 ± 3 min, *n*=4). Estes tempos de semivida também não foram diferentes dos verificados em animais que não foram submetidos a qualquer tipo de tratamento (8 ± 2 min, *n*=8) (Magalhães-Cardoso et al., 2003). O tempo de semivida do ADP (30 μM) em animais EAMG (10±1 min, *n*=4) também não foi diferente dos animais controlo (10±1 min), nem tão pouco diferiu dos tempos de semivida calculados para o ATP nas mesmas condições experimentais. Estudos anteriores do grupo, demonstram que também não existe nenhuma deficiência na atividade da ecto-5' nucleotidase que é a enzima responsável pela formação de adenosina a partir da hidrólise extracelular do AMP. Estes dados sugerem que o défice na acumulação de adenosina na JNM de animais EAMG após estimulação elétrica do nervo frénico não pode ser atribuído a alterações no metabolismo extracelular dos nucleótidos de adenina libertados.

O ATP (1 μM), aplicado por um curto período de tempo (3 minutos), reduziu a libertação de [^{3}H]-ACh induzida por estimulação elétrica do nervo frénico em 26±10% (*n*=4) e 20±6% (*n*=5) em preparações controlo e EAMG, respetivamente. O efeito inibitório do ATP não foi reproduzido pelo seu análogo estável, β,γ-imido ATP (100 μM), mas foi observado na presença de adenosina desaminase (ADA) a enzima responsável pela inativação extracelular da adenosina. Estes resultados mostram que o efeito inibitório do ATP se deve à sua pronta conversão extracelular em ADP pelas NTPDases existentes na fenda sináptica e, por conseguinte, à ativação de recetores P2 sensíveis ao ADP ($P2Y_1$, $P2Y_{12}$ e/ou $P2Y_{13}$) existentes no terminal nervoso motor. O bloqueio seletivo do recetor $P2Y_{13}$ pelo MRS 2211 (10 μM) atenuou o efeito inibitório do ATP (1 μM) sobre a libertação de [^{3}H]-ACh a partir de preparações de hemidiafragma inervado de ratazanas controlo e EAMG. A estimulação seletiva do recetor $P2Y_1$ com MRS 2365 (30 nM) produziu um efeito inibitório de maior magnitude que o ATP (1 μM) sobre a libertação de [^{3}H]-ACh tanto em animais EAMG (31±4%, *n*=5) como em ratazanas controlo (44±6%, *n*=4). Já o bloqueio seletivo deste subtipo de recetor ($P2Y_1$) com MRS 2179 (300 nM) preveniu apenas o efeito inibitório do ATP (1 μM) em preparações isoladas de animais EAMG, mas não em ratazanas do grupo controlo. Aparentemente,

a ativação de recetores do subtipo $P2Y_{12}$ parece contrariar parcialmente o efeito inibitório do ATP (1 µM), já que o bloqueio seletivo destes recetores com AR-C 66096 (100 nM) potenciou significativamente a inibição da libertação de [^{3}H]-ACh verificada após a aplicação de ATP (1 µM) para 45±7% (*n*=4) em preparações de ratazanas EAMG.

Os resultados apresentados neste trabalho sugerem que o aumento da libertação de ATP na JNM dos animais miasténicos após estimulação elétrica do nervo frénico pode conduzir à ativação de recetores P2 sensíveis ao ADP, após a sua metabolização extracelular neste nucleotídeo pelas NTPDases existentes na fenda sináptica. Os resultados preliminares obtidos até à data sugerem o envolvimento de recetores de dois subtipos, $P2Y_1$ e $P2Y_{13}$, na inibição da libertação de [^{3}H]-ACh após a aplicação de ATP. A atividade inibitória destes dois recetores parece ser parcialmente contrariada pela co-ativação de recetores do subtipo $P2Y_{12}$ nos animais miasténicos. Especula-se ainda sobre os mecanismos moleculares responsáveis para interação entre os vários recetores sensíveis ao ADP responsáveis pelo controlo da libertação de ACh na JNM e qual a preponderância de cada um deles nos animais miasténicos. Apesar desta limitação, este trabalho permitiu descobrir novos alvos farmacológicos para prevenir a falência da transmissão neuromuscular em doentes miasténicos e, com isso, talvez propor novas estratégias para o tratamento desta patologia.

Abstract

Myasthenia Gravis (MG) is a T cell–dependent antibody-mediated autoimmune disease characterized by neuromuscular contraction failure. In myasthenic individuals, contraction failure is easily achieved with continuous muscular stimulation which, in the case of respiratory muscles, could be fatal to the individual. Besides chronic immunosuppressive treatment, clinical exacerbations are usually controlled with cholinesterase inhibitors, which use has significant side effects (reviewed by Conti-Fine et al., 2006). As such, another viable therapeutic strategy might be to modulate the systems able to fine-tune control acetylcholine (ACh) release at the neuromuscular junction (NMJ), such as the purinergic system.

Recently, our group demonstrated that at motor nerve terminals of rats with experimental autoimmune *Myasthenia Gravis* (EAMG) there is a decrease of the facilitatory tonus mediated by presynaptic adenosine A_{2A} receptors ($A_{2A}R$) due to a decreased accumulation of adenosine at the synaptic cleft (Oliveira et al., 2015). Deficits on adenosine accumulation contrast with the finding that EAMG animals accumulate higher amounts of ATP after electrical stimulation of the phrenic nerve (Neves, 2015, personal communication). ATP has long been known to be co-released with ACh from motor nerve terminals (Silinsky, 1975), and that its action may be exerted either directly, through the activation of P2 purinoceptors (Salgado et al., 2000), or indirectly, via $A_{2A}R$ after its conversion into adenosine in the synaptic cleft by ectonucleotidases (Correia-de-Sá and Ribeiro, 1996). Therefore, we aimed at investigating if there was in fact a deficient activity of the ecto-nucleotidases responsible for the apparent paradox between increased amounts of ATP and decreased ADO accumulation at the NMJ of EAMG animals. Taking into consideration the accumulation of adenine nucleotides at the NMJ of myasthenic animals, the neuromodulatory role(s) of P2 receptors on ACh release in rats with EAMG, was also investigated.

The EAMG induction was proceeded in adult female rats as described by a previously established protocol at the Pharmacology and Neurobiology laboratory of ICBAS (Oliveira et al., 2015). After isolation, phrenic nerve hemidiaphragm preparations were used for experiments in order to evaluate [^{3}H]-ACh release after electrical stimulation of innervated hemidiaphragm with 5 Hz trains (750 pulses of 0.04 ms duration). Hemidiaphragm preparations were also used for enzyme kinetic experiments where the extracellular catabolism of ATP and ADP and the formation of the subsequent metabolites were evaluated by high performance liquid chromatography coupled to UV detection (HPLC-UV) system.

Data indicates that deficient accumulation of adenosine at motor endplates from EAMG rats cannot be attributed to changes in the kinetics of extracellular catabolism either of ATP (30 µM) nor ADP (30 µM) in myasthenic animals compared with the control group. The half-life of ATP (30 µM) was not significantly (P>0.05) different between EAMG (10 ± 1 min, *n*=4) and control animals (only injected with CFA) (10 ± 3 min, *n*=4). Neither was this kinetic parameter different from naïve animals (not submitted to any kind of treatment) (8 ± 2 min, *n*=8) (Magalhães-Cardoso et al., 2003). The half-life of ADP (30 µM) in EAMG animals (10 ± 1 min, *n*=4) was also not different from that calculated for CFA (10 ± 1 min, *n*=4) animals; The half-lives of ATP and ADP were also not different under the same experimental conditions. Previous studies from our group also demonstrated that there is no impairment of the activity of ecto-5'-nucleotidase that is the enzyme responsible for adenosine formation from the extracellular hydrolysis of AMP. Data suggest that the deficit in ADO accumulation at the NMJ of EAMG animals triggered by electrical stimulation cannot be attributed to changes in the extracellular metabolism of released adenine nucleotides.

Application of ATP (1 µM) during a short period of time (3 min) decreased electrically-evoked [^{3}H]-ACh release from the phrenic nerve by 26 ± 10% (*n*=4) and 20 ± 6% (*n*=5) of control and EAMG preparations, respectively. The inhibitory effect of ATP was not reproduced by its stable analogue, adenosine 5'–(β,γ- imido) triphosphate (β,γ-imido ATP, 100 µM), but it was still observed in the presence of adenosine deaminase (ADA), the enzyme responsible for the extracellular inactivation of adenosine. These results show that the inhibitory effect of ATP is most probably due to its extracellular conversion into ADP by the NTPDases present at the synaptic cleft and, consequently, to the activation of ADP-sensitive P2 receptors ($P2Y_1$, $P2Y_{12}$ and/or $P2Y_{13}$) on motoneurons. Selective blockage of $P2Y_{13}$ receptor by MRS 2211 (10 µM) attenuated the inhibitory effect of ATP (1 µM) on evoked [^{3}H]-ACh relesae from innervated hemidiaphragm preparations of EAMG and control rats. Selective stimulation of the $P2Y_1$ receptor with MRS 2365 (30 nM) produced an inhibitory effect of a greater magnitude than ATP (1 µM) on the release of [^{3}H]-ACh from both EAMG (31 ± 4%, *n*=5) and control rats (44 ± 6%, *n*=4). The selective blockage of this receptor subtype ($P2Y_1$) with MRS 2179 (300 nM) only prevented ATP (1 µM) transmitter release inhibition in EAMG animals preparations, but not in the control group.

Apparently, the activation of the $P2Y_{12}$ receptor subtype seems to partially counteract ATP (1 µM)-induced inhibition, since the selective blockage of these receptors with AR-C66096 (100 nM) significantly potentiated (to 45 ± 7%, *n*=4) the inhibitory action of ATP (1 µM) on [^{3}H]-ACh release from EAMG animal preparations.

The results presented in this work suggest that the increased release of ATP at the NMJ of myasthenic animals after phrenic nerve electrical stimulation might lead to the activation of ADP-sensitive P2 receptors after its extracellular catabolism by NTPDases present at the synaptic cleft. Preliminary results obtained so far suggest the involvement of two receptor subtypes, $P2Y_1$ and $P2Y_{13}$, in the inhibition of [^{3}H]-ACh release after the application of ATP. The inhibitory activity of these two receptors seems to be partially counteracted by the co-activation of $P2Y_{12}$ receptor subtype in myasthenic animals. There is still speculation about the molecular mechanisms responsible for the interaction between the various ADP-sensitive receptors responsible for the control of ACh release at the NMJ and concerning the preponderance of each receptor subtype in myasthenic animals. Despite this limitation, this work allowed us to discover new pharmacological targets to prevent neuromuscular transmission failure in myasthenic patients, and with that, maybe, to propose new strategies for the treatment of this pathology.

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List of Figures and Tables

List of abbreviations

[^{3}H]-ACh	Tritiated acetylcholine
[^{3}H]-choline	Tritiated choline
°C	Centigrade degrees
µCi	Microcurie
µL	Microliter
µM	Micromolar
ABC	ATP binding cassette
Abs	Antibodies
ACh	Acetylcholine
AChE	Acetylcholinesterase
ACR	Apyrase conserved regions
ADA	Adenosine deaminase
ADO	Adenosine
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
Auto-Abs	Auto-antibodies
cAMP	Cyclic adenosine monophosphate
CAT	Choline acetyl transferase
CFA	Complete Freund’s adjuvant
CNS	Central nervous system
CNT	Concentrative nucleoside transporter
DPM	Disintegrations per minute
EAMG	Experimental autoimmune Myasthenia Gravis

ENPP	Ectonucleoside pyrophosphatase/phosphodiesterase
ENT	Equilibrative nucleoside transporter
ENTPDase	Ectonucleoside triphosphate diphosphohydrolase
EOM	Extrinsic ocular muscles
EPP	Endplate potential
g	Gram
GDP	Guanosine diphosphate
GPCR	G-protein coupled receptor
GPI	Glycophosphoinositol
GTP	Guanosine triphosphate
h	Hour
HX	Hypoxanthine
Hz	Hertz
IFA	Incomplete Freund’s adjuvant
IMP	Inosine monophosphate
INO	Inosine
IP_3	Inositol triphosphate
IVIg	Intravenous immunoglobulin
kg	Kilogram
LRP4	Low density lipoprotein receptor related protein
mA	Milliampere
mAChRs	Muscarinic acetylcholine receptors
MEPP	Miniature endplate potential
MG	*Myasthenia Gravis*
mg	Milligram

min	Minute
mM	Millimolar
mm	Milimiter
MuSK	Muscle specific kinase
nAChRs	Nicotinic acetylcholine receptors
nM	Nanomolar
NMJ	Neuromuscular junction
nmol	nanomol
P1R	P1 receptors (Adenosine receptors)
P2R	P2 receptors (ATP receptors)
PBS	Phosphate buffered saline
PLC β	Phospholipase β
PNP	Purine nucleotide phosphorylase
RNS	Repetitive nerve stimulation
SCID	Severe combined immunodeficiency
SFMEG	Single fiber electromyography
$t_{1/2}$	Half-life time
TM	Transmembrane
Tyr	Tyrode
UDP	Uridine diphosphate
UV	Ultra-violet
VAChT	Vesicular Acetylcholine Transporter
β,γ - Imido ATP	Adenosine 5' – (β,γ- imido) triphosphate

Chapter I

Introduction

1. Neuromuscular Junction

The neuromuscular junction (NMJ) is a synapse specialized in the transmission of electrical impulses from the motor nerve terminal to the skeletal muscle, in order to generate an appropriate muscle contraction (reviewed by Fagerlund and Eriksson, 2009; Hughes et al., 2006).

The NMJ is formed by three major structural components that derive from different cell types: the presynaptic terminal originated from the motor neuron, the postsynaptic region of the skeletal muscle cell and the perisynaptic Schwann cell. All of these cells work together to ensure the success of neuromuscular transmission (reviewed by Fagerlund and Eriksson, 2009; Hughes et al., 2006).

Through the use of modern technologies, the NMJ structure has evolved from an apparent anatomical fusion of nerve and muscle, to the distinction of boundaries for muscle and nerve cells, creating the notion of a space between them called synapse. These findings suggest the existence of a soluble messenger molecule, which proved later to be acetylcholine (ACh) at least in vertebrate NMJs (reviewed by Bennet, 2000; Hughes et al., 2006).

1.1 Presynaptic region

The presynaptic terminal consists of the distal demyelinated part of the motor nerve axon, which has its cell body in the ventral horn of the spinal cord. This part of the nerve is encapsulated by a perisynaptic Schwann cell that anchors the nerve ending to the muscle membrane. These Schwann cells are reportedly not directly involved in the chemical transmission, but they have a key role in promoting motor neuronal survival (reviewed by Fagerlund and Eriksson, 2009; Hughes et al., 2006) and are responsible for synthesizing and releasing chemical neuromodulators (reviewed by Todd and Robitaille, 2006).

The synthesis and storage of ACh can be divided into three stages: uptake of choline into the nerve terminal resulting from the extracellular hydrolysis of released acetylcholine (ACh) by acetycholinesterase (AChE), its conversion into ACh by cytosolic choline acetyltransferase (CAT) catalyzing the transference of the acetyl group from mitochondrial acetyl coenzyme A, and packaging of ACh into synaptic vesicles through the action of the vesicular ACh transporter (VAChT) (Rang et al., 2016) (Figure 1).

$$Choline + Acetyl\ coenzyme\ A \rightleftharpoons Acethylcholine + Coenzyme\ A$$

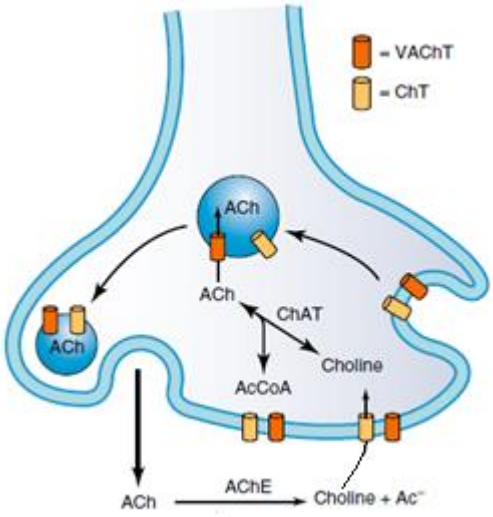

Figure 1. Schematic representation of the transport, synthesis and degradative processes in a cholinergic presynaptic nerve terminal (adapted from Siegel et al., 2006).

Once synthetized in the cytosol of the nerve terminal, ACh accumulation in synaptic vesicles is driven by a proton-pumping ATPase called vesicular ACh transporter (VAChT) (reviewed by Eiden, 1998). These vesicles are then stored in a readily releasable pool near the active zone of release (Murthy and De Camilli, 2003; Sudhof, 2004). At this site, the amount of voltage–gated calcium channels is significantly higher compared to other regions of the presynaptic terminal (Augustine et al., 1991). As such, when an action potential reaches the nerve terminal, voltage-gated calcium channels of the P/Q type are activated, allowing high amounts of calcium ions to enter the presynaptic terminal. Calcium influx leads to the rise in the concentration of this ion near the channel pore to levels high enough to promote the fusion of synaptic vesicles containing the neurotransmitter with the presynaptic membrane (exocytosis phenomenon) releasing its content into the synaptic cleft (reviewed by Hirsch, 2007; Hughes et al., 2006). Calcium acts by neutralizing the negative charges of the presynaptic membrane allowing vesicles to approach the membrane; calcium ions also bind to specific proteins (e.g. synaptotagmin) to initiate vesicular membrane fusion through a conformational change in both vesicle and plasma membrane proteins (Sudhof, 2004). After its release, ACh has two possible fates: it can be hydrolyzed back into choline by acetylcholinesterase (AChE) or it can activate cholinergic receptors existing at the skeletal muscle fiber and at the presynaptic nerve terminal.

Under resting conditions (absence of a nerve action potential), miniature endplate potentials (MEPP) can be recorded at the NMJ as a result of the spontaneous release of a small number of ACh-containing vesicles, often termed as quantal release. When an action potential reaches the nerve terminal, several hundred vesicles are released synchronously resulting in an endplate potential (EPP) which, if large enough, is able to

evoke muscle depolarization and, subsequent, contraction (this issue will be further discussed at *Post-Synaptic region* chapter) (reviewed by Fagerlund and Eriksson, 2009).

Acetylcholinesterase is anchored to the basal lamina (extracellular matrix that aids cell adhesion and neuromuscular signaling processes) (reviewed by Fagerlund and Eriksson, 2009) where it can catalyze the hydrolysis of ACh into choline and acetate. Originated choline can be then uptaken via high affinity-choline transporters (ChT) localized at the plasma membrane of the motor nerve terminal (Siegel et al., 2006). Choline uptake is the rate-limiting step in the biosynthesis of ACh, because treatment with hemicholinium-3 (a potent inhibitor of the high-affinity choline transporter) leads to a reduction in ACh release during prolonged nerve stimulation (reviewed by Eiden, 1998).

1.2 Postsynaptic region

The role of the structures located on the post synaptic surface of the skeletal muscle fiber is to optimize the action of ACh to induce an EPP.

The mammalian post-synaptic membrane region can be morphologically recognized due to the presence of deep infoldings of the plasma membrane, which are called secondary synaptic folds. This feature contributes to enhance the neuromuscular transmission as it organizes ACh receptor clusters near the synapse (primary folds) and Na^+ channels at the bottom of each synaptic fold (secondary folds) (reviewed by Hughes et al., 2006) (Figure 2).

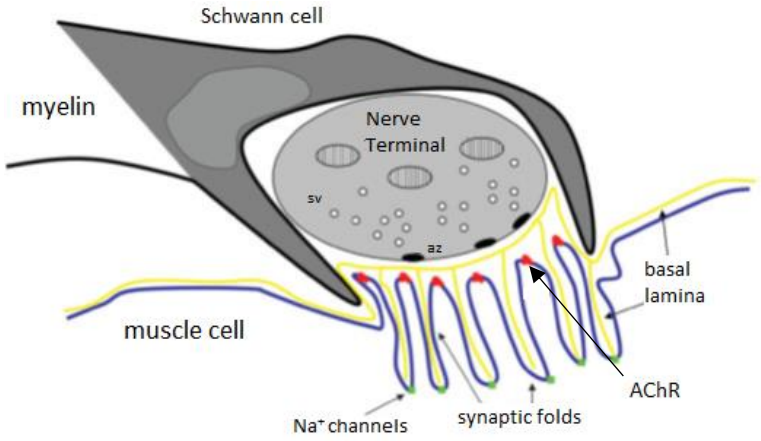

Figure 2. Representative scheme of the NMJ. The NMJ is a structure formed by three components: the perisynaptic Schwann cell, the presynaptic nerve terminal and the postsynaptic endplate region. At the presynaptic terminal, the synaptic vesicles (sv) are normally located at the active zones (az) of release. At the synapse there is a structure called basal lamina made up of laminin, agrin and collagen which provide help to neuromuscular transmission. Then, at the muscle cell region, there are multiple folds which can be identified as primary folds (shallower) where nAChRs are located (red dots) and secondary folds (deeper) where Na^+ channels reside (green dots) (adapted from Hughes et al., 2006).

1.3 ACh receptors

There are two types of ACh receptors: ionotropic nicotinic (nAChRs) and metabotropic muscarinic (mAChRs) receptors (Figure 3). The nicotinic receptors are found at neuromuscular junctions, autonomic ganglia and in several regions of the central nervous system (CNS), whereas muscarinic receptors were identified in the heart, smooth muscle fibers and exocrine glands, in addition to their presence in the CNS and autonomic ganglia (Rang et al., 2016).

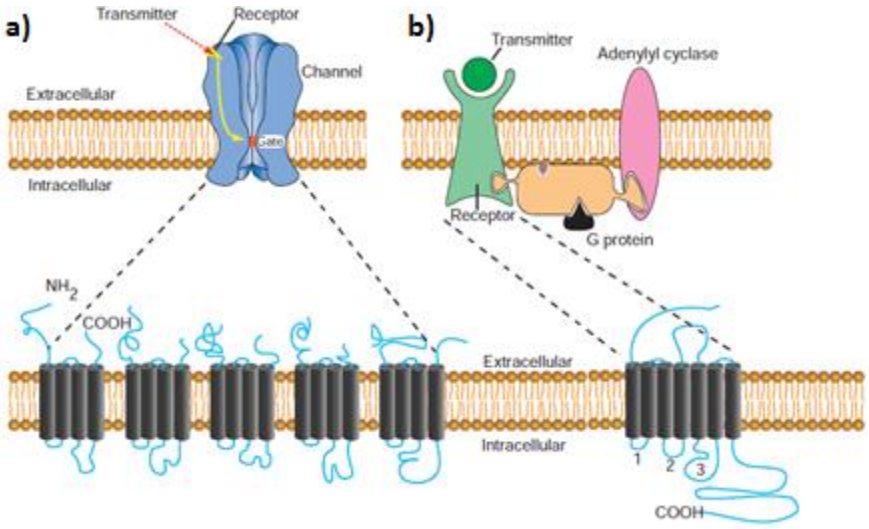

Figure 3. Structural comparison between ionotropic and metabotropic receptors. a) Ionotropic receptors are composed by five transmembrane peptide subunits forming a pentameric pore; activation of this pore requires binding of 2 molecules of ACh to extracellular loops of two α-subunits. b) Metabotropic receptors are G-protein-coupled receptors formed by a protein with seven transmembrane domains with only one binding site to ACh in the extracellular region; the third intracellular loop is essential for the interaction with a specific G-protein (adapted from Squire et al., 2008).

Like many other metabotropic receptors, the muscarinic receptor has seven transmembrane spanning domains, connected by peptide loops. The third intracellular loop has critical sequences that allow it to couple to a GTP binging protein (G-protein), explaining the GPCR designation (G-coupled protein receptors) (reviwed by Hulme et al., 1990). Once this receptor is activated, it couples to a G-protein initiating the exchange of GDP by GTP, activating the G-protein. Activated G-proteins then couple to many downstream effectors that will ultimately produce cellular effects (Squire et al., 2008).

There are five subtypes of muscarinic receptors identified from M_1 to M_5. The odd-numbered receptors (M_1, M_3) are coupled to $G_{q/11}$ proteins leading to activation of phospholipase C-β (PLCβ) and the inositol trisphosphate (IP_3) pathway. The even-numbered receptors (M_2, M_4) are coupled to $G_{i/o}$ proteins resulting in the inhibition of adenylyl cyclase and in the reduction of intracellular cyclic adenosine monophosphate (cAMP) levels; these receptors may also couple to the opening of potassium channels

causing membrane hyperpolarization (Rang et al., 2016). At the NMJ, excitatory M_1 and inhibitory M_2 receptors can act as presynaptic autoreceptors modulating the release of ACh (Wessler, 1989). The balance between M_1 excitation and M_2 inhibition to control neurotransmitter release at the rat motor endplate is highly dependent on the rate of neuronal activity and on the crosstalk with pre-synaptic adenosine A_1 and A_{2A} receptors (Oliveira and Correia-de-Sá, 2005; Oliveira et al., 2002; 2009). The M_1 positive feedback control of ACh release predominates during low frequencies (e.g. 5Hz) of motor nerve stimulation. Under these conditions small amounts of adenosine are found at the neuromuscular synapse, which favors activation of inhibitory A_1 receptors shutting-down the inhibitory control of ACh operated by muscarinic M_2 receptors. Upon increasing the neuronal firing rate (to 50 Hz) adenosine accumulated at the neuromuscular junction reaches levels high enough to activate facilitatory A_{2A} receptors, which contribute to downmodulate M_1 receptors activation favoring the negative control of the neuromuscular transmission operated by the M_2 receptor subtype (Oliveira et al., 2002).

Ionotropic nicotinic ACh receptors are pentameric transmembrane protein complexes arranged around a pseudoaxis of symmetry forming an ion pore (Figure 4). At the neuromuscular junction, these receptors are present both pre- and post-synaptically and they mediate fast neurotransmission by ACh. Our group showed for the first time that the pre-synaptic nicotinic receptor responsible for ACh autofacilitation during high-frequencies of nerve stimulation contained α3β2 subunits (Faria et al., 2003). Fast desensitization of this receptor is also controlled by tonic adenosine A_{2A} receptors activation in order to prevent excess of transmitter release and nerve fiber damage during intense neuronal firing (Correia-de-Sá and Ribeiro, 1996; Timóteo et al., 2003).

The muscle-type nicotinic receptor is formed by two α subunits assembled together with one copy of β, γ and δ subunits. Structural studies showed that the subunits are arranged around a central cavity that is believed to lead to the ion channel, which in the resting state is impermeable to ions; upon activation however, it opens selectively for cations. The sites for ligand binding are located toward the external perimeter of each ligand binding α-subunit interface and to activate the receptor two ACh molecules are necessary.

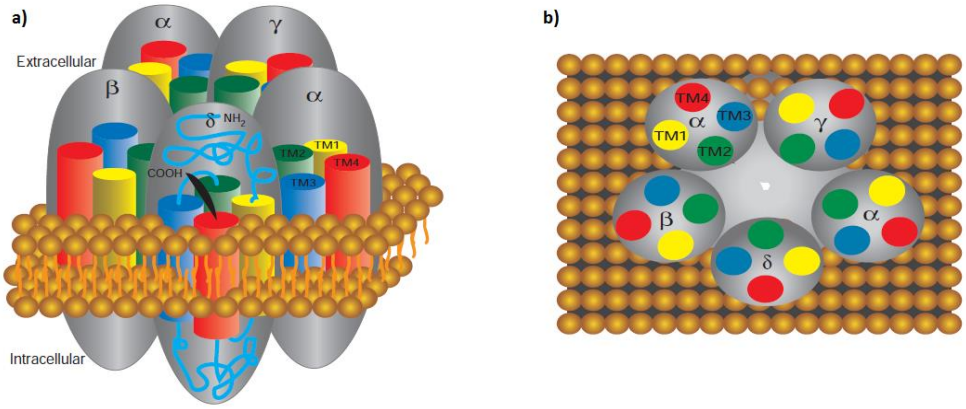

Figure 4. Schematic representation of the molecular organization of nAChRs in the membrane. a) The amino and carboxy terminal extend in the extracellular space. The four membrane-spanning domains termed TM1-TM4 assume a α-helix structure to traverse the membrane. b) Top view of all five units highlighting the relative positions of all transmembrane domains, and the presence of two α subunits (adapted from Squire et al., 2008).

Binding of ACh to the nicotinic receptor triggers the influx of Na^+ into the muscle fiber to initiate depolarization of the muscle (Siegel et al., 2006). The postsynaptic total depolarization produced by ACh exocytosis triggered by a nerve action potential is the end-plate potential (EPP) (Hughes et al., 2006). This depolarization is perceived by voltage-sensitive Na^+ channels ($Na_v1.1$), which allow the diffusion of Na^+ down their concentration gradient, spreading the depolarization phenomena throughout the muscle fibers, leading to calcium release within the muscle and the actin-myosin interaction that ultimately results in its contraction (reviewed by Conti-Fine et al., 2006; Fagerlund and Eriksson, 2009).

1.4 Neuromuscular Safety Factor

Neuromuscular transmission safety factor is defined as the ratio between the actual EPP and the threshold potential required to generate the muscle action potential (Figure 5). The quantal content of an impulse, the conduction properties, the architecture of synaptic folds, the density of post-synaptic nAChR and the activity of AChE in the synaptic cleft all contribute to the EPP. Normally, quantal ACh released to induce an EPP is greater than the threshold necessary to generate a muscular action potential, which accounts to a large safety factor. The current flow created after activation of nAChRs has its maximal depolarizing effect at the depths of the synaptic folds where there is a high density of voltage-gated Na^+ channels. The opening of these channels after the initial depolarization increases the effect of the transmitter release and reduces the threshold for action potential generation, thereby enhancing the safety factor. In the case of repetitive stimulation, neurotransmitter release decreases gradually in the course of a train thus reducing the amplitude of EPPs but, under normal conditions, this phenomenon is not enough to prevent action potentials generation (reviewed by Fagerlund and Eriksson, 2009; Hughes et al., 2006). However, neuromuscular transmission failure may occur in pathological conditions, such as *Myasthenia Gravis*, which will be further discussed in the following chapter.

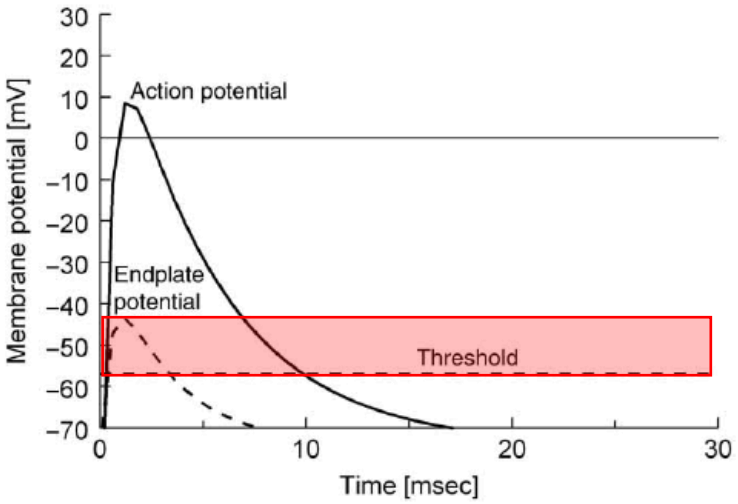

Figure 5. Schematic representation of the muscle action potential. The stippled line indicates the shape of the EPP, as the full line indicates the shape of the action potential. The firing threshold of the muscle is indicated by the horizontal dashed line. The EPPs capable of surpassing the threshold potential are able to generate a muscle action potential. The safety factor is represented by the red colored rectangle and it represents the magnitude of the EPP needed to trigger muscle contraction.

2. *Myasthenia Gravis*

Myasthenia Gravis (MG) is the most common primary disorder of neuromuscular transmission (reviewed by Sathasivam, 2014; Turner, 2007). The major characteristic feature of MG is painless, fatigable weakness (reviewed by Vincent et al., 2001). These symptoms are the result of autoantibodies (auto-Abs) attack to proteins of the post-synaptic region of NMJ which are involved in the neurotransmission phenomenon (reviewed by Berrih-Aknin et al., 2014). Among all the myasthenic patients, 85% of them are seropositive for muscle nAChR and 5% have autoreactive antibodies directed against a protein called muscle specific kinase (MuSK), which plays a central role in the clustering of nAChRs at the NMJ (reviewed by Berrih-Aknin et al., 2014; Hoch et al., 2001). Recently, the agrin receptor, low-density lipoprotein receptor-related protein 4 (LRP4), a protein that forms a complex with MuSK helping AChRs clustering, has been identified as a novel autoantigenic target (Higuchi et al., 2011; Pevzner et al., 2012) (Figure 6).

The development of those antibodies is apparently caused by the breakdown of self-tolerance in the thymus (Newsom-Davis et al., 1981) with activation of AChR-specific CD4+ T helper cells and production of pro-inflammatory cytokines, leading to the synthesis of high affinity Abs (Vincent, 2002). MG is considered a prototypical antibody-mediated autoimmune disease because it has the characteristics that fulfill all the strict criteria (reviewed by Conti-Fine et al., 2006; Vrolix et al., 2010):

a. Auto-antibodies of the blood plasma are found at the site of pathology, the NMJ in this case (Engel et al., 1977);
b. These antibodies are capable of causing the same symptoms when injected in rodents (Toyka et al., 1977);
c. Immunization of animals with muscle nAChR reproduces the disease (Patrick and Lindstrom, 1973);
d. Therapies based on the removal of blood Abs (plasma exchange) attenuates the symptoms (Newsom-Davis et al., 1978);

2.1 Pathophysiology

There are at least three mechanisms by which anti-nAChR antibodies may affect neuromuscular transmission (reviewed by Vrolix et al., 2010) (Figure 7):

a. Binding and activation of complement at the NMJ, which leads to focal lysis of the post-synaptic folds by the membrane attack complex (Engel et al., 1977; van der Neut et al., 2007);

b. Antigenic modulation: Abs bind and crosslink the nAChRs, inducing the endocytosis of the complex and further degradation of the nAChRs by the muscle cell (Heinemann et al., 1977; van der Neut et al., 2007);
c. Functional nAChR interference by blocking ACh binding (Almon et al., 1974).

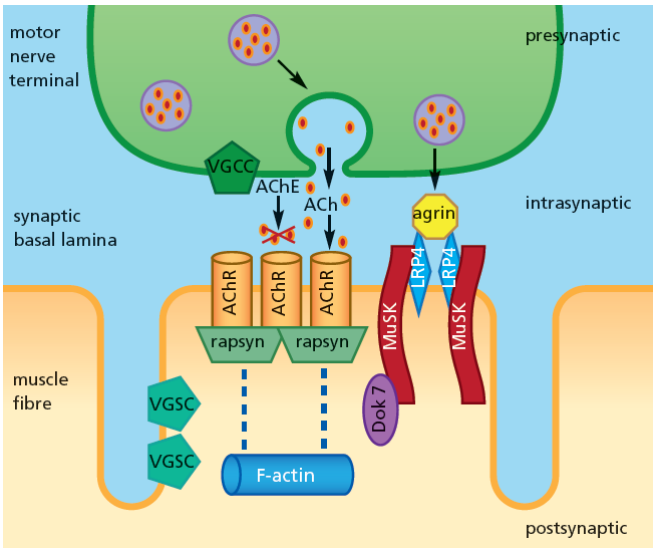

Figure 6. Postsynaptic organization of muscle nAChR clusters. MuSK is present predominantly at the NMJ, where it is part of the receptor for agrin. Agrin is a protein synthesized by motor neurons and secreted into the basal lamina. Binding of this protein to LRP4 activates MuSK, which will trigger downstream transduction pathways culminating in the coclustering of AChRs with rapsyn. Rapsyn, is a peripheral membrane protein that attaches AChRs to the contractile F-actin protein, leading to dense AChR clustering at the primary synaptic folds.(Ruegg and Bixby, 1998; adapted from Sathasivam, 2014).

These mechanisms are, therefore, responsible for the decrease in the amount of nAChRs and for the loss of functional and anatomical structure of the NMJ. As a consequence, the EPPs induced present a lower magnitude than the threshold potential for activating the voltage-gated calcium channels responsible for the development of the muscle action potential. This is particularly evident with repetitive muscular activity since in this condition the quantal content of neurotransmitter release decreases as a function of time. In a normal situation, the neuromuscular safety factor is enough to surpass this decrease. In myasthenics, the decrease of EPP associated to the changes in the architecture of the NMJ leads to an increased probability of neuromuscular failures (reviewed by Gomez et al., 2010).

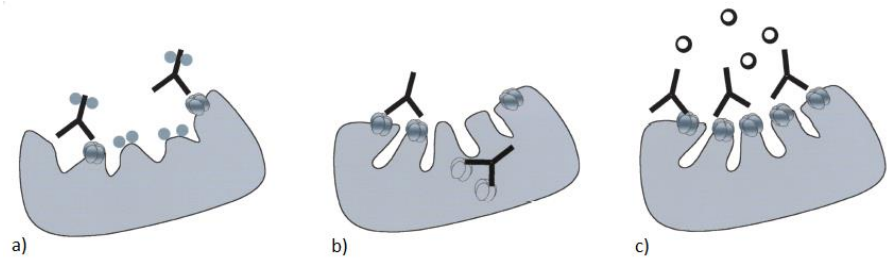

Figure 7. Mechanisms of action of anti-nAChRs antibodies on the pathophysiology of MG. a) Abs towards AChRs that bind complement result in destruction of the muscle endplate and a reduction in the number of available AChRs. b) Anti AChR Abs cross-link adjacent AChRs increasing their rate of internalization into muscle. c) Antibodies block the binding site for ACh at the postsynaptic nicotinic receptor (adapted from Nicolle, 2002).

2.2 Epidemiology

The incidence rate of this disease varies between 1.7 and 21.3 per million inhabitants depending on the location of the study (Carr et al., 2016). From epidemiological studies, we learned that the incidence rate of MG has a bimodal distribution, which suggests a hormonal or environmental influence on the disease onset. MG affects both sexes, at all ages and in all races (reviewed by Turner, 2007). Before the age of 50, studies show that early-onset MG is characterized by female predominance (60-70%) (Carr et al., 2016). On the other hand, between the ages of 50 and 60, there is no gender difference. After 60 years old, a very late onset MG appears and is known to be constituted mostly of male patients (Oger and Alkhawajah, 2013). Juvenile MG is variable among different races. It is uncommon in Europe and North America (10-15% of total Caucasian MG patients) and is much more frequent in Asia (50% of MG patients in China) (reviewed by Berrih-Aknin et al., 2014)

In adulthood, acquired autoimmune MG is the commonest form (reviewed by Turner, 2007). On the other hand, transient neonatal MG is caused by passive transfer of maternal Ab, especially the Anti-AChR antibodies (Béhin et al., 2008).

2.3 Clinical features

In about 2/3 of myasthenic patients, the initial symptoms are ptosis and diplopia which reflects that the extrinsic ocular muscles (EOM) and the levator palpebrae muscles are the first to show muscular weakness. The symptoms then progress in a craniocaudal direction to the facial and bulbar muscles causing reduced facial expression and speech, chewing and swallowing difficulties. Finally limb muscles are affected resulting in generalized MG (reviewed by Conti-Fine et al., 2006; Sathasivam, 2014). Weakness of the intercostal muscles and diaphragm can cause dyspnea (shortness of breath), which can evolve to severe respiratory failure. Patients with these symptoms should be

monitored for forced vital capacity and for blood gas because intubation and mechanical ventilation may be needed (reviewed by Turner, 2007).

Between different muscles, the susceptibility to MG is not the same. EOMs are particularly susceptible to show myasthenic weakness because the NMJ have less synaptic folds and, therefore, fewer postsynaptic AChRs and Na^+ channels, which results in a decreased safety factor. Also, they are more often subjected to a high frequency of neuronal firing, making them more susceptible to fatigue. Taken together, these characteristics explain why the first symptom is ocular muscle weakness. In skeletal muscles, the NMJs of the fast-twitch fibers release higher amounts of ACh, have more postsynaptic folding, and higher postsynaptic sensitivity to ACh than slow twitch fibers. These properties make fast twitch skeletal muscle fibers less prone to myasthenic failure tan slow-twitch fibers (reviewed by Conti-Fine et al., 2006).

2.4 Diagnosis

A complete medical and neurological evaluation is necessary to diagnose MG. Several number of tests may be used to establish a diagnosis of MG:

a) Tensilon test: this test consists of an intravenous injection of edrophonium, a short-acting AChE inhibitor that transiently increases the amount of ACh available, improving weakness caused by an impairment of the neuromuscular transmission. This test is sensitive in diagnosing a defect on neuromuscular transmission, but it is not specific for MG, neither it is recommended in elderly patients or patients with cardiac disease and/or pulmonary disease (reviewed by Juel and Massey, 2007).

b) Antibody tests: these tests evaluate the presence of anti-AChRs Abs in the patient's blood. If these are not detected, anti-MuSK antibodies should be tested. The detection of serum AChR antibodies is highly specific for MG but its sensitivity is low in the presence of ocular symptoms only. Also, the absolute titer of Abs does not correlate with differences in disease severity.

c) Neurophysiology tests: these electrodiagnosis tests can be performed in two exams: repetitive nerve stimulation (RNS) and single fiber electromyography (SFMEG) (reviewed by Nicolle, 2002; Sathasivam, 2014). In RNS, the amplitude of the compound muscular action potential induced by repetitive nerve stimulation with frequencies of 3-10 Hz is measured and MG is diagnosed if a decrease in the compound muscular action potential is observed. Although it is technically easy and sensitive with the generalized disease, it is non-specific leading to false-positives (Keesey, 1989). SFEMG is the most sensitive diagnostic test and it

should be performed if RNS is normal and a NMJ disorder is suspected. It consists in an electromyography where action potentials of individual muscle fibers are recorded due to its 25 µm diameter recording surface (Selvan, 2011). Despite its high sensitivity, abnormalities detected are not specific for MG (Oh et al., 1992)

d) Ice pack test: An ice cube is placed over the drooping eyelid for about two minutes and, if there is ptosis improvement, a neuromuscular transmission disorder is suggested (Czaplinski et al., 2003).

2.5 Treatment

There are generally four options regarding treatment of MG which have different time courses:

a) Acetylcholinesterase inhibitors: These compounds are the first-line treatment in MG patients. They slow the hydrolysis of ACh at the NMJ, increasing the amount of ACh, which allows patients to recover from tetanic blockade and to improve muscular strength (Drachman, 1994). Pyridostigmine is the most commonly used drug, except for anti-MuSK MG patients which are insensitive to this treatment (Hatanaka et al., 2005). This is simply a symptomatic treatment, meaning that it does not retard the underlying immune attack. This treatment may have side effects due to increased muscarinic activity and a cholinergic crisis may develop if there is an excessive dosing in patients with severe MG, worsening muscle weakness (reviewed by Juel and Massey, 2007; Trouth et al., 2012).

b) Immunomodulation treatments: These treatments are used for crisis intervention, and they are seldom used for prolonged therapy (reviewed by Sieb, 2014). Two treatments belong to this category: Plasmapheresis, which consists in the direct removal of Abs against AChR from circulation (Batocchi et al., 2000) and intravenous immunoglobulin therapy (IVIg) which involves the isolation of immunoglobulins from human plasma and administration for 5 days. It's mechanism of action is complex but it involves inhibition of complement deposition, interference with antigen recognition by sensitized Tcells and others (Samuelsson et al., 2001). These two are equal first-line treatments, however, IVIg is easier to administer and associated with less adverse events which makes it a more popular choice among physicians.(reviewed by Sathasivam, 2014).

c) Chronic immunosuppression: Corticosteroids, like prednisolone are the immunosuppressive agents most frequently used for the treatment of MG and the most effective. These may be used at low doses for years or, at high doses for

months. Anti-AChR Abs levels reduce in the first months of therapy and most patients report to have clinical benefit (reviewed by Conti-Fine et al., 2006).

i. Azathioprine is the non-steroidal immunosuppressant most frequently used. It is a purine analog that reduces nucleic acid synthesis, interfering with T and B cell proliferation (reviewed by Conti-Fine et al., 2006; Trouth et al., 2012). It usually takes up to 15 months to evaluate a clinical response, which is why it is normally used in combination with prednisone. (Palace et al., 1998).

ii. Mycophenolate mofetil blocks purine synthesis, suppressing both T and B cell proliferation and it might be used if treatment with azathioprine is not tolerated (reviewed by Trouth et al., 2012)

iii. Cyclophosphamide interferes with DNA synthesis and may preferentially suppress B lymphocytes (reviewed by Nicolle, 2002).

iv. Cyclosporine blocks the synthesis of IL-2 cytokine receptor and other key-proteins to the function of CD4+ T cells. This is the mainly used treatment in patients who do not respond/tolerate to azathioprine (reviewed by Trouth et al., 2012)

MG patients resistant to therapy have been successfully treated with cyclophosphamide in combination with bone-marrow transplant or with rituximab, a monoclonal antibody against the B-cell surface marker CD20 (reviewed by Trouth et al., 2012).

d) Surgical treatment: In early onset MG, thymic follicular hyperplasia is frequently found in women and in late onset MG, thymoma is frequently present (reviewed by Berrih-Aknin et al., 2014). As such, thymectomy is strongly recommended for patients with thymoma, because its efficacy in other situations has been questioned for lack of solid controlled prospective studies (reviewed by Trouth et al., 2012). Thymectomy may not be a viable therapeutic strategy in anti-MuSK MG because they lack the thymic characteristics that anti-AChR MG have, also suggesting different pathologic mechanisms for both forms of MG.

MG treatment should be individualized according to patient characteristics and severity of disease using the array of treatment options available. When the treatment is well fit, the majority of the patients can have largely normal lives. Even complete stable remission can be achieved in some patients (Baggi et al., 2013). Unfortunately, treatment is less effective in anti-MuSK, with a significantly increased risk of myasthenic crisis (Deymeer et al., 2007). Nonetheless, these treatments are hardly proven by controlled studies. It is largely based on serendipity and retrospective studies (reviewed by Juel

and Massey, 2007; Sieb, 2014) which emphasizes the need for new therapeutic strategies.

2.6 Animal models of *Myasthenia Gravis*

The development of animal models is crucial to the study of any disease because it allows the investigator to better understand the characteristics of the disease. Thirty years ago, Lindstrom and Patrick reported for the first time that rabbits immunized with AChRs purified from the electric organ of *Electrophorus electricus* develop MG-like symptoms (Patrick and Lindstrom, 1973).

Although there are other animal species suitable for experimental autoimmune *Myasthenia Gravis* (EAMG) induction, rats are the most often used model (65%), with mice accounting for 35% of the cases. Despite the fact that mice were expected to represent the most suitable model for EAMG induction, due to availability of mutant strains and to access to a huge variety of monoclonal antibodies specific for cell markers, these animals are less susceptible to EAMG induction due to a higher safety factor (high quantal amount of released ACh from the nerves) making it difficult to detect neuromuscular transmission failure than in rats (reviewed by Baggi et al., 2012). From the different rat strains, Lewis rats exhibit intermediate susceptibility, but the clinical manifestations in this strain are similar to those in human MG (Biesecker and Koffler, 1988). As such, rats have been the animal species most often used to induce EAMG, which can be achieved by active immunization, passive transfer or adoptive transfer (Figure 8):

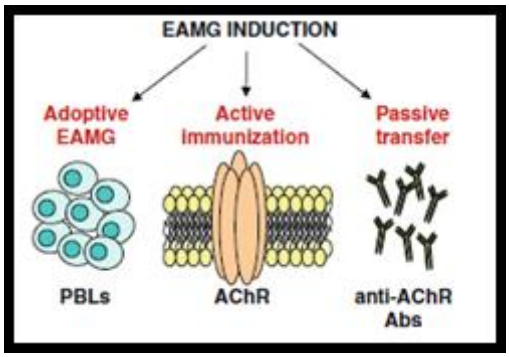

Figure 8. Schematic representations of the different EAMG induction modes.

a) Active immunization: It is either performed by a single immunization with purified AChR in complete Freund's adjuvant (CFA) (Lindstrom, 1980) or by immunization with a synthetic peptide which corresponds to the aminoacidic region between positions 97-116 of rat-AChR α subunit, made up in CFA followed by a second

immunization boost of the peptide in incomplete Freund's adjuvant (IFA), 30 days after the beginning of the immunization protocol (Baggi et al., 2004). CFA and IFA are both constituted essentially by paraffin oil containing mannide mono-oleate as a surfactant. In addition, CFA contains heat-killed mycobacteria (*Mycobacterium tuberculosis*, in our case). These adjuvants generally act by prolonging the lifetime of injected autoantigen, by stimulating its effective delivery to the immune system, and by providing a complex set of signals to the innate compartment of the immune system (reviewed by Billiau and Matthys, 2001). The last one is the induction protocol used in this work since the anti-AChR Abs produced derive from the breakdown of self-tolerance, almost mimicking an autoimmune disease (Baggi et al., 2004).

b) Passive transfer: this induction method is basically the passive transfer of auto-Abs via daily injections of serum IgG isolated from MG patients or anti-AChR antibodies from donor animals with chronic EAMG. Also, monoclonal Abs directed to the α subunit of AChR derived from immunized animals or from cell line culture supernatants (reviewed by Baggi et al., 2012).

c) Adoptive transfer: this method is based in the transplantation of human tissues or cells in severe combined immunodeficiency (SCID) mice, lacking mature B and T cells (Schönbeck et al., 1992).

MG and EAMG have several similar characteristics: muscle weakness, fatigability, decreased response upon repetitive nerve stimulation, improvement of muscular strength following treatment with anti-cholinesterase drugs. The immunopathological features are also quite similar, such as the presence of anti-AChR Abs in serum, deposition of complement components at the NMJ, and others.

Despite their similarities, EAMG and human MG seem to have one big difference. In EAMG animals, the auto-sensitization process seems to occur only in lymph nodes apparently not affecting the thymus, yet in human MG thymic alterations are frequent (reviewed by Baggi et al., 2012; Christadoss et al., 2000).

3. Purinergic signaling

More than 30 years ago, scientists discovered that adenosine triphosphate (ATP) was a constituent of cholinergic synaptic vesicles (Dowdall et al., 1974). A year later, Eugene M Silinsky found that ATP was coreleased with ACh at the NMJ of rat phrenic nerve-hemidiaphragm upon nerve stimulation (Silinsky, 1975; Silinsky and Hubbard, 1973). These findings opened new avenues on purinergic signaling roles in the peripheral nervous system (PNS), since they make ATP a likely neurotransmitter, co-transmitter or presynaptic modulator in cholinergic (as well as many other) synapses.

Besides motor nerve endings, purines can also be released from skeletal muscle fibers (Santos et al., 2003) and Schwann cells (Liu et al., 2005; Shin et al., 2012). It is estimated that about 60% of ATP released at the motor endplate is derived from activated skeletal muscle fibers, as determined in the rat hemidiaphragm using α-bungarotoxin as paralyzing agent (Santos et al., 2003). Different release sites seldom correspond to distinctive release modes. As a matter of fact, ATP can be released together with ACh by vesicle exocytosis, but the nucleotide can also be translocated to the extracellular space secondary to cell lysis and by facilitated diffusion through nucleotide transporters or electrodifusional transport through specific release channels, namely pannexin-1 hemichannels (reviewed by Yegutkin, 2008). Once in the extracellular milieu, ATP can act either directly, via P2 purinoceptors (P2R) (Salgado et al., 2000), or indirectly, via adenosine (ADO) receptors (P1 receptors, P1R) after its extracellular catabolism by ectonucleotidases (Cunha et al., 1996).

3.1 ATP release

Since ATP is a highly polarized molecule, it cannot pass freely the cell membrane. As such, ATP outflow to the extracellular space may occur by a series of mechanisms:

a) Vesicular exocytosis: As previously stated, ATP is able to be co-released with ACh from cholinergic nerve terminals. Like ACh, ATP is taken up and stored in synaptic vesicles, which can be later on released in a Ca^{2+}-dependent manner.
b) Carrier-mediated release: Although this kind of mechanism is yet to be molecularly identified in the nervous system, specific transporters such as ATP binding cassettes (ABC) are able to translocate ATP across the plasma membrane (reviewed by Sperlagh et al., 2007; Wang et al., 1996).
c) Channels and pores: Connexin and pannexin hemichannels are also potential candidates to drive ATP across the plasma membrane in non-neuronal cells (reviewed by Sperlagh et al., 2007).

d) Cellular lysis: ATP can be released in huge amounts after cell damage or whenever the integrity of the plasma membrane is compromised (reviewed by Yegutkin, 2008).

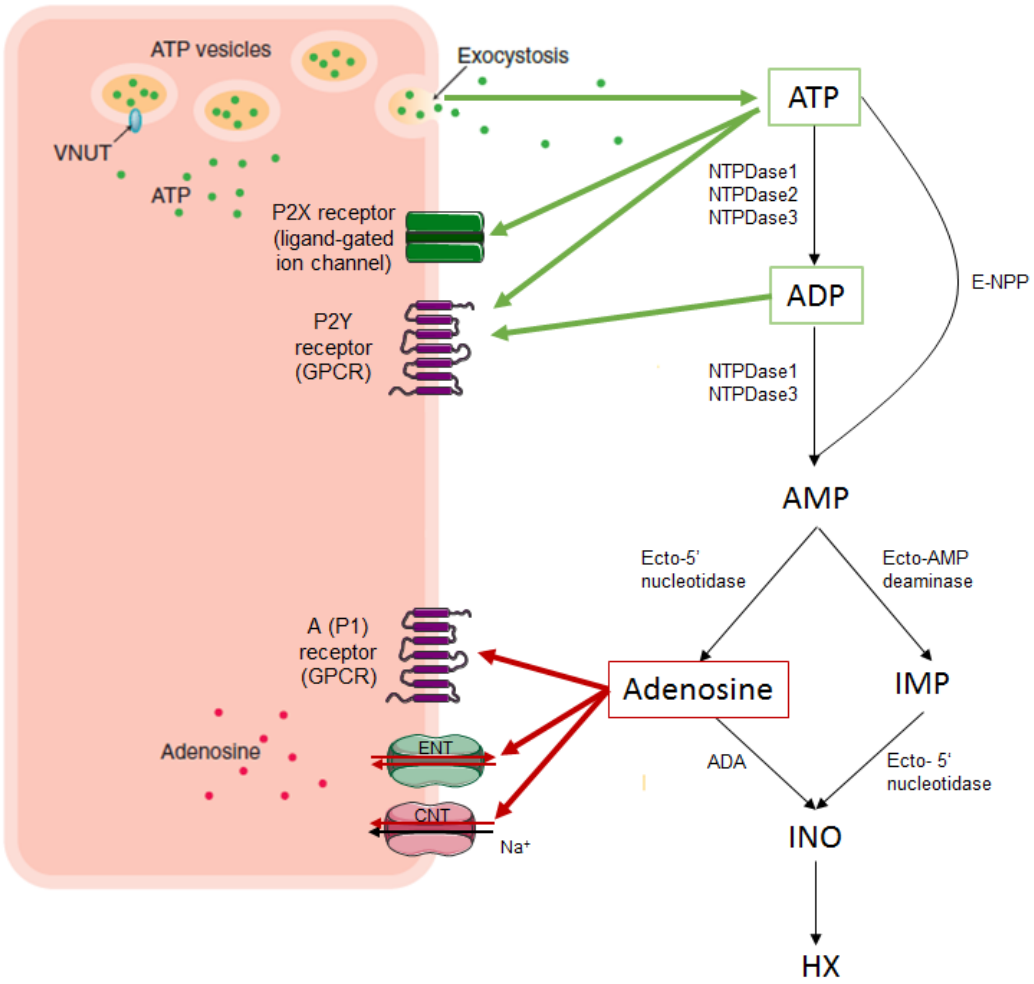

Figure 9. The purinergic signaling cascade. ATP and adenosine release sites, metabolic enzymes and P1/P2 purinoceptors. Green arrows represent interactions with adenine nucleotides, ATP or ADP; Red arrows represent interactions with adenosine.

3.2 Metabolism of purines

The extracellular accumulation of nucleotides is regulated by several groups of ectonucleotidases located on the cell surface; some of these membrane-bound enzymes break its GPI anchor and become soluble in the interstitial medium or within body fluids. Ectonucleotidases contribute to the extracellular hydrolysis of nucleotides for which they have substrate specificity (reviewed by Robson et al., 2006; Zimmermann, 2001):

a) The E-NTPDase (ectonucleoside triphosphate diphosphohydrolase) family hydrolyzes nucleoside 5'-tri- and di-phosphates with different characteristics. They share five highly conserved sequence domains called "apyrase conserved regions" (ACRs) which are of major

importance for their catalytic activity. Maximal catalytic activity requires the presence of divalent cations, like calcium or magnesium, and an alkaline pH. The K_m values for ATP of the purified enzymes are in the lower micromolar range. There are 8 different NTPD genes (Figure 9). Regarding to their location, NTPDases1, 2, 3 and 8 are typical cell-surface enzymes with the catalytic site facing the extracellular medium (true E-NTPDases), NTPDases 5 and 6 are intracellularly located but after heterologous expression, they undergo secretion, and NTPDases 4 and 7 are entirely intracellularly located facing the lumen of cytoplasmic organelles such has the Golgi apparatus and lysosomal/autophagic vacuoles.

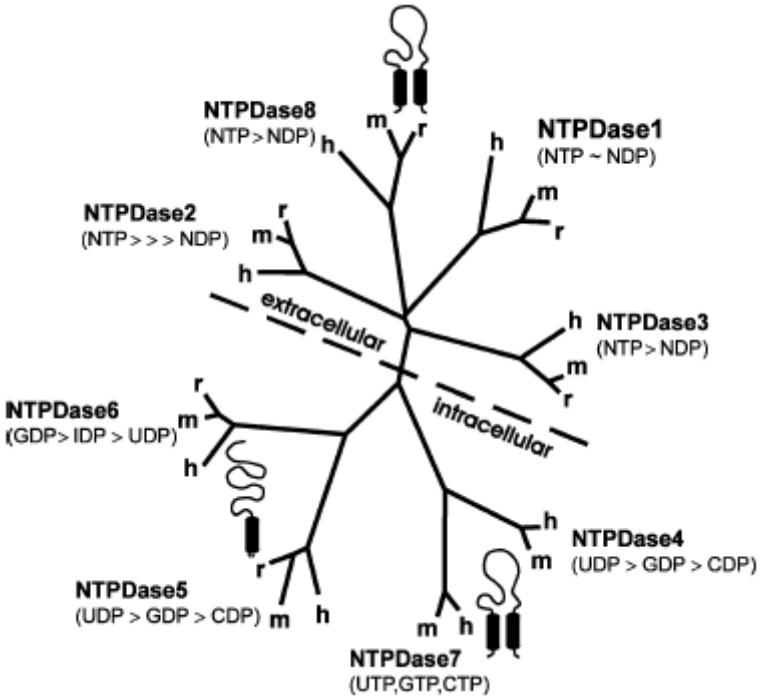

Figure 10. The graph shows the different properties of all eight members of the NTPDase family. In terms of location, there is a difference between surface-located (top) and intracellular (bottom) NTPDases. In addition, the major substrate preferences of individual subtypes and the predicted membrane topography for each group of enzymes is given (one or two transmembrane domains, indicated by barrels) (reviewed by Robson et al., 2006).

There is a considerable difference in substrate specificity between E-NTPDases: NTPDase1 (or CD39, apyrase) hydrolyzes ATP and ADP almost equally well; NTPDase2 has a 30-fold preference for the hydrolysis of ATP over ADP; NTPDase3 and 8 are functional intermediates and hydrolyze ATP approximately three times better than ADP.

b) The E-NPP (ectonucleotide pyrophophatase/phosphodiesterase) family, which comprises NPP1 to NPP3, have alkaline phosphodiesterase and

nucleotide pyrophosphatase catalytic activities and they are capable to form AMP from 3',5'-cAMP, ATP, ADP, NAD^+ and diadenosine polyphosphates (e.g. Ap_nA). Their activity depends on divalent cations and alkaline pH for maximal activity. The K_m values for ATP are between 20 to 50 µM.

c) Ecto-5'-nucleotidase (CD73) is a glycophosphoinositol (GPI)-anchored enzyme which hydrolyses nucleoside 5'-monophosphate, like AMP, and can be feed-forward inhibited by ATP and ADP (Cunha et al., 1996). Ecto-5'-nucleotidase is the rate-limiting enzyme for adenosine formation and P1 receptors activation in most tissues, including the NMJ (Magalhães-Cardoso et al., 2003).

d) Alkaline phosphatases reveal a broad substrate specificity because they hydrolyze 5'-tri-, -di- and –monophosphatase, being thus able to catalyze the entire hydrolysis chain. Nonetheless, computational kinetic assays for alkaline phosphatase of E. coli demonstrated that the K_m for these substrates is in the millimolar range, making ENTPDases most likely to participate in the catabolism (Pétursson, 2014). Like the ecto 5'-nucleotidase, they are anchored to the plasma membrane via a GPI-anchor.

e) Adenylyl kinase is an ecto-ATP:AMP phosphotransferase capable of mediating the production of ATP and AMP from ADP, and vice-versa.

f) Adenosine deaminase (ADA): this enzyme catalyzes the irreversible deamination of adenosine into inosine, terminating ADO signaling due to P1 receptors activation (reviewed by Yegutkin, 2008).

g) Purine Nucleotide Phosphorylase (PNP) is the enzyme that terminates purine signaling by phosphorylating inosine into hypoxantine (HX) (reviewed by Yegutkin, 2008)

At the NMJ, besides the production of adenosine via ecto-5'-nucleotidase, AMP can also be deaminated extracellularly by ecto-AMP deaminase, which is the enzyme that converts extracellular AMP into IMP (Cunha and Sebastião, 1991) bypassing the formation of ADO (Magalhães-Cardoso et al., 2003). Experimental data clearly showed that dephosphorylation of AMP leading to adenosine formation was more effective than AMP deamination forming IMP at the rat innervated hemidiaphragm (Magalhães-Cardoso et al., 2003). Nonetheless, AMP deamination might gain particular relevance to uncouple adenosine formation from the extracellular catabolism of adenine nucleotides, whenever ATP and ADP reach high enough levels at the neuromuscular synapse to feed-forwardly inhibit ecto-5'-nucleotidase activity.

3.3 Nucleoside transporters

Since adenosine is not stored in synaptic vesicles nor it is released in quanta, the nucleoside is not classically termed a neurotransmitter and it is generally thought of as a neuromodulator. Because of its low hydrophilicity, adenosine is only able to reach the extracellular space either as a result from the extracellular catabolism of released adenine nucleotides (see above) or via transmembrane nucleoside transporter proteins. Nucleoside transporters acting together with ADA are the main responsibles for termination of adenosine signaling at the NMJ (Siegel et al., 2006). Using radiolabeled nucleosides it was possible to discover the existence of two types of nucleoside transporters: the SLC28 family of cation-linked concentrative nucleoside transporters (CNTs) and the SLC29 family of energy-dependent equilibrative nucleoside transporters (ENTs) (reviewed by Young et al., 2013).

a) CNTs: there are three subtypes of membrane-bound transporters in this family (see Table 1); they all profit from the inwardly directed sodium gradient to actively transport adenosine into the cells. The current topological model consists of 13 TM domains with the cation and the substrate recognition sites in the carboxy half of the protein (reviewed by Gray et al., 2004). Ouabain is able to inhibit these transporters due to its ability to inhibit Na^+/K^+ ATPase (Benfenati et al., 1984).

Table 1. Characteristics of the SLC-28 sodium coupled nucleoside transport family (adapted from Gray et al., 2004)

Protein Name	Predominant substrates	Tissue distribution	Cloning characteristics
CNT1	Pyrimidine nucleosides, adenosine	Liver, kidney, small intestine	hCNT1 and rCNT1 are 83% identical at protein level
CNT2	Purine nucleosides, uridine	Kidney, liver, heart, brain, placenta, pancreas, **skeletal muscle**, colon, rectum, small intestine	hCNT2 and rCNT2 are 81% identical at protein level
CNT3	Broad selectivity for pyrimidines and purines	Pancreas, trachea, bone marrow, mammary gland, intestine, lung, placenta, prostate, testis and liver	hCNT3 and mCNT3 are 78% identical at protein level.

b) ENTs: all four members of the SLC29 family are able to transport ADO through the membrane by facilitated diffusion, whether inwardly or outwardly (see Table 2). Topology studies suggest the presence of 11 TM helices with the amino

terminal in the cytoplasm and the carboxi-terminus at the extracellular compartment. Before the identification of the genes encoding for these proteins, they were classified has equilibrative sensitive or insensitive to nitrobenzylthioinosine (NBTI). NBTI and dipyridamole are competitive inhibitors of these transporters since they compete with the endogenous ligand for the substrate binding site (reviewed by Baldwin et al., 2004; Young et al., 2013).

Table 2. Characteristics of the SLC-29 nucleoside transport family (adapted from Baldwin et al., 2004)

Protein Name	Predominant substrates	Tissue distribution	Cloning characteristics
ENT1	Purine and pyrimidine nucleosides.	Ubiquitous, plasma membrane and perinuclear membranes.	hENT1 and rENT1 are 78% identical at protein level
ENT2	Purine and pyrimidine nucleosides and nucleobases	Ubiquitous, plasma membrane. Particularly abundant in **skeletal muscle.**	hENT2 and rENT2 are 88% identical at protein level
ENT3	Purine and pyrimidine nucleosides and some nucleobases. Adenosine	Widely distributed, possibly intracellular	hENT3 and mENT3 are 74% identical at protein level.
ENT4		Widely distributed	hENT3 and mENT3 are 86% identical at protein level.

3.4 Purinoceptor subtypes

The existence of purinergic receptors was first defined 30 years ago, but only two years later a basis for distinguishing two types of purinoceptors was established, naming P1R to adenosine receptors and P2R to ATP receptors. After the finding that there were more than one receptor in each family, it was proposed that P1R should be divided into A_1, A_{2A}, A_{2B} and A_3 and that P2R should be divided into a family of seven P2X ligand-gated ion channel receptors ($P2X_1$-$P2X_7$) and another family of eight P2Y metabotropic G-coupled protein receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$) (see Table 3) (reviewed by Burnstock, 2007). At the NMJ, P1R are located on the presynaptic terminal (Correia-de-Sá et al., 1991) and on perysynaptic Schwann cells, whereas P2R

can be found in PSCs, nerve terminals and muscle fibers (reviewed by Todd and Robitaille, 2006).

Table 3. Compilation of all purinergic receptors, its intracellular signaling mechanisms and principal endogenous ligands (Burnstock et al.)

Receptor subtype	Mechanism	Principal endogenous ligand
P1 receptors		
A_1	$G_{i/o}$ – lowers cAMP	Adenosine (high-affinity)
A_{2A}	G_s – raises cAMP	
A_{2B}	G_s – raises cAMP	Adenosine (low-affinity in rodents)
A_3	$G_{i/o}$ – lowers cAMP	
P2Y receptors		
$P2Y_1$	$G_{q/11}$ – activates PLCβ; mobilizes Ca^{2+}; sometimes alters cAMP.	ADP > ATP
$P2Y_2$		UTP = ATP
$P2Y_4$		UTP > ATP
$P2Y_6$		UDP >> UTP > ATP
$P2Y_{11}$		ATP > UTP
$P2Y_{12}$	$G_{i/o}$ – lowers cAMP	ADP
$P2Y_{13}$		ADP>>ATP
$P2Y_{14}$		UDP-glucose
P2X receptors		
$P2X_1$	Receptor gated cation selective ion channels	ATP
$P2X_2$		
$P2X_3$		
$P2X_4$		
$P2X_5$		
$P2X_6$		
$P2X_7$		

3.4.1 P1 receptors (ADO receptors)

As mentioned above, four types of P1 receptors have been identified: A_1, A_{2A}, A_{2B} and A_3. They all couple to G proteins and posess seven transmembrane (TM)

hydrophobic domains, with the NH_2 terminus facing the extracellular side and the COOH terminus facing the cytoplasmic side. The third cytoplasmic loop interacts with the appropriate G protein, initiating the signaling cascade leading to a final event. These receptors also have histidine residues in TM6 and TM7 facing the extracellular milieu, which are important for ligand binding (reviewed by Burnstock, 2007). A_{2A} and A_{2B} receptors couple to G_s, activating adenylyl cyclase, which catalyzes the conversion of ATP to cAMP. A_1 and A_3 receptors, couple to $G_{i/o}$, generally inhibiting adenylyl cyclase and cAMP production, but can also facilitate outward K^+ currents leading to hyperpolarization of excitable cells (reviewed by Cobb and Clancy, 2003) (Figure 11).

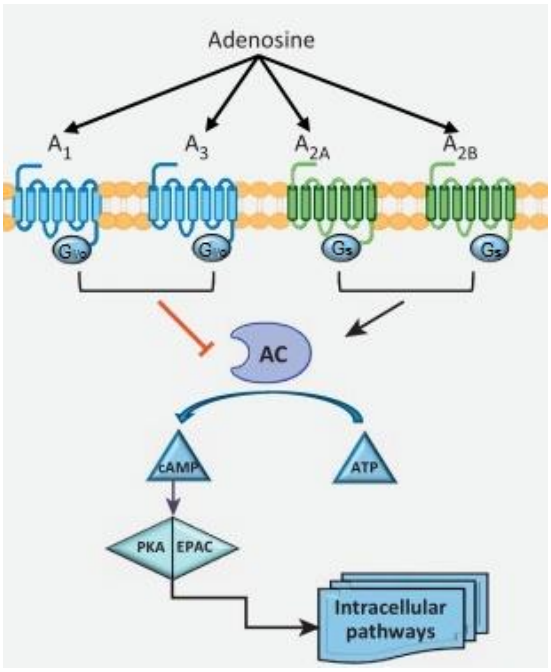

Figure 11. Illustrative image of all adenosine receptors and their intracellular coupling system. A_1R and A_3R inhibit adenylyl cyclase (AC) decreasing cAMP production, whereas $A_{2A}R$ and $A_{2B}R$ activate AC, increasing cAMP levels. All of the receptors have seven TM region as depicted (adapted from Mediero and Cronstein, 2013).

3.4.2 P2 receptors (ATP receptors)

There are two families of P2 receptors: P2X and P2Y. The seven P2X receptors ($P2X_{1-7}$) are ligand-gated cation channels with intracellular NH_2 and COOH termini. They have in common two transmembrane domains, one involved with channel gating and the second delimiting the ion pore, and a large extracellular loop containing the ATP binding site. The pore is formed by three subunits which can be equal (homomultimers) or different (heteromultimers), constituting a stretched trimer. At the peptide level, P2X receptors show 30-50% homology. Their molecular physiology is variable, since their permeability and activation/inactivation/deactivation kinetics are very different among

different receptors (reviewed by Burnstock, 2007). These receptors, like all ligand-gated ion channels, once activated are capable of increasing their permeability to cations such as Na^+, Ca^{2+} and K^+. For instance, Na^+ and Ca^{2+} are allowed to enter the cell and K^+ to leave the cell into the extracellular medium, mediating thus the P2XR effects (Figure 12).

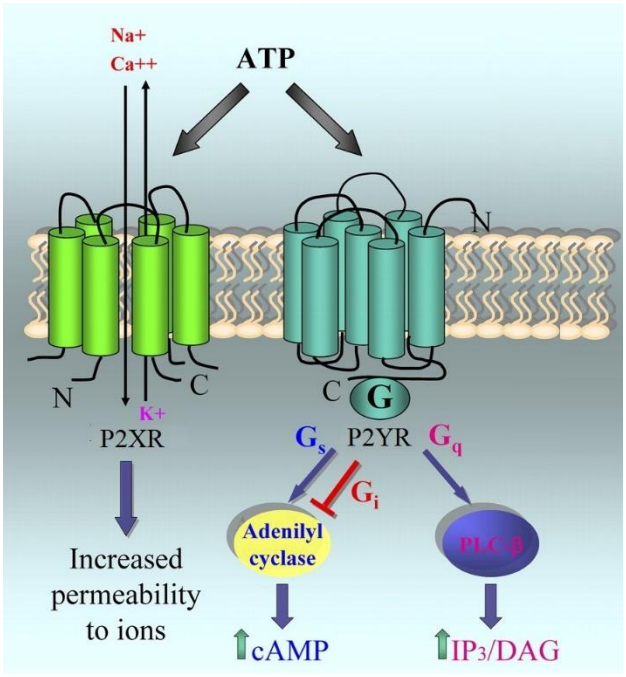

Figure 12. Prototypic representation of P2XR and P2YR and their intracellular transduction pathways. P2XR, which is formed by 3 peptide subunits of 2 transmembrane domain each, forms a pore that once activated by ATP increases it's permeability to cations, allowing sodium (Na^+) and calcium (Ca^{2+}) to flow into the cell and potassium (K^+) to flow out of the cell. P2YR, 7 membrane spanning, G-protein coupled receptors, once activated by ATP may induce different responses according to receptor type (adapted from Vitiello et al., 2012).

There are 8 P2YR subtypes: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$). Structurally, P2YR are characterized by an extracellular NH2 terminus and an intracellular COOH terminus containing consensus binding motifs for protein kinases. As all of them are G-protein-coupled receptors, they have seven membrane-spanning domains, which help to form the ligand-docking pocket. In fact, site mutagenesis of the $P2Y_1$ and $P2Y_2$ receptors have shown that there are positively charged residues in TM 3, 6 and 7 which are crucial for receptor activation by nucleotides (Erb et al., 1995; Jiang et al., 1997). These positively charged residues probably interact with the negative charges of nucleotides phosphate groups, since its ligands are nucleotides uncomplexed to magnesium or calcium (reviewed by Abbracchio et al., 2006). Regarding the intracellular loop, the structural diversity influences the coupling to different G proteins: $G_{q/11}$ couples to $P2Y_{1,\ 2,\ 4,\ 6}$, $G_{i/o}$ couples to $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$, and the $P2Y_{11}$ may couple to $G_{q/11}$ or G_s. Once activated, the receptors coupled to G_q protein lead to the

stimulation of phospholipase C followed by increases in inositol phosphates and mobilization of Ca^{2+} from intracellular stores. Besides G_q, the $P2Y_{11}$ receptor is also able to couple to G_s, and once activated it is the only P2 receptor subtype capable of activating adenylyl cyclase activity and increasing cAMP production. The receptors coupled to $G_{i/o}$ inhibit adenylyl cyclase activity and decrease cAMP levels. The pharmacological profile of P2 purinoceptor subtypes differ substantially. Broad differences in ligand specificity can be explained by the fact that they show little peptide homology (19-55%). $P2Y_1$, $P2Y_6$, $P2Y_{12}$ and $P2Y_{13}$ are mainly activated by nucleoside diphosphates, which is UDP instead of ADP in the case of the $P2Y_6$ receptor. $P2Y_2$ and $P2Y_4$ are activated primarily by nucleoside triphosphates, ATP and UTP, respectively. Some P2Y receptors are activated by both purine and pyrimidine nucleotides, such as $P2Y_2$, $P2Y_4$ and $P2Y_6$. $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors are activated only by purine nucleotides, in particular ADP. The $P2Y_{14}$ receptor is activated by UDP-glucose (reviewed by Burnstock, 2007) (see Table 3).

3.5 Purinergic modulation of neuromuscular transmission

Both adenine nucleotides and adenosine are able to modulate transmitter release via the activation of presynaptic P2R (Giniatullin and Sokolova, 1998) and P1 (Correia-de-Sá et al., 1991) receptors, respectively.

In 1991, it was demonstrated that adenosine exerts a dual neuromodulatory role at the rat NMJ, it can either inhibit via A_1R or facilitate via $A_{2A}R$ the release of ACh from stimulated nerve terminals (Correia-de-Sá et al., 1991). The way adenosine builds its influence to control neurotransmitter release at the NMJ depends on the concentration of the nucleoside at the synapse. At low concentrations (10-100 µM), adenosine inhibits ACh release through the activation of A_1R, while at concentrations higher than 100 µM, the nucleoside facilitates the transmitter release by acting at $A_{2A}R$ (Correia-de-Sá and Ribeiro, 1996). The amount of adenosine accumulated at the NMJ depends on paradigm of nerve stimulation (frequency, duration of pulses and train length). During periods of low stimulation frequencies (*e.g.* 5 Hz, 40 µs pulse duration), the amount of adenosine generated at the synapse is only sufficient to activate inhibitory A_1R, but upon increasing the duration of the pulses (to 1 ms) or the stimulation frequency (to 50 Hz) adenosine levels increase reaching concentrations high enough to activate facilitatory $A_{2A}R$ (Correia-de-Sá et al., 1996). As mentioned above, the rate limiting enzyme for adenosine formation from released adenine nucleotides is ecto-5'-nucleotidase, which can be feed-forwardly inhibited by high levels of ATP and ADP (Magalhães-Cardoso et al., 2003). This may explain why high-frequency stimulation trains (50 Hz, 500 pulses) led to the

accumulation of smaller amounts of adenosine at the neuromuscular synapse and, thus, less facilitation of ACh release due to A_{2A} receptors activation, than high-frequency bursts (50 Hz, 5 trains of 100 pulses, with 20s intertrain interval). Massive ATP accumulation during high-frequency trains must dissipate, at least partially, before adenosine can be formed in sufficient amounts by the ecto-5'-nucleotidase. This feature clearly suggests that the effects mediated by nucleotide-sensitive P2 receptors do not coincide with adenosine A_{2A} receptor-mediated facilitation of neuromuscular transmission. This theory is supported by the finding that adenosine A_{2A} receptor is preferentially activated by adenosine formed from the extracellular catabolism of released nucleotides by ecto-5'-nucleotidase (Cunha et al., 1996), while tonic A_1 receptor-mediated inhibition of ACh release results, most probably, from the activity of adenosine released as such via equilibrative nucleoside transporters (Correia-de-Sá and Ribeiro, 1996).

Thus, adenosine acts predominantly as an inhibitory signal (via A_1 receptors) under resting conditions, but it can stimulate transmitter release during high-frequency long-lasting stimuli through the activation of facilitatory A_{2A} receptors. The inhibitory action of the A_1 receptor is mostly due to the direct inhibition, via G proteins, of P/Q-type calcium channels that are responsible for Ca^{2+}-dependent transmitter exocytosis in resting conditions (Silinsky, 2004). Conversely, activation of A_{2A} receptors by adenosine operates a coordinated shift in Ca^{2+} channel dynamics leading to facilitation of ACh release, from the "prevalent" $Ca_v2.1$ (P-type) to the "facilitatory" Ca_v1 (L-type) channel (Correia-de-Sá et al., 2000) which may contribute to overcome tetanic depression during high-frequency neuronal firing (Oliveira et al., 2004). Using a toxicological rat model of MG our group observed a significant impairment on the adaptive shift from fast-inactivating $Ca_v2.1$ (P-type) channels to high-capacity long-lasting Ca_v1 (L-type) channels, which may contribute to tetanic failure of neurotransmission in myasthenics (Noronha-Matos et al., 2011). Additionally, data from the same study demonstrate that endogenous adenosine generated in myasthenic motor endplates during repetitive nerve firing was insufficient to activate facilitatory A_{2A} receptors, but this situation could be overcome using the adenosine nucleotide precursor, AMP, whose activity that does not interfere with the ecto-5'-nucleotidase enzymatic performance (Noronha-Matos et al., 2011). Interestingly, similar results were also obtained with an EAMG rat model (Oliveira et al., 2015).

Although the role of ATP at the NMJ is better documented by its action on P1R after its breakdown into adenosine, ATP is also capable of modulating the neuromuscular transmission directly through the activation of P2R (Giniatullin and Sokolova, 1998;

Guarracino et al., 2016). Both facilitatory and inhibitory actions of ATP via P2 receptors have been identified at the NMJ.

The facilitatory role of ATP on neurotransmitter release has been attributed to the activation of P2X7-like receptors (Moores et al., 2005; Salgado et al., 2000). This was hypothesized because this receptor subtype exhibits high Ca^{2+} permeability which may contribute to facilitate ACh release. Likewise, facilitation of transmitters release operated by ATP via P2X receptors have also been found in *Xenopus* developing neuromuscular synapses (Fu et al., 1997), chicken ciliary ganglia (Sun and Stanley, 1996) and mouse motor nerve terminals (Hong and Chang, 1998).

The inhibitory role of ATP has been assigned to P2Y receptors in the frog (Giniatullin and Sokolova, 1998) and the mouse (De Lorenzo et al., 2006; Galkin et al., 2001) NMJs. At the frog NMJ, the inhibitory action of ATP was blocked by pertussis toxin and N-ethylmaleimide, suggesting that this effect is mediated by P2Y receptors coupled to $G_{i/o}$, but paradoxically the authors ascertain that the ATP effect was dependent on PC-PLC (phosphatidylcholine specific PLC) and PKC (Sokolova et al., 2003). At the mouse NMJ, both ATP and its stable analog, β,γ-imido ATP, were able to decrease spontaneous secretion of the neurotransmitter by reducing Ca^{2+} entry through L-type and N-type voltage-gated channels (De Lorenzo et al., 2006). Recently, the Giniatullin's group presented results suggesting that ATP inhibits ACh release from motor nerve terminals of the frog via the activation of $P2Y_{12}$ receptors, involving NADPH oxidase activity and lipid rafts (Giniatullin et al., 2015). Species differences may determine the P2Y receptor subtype predominantly involved in the inhibitory role of ATP because the $P2Y_{13}$ receptor subtype has been recently at mouse NMJ (Guarracino et al., 2016). Thus, this contention is far from being resolved prompting for new and more accurate studies to determine which receptor subtypes are present at the motor endplate and in order to clarify their role in the control of neuromuscular transmission both in healthy tissues and in pathological conditions, like MG.

Chapter II

Aim

Recently, our group demonstrated a disparity between the amounts of endogenous ATP (Neves, 2015, personal communication) and adenosine accumulation (Oliveira et al., 2015) in EAMG animals. In myasthenic animals, the amount of ATP released during electrical stimulation of the rat phrenic nerve is increased compared to control preparations (Neves, 2015, personal communication), but this is not reflected in higher adenosine accumulation at the myasthenic neuromuscular synapse; on the contrary, stimulation of the phrenic nerve released smaller amounts of adenosine in myasthenic hemidiaphragms than in control preparations, while no changes were observed between the two groups regarding baseline levels of the nucleoside (Oliveira et al., 2015). This feature explains, at least in part, why the A_{2A}-receptor-mediated facilitation of ACh release from stimulated motor nerve terminals is deficient in EAMG rats increasing the probability of neuromuscular transmission failure (Oliveira et al., 2015).

Interestingly, rehabilitation of the A_{2A}-receptor-mediated facilitation of transmitter release was observed by incubating the myasthenic preparations with the adenosine precursor, AMP, which is the substrate of ecto-5'-nucleotidase. This suggests that if a deficiency in the conversion of ATP into adenosine exists it must be upstream the ecto-5'-nucleotidase. To elucidate this possibility, we evaluated the extracellular catabolism of ATP and ADP in phrenic nerve-hemidiaphragm preparations isolated from healthy and EAMG rats by RP-HPLC-UV.

To our knowledge, no studies have been made to investigate the role of adenine nucleotides in conditions exhibiting neuromuscular transmission deficits, like those verified in MG. This prompted us to characterize the role of ATP and the P2 receptor subtype(s) involved in the control of ACh release induced by phrenic nerve stimulation in healthy and EAMG rats, excluding the effect mediated by ATP conversion into adenosine using enzymatically-stable nucleotide analogues and a strategy that allows to assess the effect of only ATP and its active metabolite ADP.

Chapter III

Materials and Methods

1. Induction and Clinical assessment of EAMG

Female Wistar Han rats with approximately 100 g (about 4/5 weeks of age) (Charles River, Barcelona, Spain), kept at a constant temperature (21°C) and a regular light (06.30–19.30 h) - dark (19.30–06.30 h) cycle, with food and water *ad libitum*, were randomly divided into two groups CFA (control), and EAMG. The procedure was carried out under general anesthesia by intraperitoneal injection of ketamine (75 mg/kg) and medetomidine (100 mg/kg). EAMG rats were immunized by subcutaneous injection with 50 µg of the immunogenic peptide R97-116 (DGDFAIVKFTKVLLDYTGHI, JPT Peptide TechnologiesGmbH), a synthetic peptide comprehending the aminoacidic sequence between positions 97 and 116 from the α subunit of the rat nicotinic AChR made up in Complete Freund's Adjuvant (CFA) (Sigma, St Louis, MO, USA). CFA is composed of paraffin oil containing mannide mono-oleate as a surfactant and heat-killed mycobacteria and it is used as an adjuvant to the induction protocol because it helps to extend the lifetime of injected autoantigens, stimulates its effective delivery to the immune system and provides a set of signals to the innate immunity that result in altered leukocyte proliferation and differentiation (reviewed in Billiau & Matthys, 2001). Injections were performed at day 0 and boosted on day 30 with the same peptide dissolved in Incomplete Freund's Adjuvant (IFA). IFA is essentially composed by the surfactant. Control animals were only injected with CFA (or IFA in boost case) and phosphate buffer solution (PBS) instead of the peptide. Evaluation of disease manifestations in immunized rats was performed by testing muscular weakness. Clinical scoring was based on the presence of tremor, hunched posture, general behavior, fatigability and the overall appearance of the animal. Muscle strength was assessed by the grip strength test (Bioseb, France) and fatigability was evaluated after exercise (repetitive paw grips on the cage grid). Disease severity was graded as follows: grade 0, normal strength and no fatigability; grade 1, mildly decreased activity and weak grip or cry; grade 2, clinical signs present at rest; grade 3, severe clinical signs at rest, no grip, moribund; and grade 4, dead (Baggi et al., 2004).

1.1 Evaluation of animal health conditions

It is known that within days after the subcutaneous injection of the immunogenic peptide R97-116, a strong and long-lasting inflammatory reaction appears at the site of injection and in the draining lymph nodes, demonstrating that the inflammatory process has been initiated (reviewed in Billiau and Matthys, 2001). Thus, after the induction, each animal was weighted and evaluated for wellbeing and disease manifestations twice a week, or even more, until sacrifice on day 42, depending on the degree of discomfort

presented by the animals. Evaluated parameters included general appearance (anatomical changes, dehydration, wounds, hypothermia, hyperthermia, pain and alopecia), fur, skin and eyes appearance (granulomas, lack of fur maintenance, slight chromodacryorrhea), footpad appearance (anatomical alterations, edema, erythema, heat, pain and wound) and spontaneous and induced behavior (grooming, curiosity, mobility, state of alertness, aggressiveness, tremors, and vocalization).

Animal handling and experiments were in accordance with the guidelines prepared by Committee on Care and Use of Laboratory Animal Resources (National Research Council, USA) and followed the European Communities Council Directive (86/609/EEC). All animals were euthanized by decapitation. Then, they were submitted to surgical isolation of the phrenic nerve hemidiaphragm as described below. Each muscle was superfused (5 mL/min, 37°C, pH 7.4) with gassed (95% O2; 5% CO2) Tyrode's solution (pH 7.4) containing (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1, NaH_2PO_4 0.4, $NaHCO_3$ 11.9 and glucose 11.2, at 37°C.

2. Isolation of the phrenic nerve-hemidiaphragm

After sacrifice of the animals by decapitation, a thoracotomy was preformed which allowed the identification and dissection of both right and left phrenic nerves until their insertion into ipsilateral hemidiaphragms. The parietal pleura that covers the hemidiaphragm was carefully removed to facilitate muscle isolation. These procedures were performed rapidly while post mortem cardiac reflex contractions were still present, always keeping the preparation humidified with gassed Tyrode's Solution heated at 37 °C. After its removal from the animal body, the innervated hemidiaphragm was then put into a dissection plate (Figure 13 A) filled with gassed Tyrode's solution to isolate the portion of the diaphragm presenting more nerve terminals. After its identification, the skeletal muscle fibers were cut tangentially at a distance of 3-4 mm from both sides of the phrenic nerve insertion into the muscle (Figure 13 B).

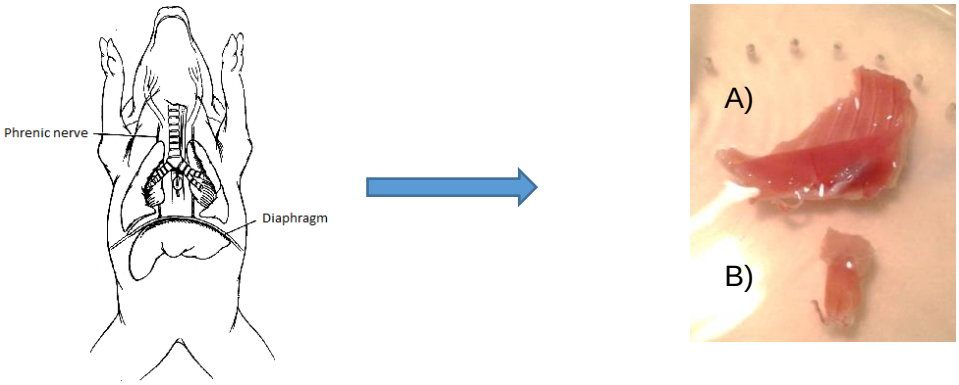

Figure 13. Scheme of the anatomy of both left and phrenic nerves innervating the rat diaphragm. After thoracotomy, the left phrenic nerve is easily seen and isolated, whereas the right phrenic nerve is located beneath the lung and requires more attention whilst performing the dissection. After cleaning out the parietal pleura from the phrenic nerve and diaphragm, the diaphragmatic region with the two phrenic nerves was separated from the body of the animal (a). Then the section of each hemidiaphragm mostly enriched with cholinergic terminals is isolated by tangential cut of the muscle, leaving the preparation looking like (b).

3. Kinetics of the extracellular catabolism of adenine nucleotides

Phrenic nerve-hemidiaphragm preparations from CFA and EAMG rats were mounted in a horizontal organ chamber (2 mL capacity) and pinned to a thin Sylgard® layer with chirurgical pins so that optimal muscular tension is reached. Preparations were heated to 37°C through the circulation of heated water pumped from a thermostatized bath (SBS®) into the compartment beneath the preparation. The preparation was continuously superfused with gassed Tyrode's solution with the help of by a peristaltic pump (Gilson Maniplus3) (Figure 15).

Washout	Equilibrium	Control	Washout	Kinetic	Washout	Control
15 mL/min	5 mL/min		5 mL/min		5 mL/min	
5 min	30 min	45 min	15 min	45 min	15 min	45 min
				ATP/ADP 30 µM		

Figure 14. Protocol used in the experiments to evaluate the kinetics of the extracellular catabolism of adenine nucleotides.

After mounting the preparations in the organ bath, they were washed out of cell debris and were warmed with physiological Tyrode's solution during 5 min with a flow rate of 15 mL/min (Figure 14). Next, the preparations were equilibrated for 30 minutes with the physiological Tyrode's solution (5 mL/min flow rate), which allowed the preparations to adjust to the new experimental conditions. The first control kinetic was initiated by

incubating the preparations with 2 mL of Tyrode's solution for 45 minutes. Bath samples are collected at given time-points (0, 1, 2, 5, 10, 15, 30 and 45 min) and were snap-frozen in liquid nitrogen before storage at –80°C until analysis by HPLC with UV detection (HPLC-UV, LaChrome Elite, Hitachi, Merck, Germany). Before the test kinetic, a 15-min washout period was performed where the preparations were superfused with Tyrode's solution at a 5 mL/min flow rate. The test kinetic was performed as the first control kinetic, but in this case the nucleotide substrate (ATP or ADP) was added to the Tyrode's solution. A second control kinetic in the absence of added adenine nucleotides was performed at the end of the experimental protocol to ensure that damage of cells during the experimental period was not affecting the results by contamination with intracellular enzymes.

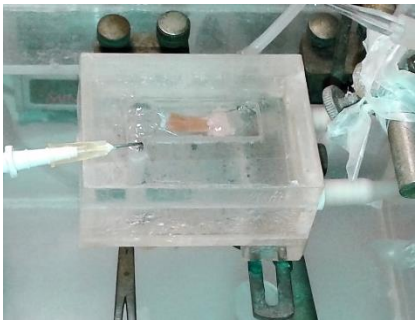

Figure 15. Phrenic nerve-hemidiaphragm preparation mounted in a horizontal organ bath where the enzymatic kinetic assays were performed.

Neuromuscular preparations time-dependently release spontaneously IMP, inosine (INO) and hypoxanthine (HX) in concentrations up to 1 µM (Magalhães-Cardoso et al., 2003). Therefore, there is a need to discount in each experimental procedure the spontaneous release of these purine metabolites. To circumvent this problem, we performed two control kinetics, one before and another after the kinetic where we tested the extracellular catabolism of ATP and ADP. The interpolated (average) values of IMP, INO and HX calculated from the two control kinetics were discounted from the concentrations determined at corresponding time-points in the test kinetic (Magalhães-Cardoso et al., 2003). The concentration of the substrate and the metabolic products were plotted as a function of time (progress curves). The following parameters were analyzed for each progress curve: half-life time ($t_{1/2}$) of the initial substrate and time of appearance of the highest product concentration.

4. Separation and quantification of adenine nucleotides and its metabolites by HPLC

4.1 Instruments

Separation and quantitative analysis of purines in collected samples was performed using a chromatographic LC/UV system composed by a quaternary gradient pump (L-2130, Elite LaChrom), equipped with a manual injection valve (Rheodyne 7725) with 20 µL and 200 µL loops, coupled to a UV detector (L-2400, Elite LaChrom), where the wave length was held at 254 nm. This chromatographic system was coupled to a software program (EZ-ChromElite, VWR international) for data processing (recording and quantitative analysis of chromatograms). The separation was realized at room temperature with a reverse phase, 12.5 cm long and 4 mm internal diameter column made up with 5 µm diameter particles (LiChrospher 100 RP-18, Merck).

4.2 Separation process

Separation of all metabolites was carried out by reverse-phase chromatography with an elution gradient. The eluents were composed of (100 mM) KH_2PO_4, pH7 (Solution A) and (100 mM) KH_2PO_4 with 30% (v/v) methanol (Solution B). The elution program was as follows: a linear elution of solution A during 4 min followed by an increasing gradient of solution B over a period of 10 min. At 14 min, the gradient reaches 100% of solution B. To reestablish the initial elution conditions of absorbance values and internal pressure of the system, the gradient was decreased for 8 min, reaching 100% of solution A at the end of 22 min. The mobile phase flow was 1.25 mL/min. Chromatographic identification of metabolites in samples was made by comparison with the retention time of high purity standard solutions under the same chromatographic conditions. Typical retention times were as follows: IMP (1.4 min), ATP (2 min), ADP (2.2 min), AMP (2.7 min), HX (3.4 min), INO (8.5 min) and ADO (11.4 min) (Figure 16).

Some of the samples were also separated and analyzed by ion pair-reversed phase chromatography (IP-RP-HPLC) in order to obtain a better resolution in separation of adenine nucleotides (according to Pinheiro et al., 2013). The retention times of all metabolites using this separation method was: HX (1.2 min), INO (1.7 min), IMP (2.4 min), ADO (3.6 min), AMP (4.5 min), ADP (6.6 min), ATP (8.1 min).

4.3 Quantification Process

The chromatograms were obtained after the injection of 25µL of either standard solutions or collected samples. Both were quantified through the external standard method (Cassiano et al., 2009). For each component of the mixture (ATP, ADP, AMP, IMP, ADO, INO, HX) a calibration curve was prepared (graph peak area versus concentration) with a linear slope within the expected range of concentrations for each compound, and interception with the ordinate axis at zero or near zero. The concentration of each compound of the sample was determined through the mathematical expression of the straight lines calibration (y= mx+b), given that it is proportional to the analytical signal (area) since the injected volumes are accurately known. This method requires a strict control of technical and instrumental conditions (separation conditions, mobile phase flow, and injection volume) to obtain the calibration curves of the compounds (ATP, ADP, AMP, IMP, ADO, INO, HX) used in the adenine nucleotides/nucleosides quantification. Standard solutions of the compounds were injected (25 µL) with increasing concentrations (1.9µM – 30µM).

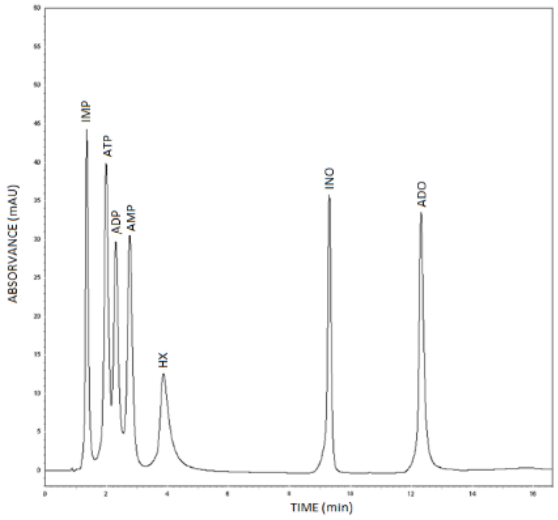

Figure 16. RP-HPLC-UV chromatogram illustrating the separations

4.4 Half-life time ($t_{1/2}$) determination

The half-life time ($t_{1/2}$) expresses the period of time required for the amount or concentration of the compound to decrease by one-half.

In the presence of a first-order kinetics, the graphical representation is linear [log (concentration) = f (time)].

The mathematical expression of the line is given by the following equation (Shargel and Yu, 1982):

$$logA = -\frac{Kt}{2,3} + logA_0$$

(A: compound concentration; $-\frac{K}{2,3}$: slope of the straight; A_0: y-axis interception; t: time)

The half-life time of this equation results on:

$$T_{1/2} = \frac{0,693}{K}$$

K is a constant expressed as time^{-1} (-K= slope of the straight × 2,3).

5. [^{3}H]-ACh release experiments

The procedures used for labeling the preparations and measuring evoked [^{3}H]-ACh release have been previously described (Correia-de-Sá et al., 1991; 1996; Wessler and Kilbinger, 1986) and were used with minor modifications (Figure 17).

5.1 Setting up the preparation in the organ bath

Phrenic nerve-hemidiaphragm preparations were mounted in 3-mL capacity thermostatized organ bathes; muscles stretched to approximately 1.1 times its resting size were fixated with 4 surgical pins to a thin Sylgard® layer coating the bottom of organ bathes. Four preparations were used in parallel. Each phrenic nerve was inserted in a suction electrode and the integrity of the preparation was tested by electrical stimulation of the phrenic nerve trunk with 1 Hz frequency, which must trigger readily observable muscular contractions.

5.2 Experimental period

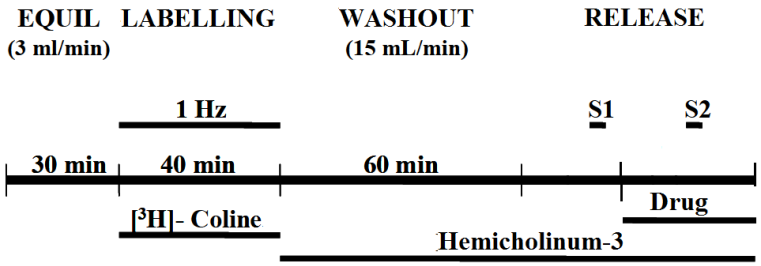

Figure 17. Illustration of the experimental protocol for measuring [^{3}H]-ACh release.

5.2.1 Equilibrium

To allow the preparations to adapt to the new experimental conditions (stretch and medium composition) and to eliminate tissue debris resulting from surgical manipulation, the preparations were equilibrated for 30 min under continuous superfusion with warmed (37ºC) Tyrode's solution at a constant flow rate of 3 mL/min.

5.2.2 Labeling

Nerve terminals were loaded with [^{3}H]-Choline (2,5 µCi/nmol) for 40 min. During this procedure, the phrenic nerve trunk was stimulated electrically with supramaximal rectangular pulses of 40-µs duration delivered at 1-Hz frequency to allow the release of unlabeled ACh and the incorporation of newly synthesized [^{3}H]-ACh into the synaptic vesicles.

5.2.3 Washout

Washout of the preparations was performed for 60 min, by superfusion (15 mL/min) with Tyrode's solution supplemented with hemicholinium-3 (10 µM), which remained present in the incubation/superfusion solutions from this onwards. Hemicholinium-3 is a high-affinity choline uptake inhibitor that is required to prevent re-synthesis of unlabeled ACh, thus increasing the signal-to-noise ratio of [^{3}H]-ACh being measured in the incubation fluid following electrical stimulation of the phrenic nerve.

5.2.4 Release

After the washout, superfusion of the preparations was stopped and the collecting period started at zero time. During the release period, samples were collected every 3 min with the help of a peristaltic pump (Minipuls 3; Gilson) coupled to a programmable fraction collector (FC 203B; Gilson). Refilling of the organ chamber was performed manually with the adequate solution. 400 µL-aliquots of each collected sample were added to 3.5 mL of scintillation cocktail (Packard Insta Gel II) to allow measurement of of the radioactivity released in each time point.

5.2.5 Stimulation conditions

The release of [^{3}H]-ACh was evoked by electrical stimulation of the phrenic nerve with 750 rectangular pulses of 40 µs duration and 8 mA intensity delivered at 5-Hz frequency. Two stimulation periods were used: at 12 min (S1) and at 39 min (S2) after the end of the washout period (time zero). Electrical stimulus were delivered using two

Grass S48 stimulators coupled to stimulus isolation units (Grass SIU5) operating in a current constant mode.

5.2.6 Released [^{3}H]-ACh quantification

Tritium content in the collected samples was quantified by liquid scintillation spectrometry (Beckman, LS3801) (% counting efficiency = 40±2%) after appropriate background subtraction. The evoked release of [^{3}H]-ACh was calculated by subtracting the basal tritium outflow from the total tritium outflow during the stimulation period. The change in the ratio between the evoked [^{3}H]-ACh released during the two stimulation periods (S2/S1) relative to that observed in control situations (in the absence of test drugs) was taken as a measure of the effect of the tested drugs. The positive values represent facilitatory effects and the negative values correspond to inhibitory effects on evoked [^{3}H]-ACh release.

6. Drugs and Solutions

ATP, ADP, AMP, ADO, IMP, INO, HX, picA reagent (Tetramethylammonium bromide), potassium phosphate (pro HPLC) and β,γ-imido ATP were obtained from Sigma (St. Louis, MO, USA). MRS 2211 and MRS 2365 were purchased to Tocris Bioscience (Bristol, UK). MRS 2179 was purchased to Abcam (Cambridge, UK). The scintillation cocktail (Insta-gel Plus) was acquired from Perkin Elmer (Boston, USA) and the R97-116 peptide (DGDFAIVKFTKVLLDYTGHI) was obtained from JPT Peptide Technologies GmbH. All stock solutions were made up in water and stored as frozen aliquots at -20ºC. Dilutions of these stock solutions were made daily.

Chapter IV

Results and Discussion

1. Kinetics of the extracellular catabolism of adenine nucleotides (ATP and ADP) in the diaphragm of control and EAMG rats.

Recently, our group demonstrated a disparity between the amounts of endogenous ATP (Neves, 2015, personal communication) and adenosine accumulation (Oliveira et al., 2015) in EAMG animals. In myasthenic rats, the amount of ATP released during electrical stimulation of the rat phrenic nerve was increased compared to control preparations (Neves, 2015, personal communication), but this was not reflected in higher adenosine accumulation at the myasthenic neuromuscular synapse; on the contrary, stimulation of the phrenic nerve released smaller amounts of adenosine in myasthenic hemidiaphragms than in control preparations, but no changes were observed between the two groups regarding baseline levels of the nucleoside (Oliveira et al., 2015). Despite these features one could restore the A_{2A}-receptor-mediated facilitatory tone of transmitter release in EAMG rats using the adenosine precursor, AMP, which is the substrate of ecto-5'-nucleotidase. Thus, if a deficiency in the conversion of ATP into adenosine exists it must be upstream the ecto-5'-nucleotidase. To elucidate this possibility we evaluated the extracellular catabolism of ATP and ADP in phrenic nerve-hemidiaphragm preparations isolated from control and EAMG rats by HPLC.

No significant differences ($P>0.05$) were observed in the half-degradation time of ATP (30 µM) between EAMG (10±1 min, *n*=4) and control (10±3 min, *n*=4) animals. We also found no significant differences ($P>0.05$) in the in the half-degradation time of extracellular ADP (30 µM) among EAMG (10±1 min, *n*=4) and control (10±1 min, *n*=4) animals. Results show that the half-degradation times of the two adenine nucleotides, ATP and ADP (30 µM), were very similar in hemidiaphragms of control and EAMG rats.

Extracellular ATP (30 µM) was metabolized sequentially into ADP, AMP, ADO, INO and hypoxanthine in both EAMG and control animals (Figure 18 B, C, D, F and G). No changes were detected in the formation rate of ATP metabolites among control and EAMG animals. During the first 5 min, ATP was preferentially converted into ADP (Figure 18 B) without any significant formation of AMP (Figure 18 C) and its metabolites, ADO (Figure 18 D), INO (Figure 18 F) and hypoxanthine (Figure 18 G). ADP accumulation in the incubation fluid as a consequence of the extracellular ATP catabolism progressively increased during the first 5 min followed by a plateau lasting from 5 to 15 min and decreased thereafter (Figure 18 B).

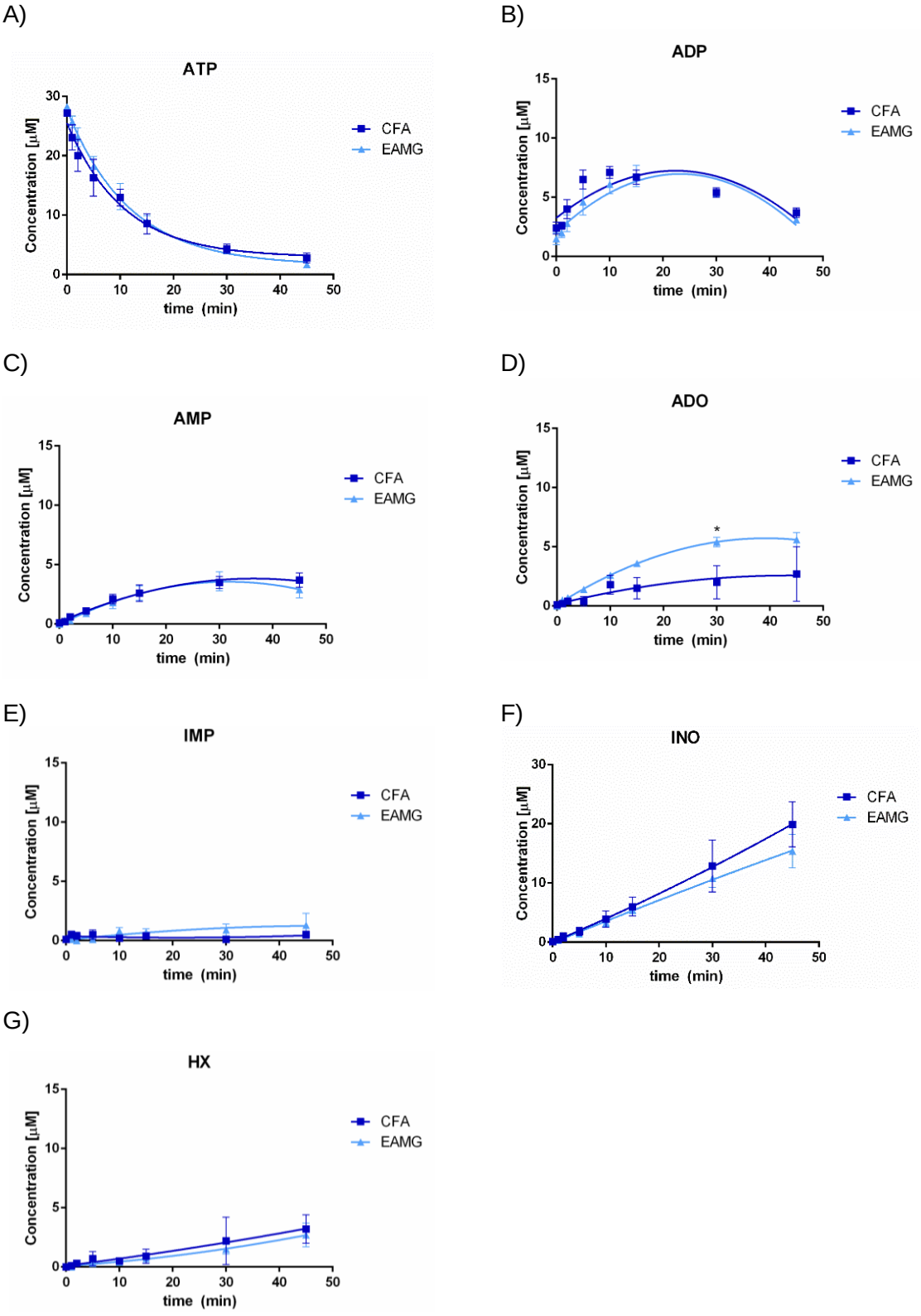

Figure 18. Time-course of extracellular ATP (30 µM) catabolism in phrenic nerve hemidiaphragm preparations from control (CFA) and EAMG animals. ATP (30 µM) was added at zero time and samples were collected at times 0, 1, 2, 5, 10, 15, 30, 45 and the abscissa represents the time scale. Quantification of ADP and all other metabolites was performed by HPLC analysis. (A) ,(B), (C), (D), (E), (F) and (G) shows the kinetics of extracellular ATP, ADP, AMP, ADO, IMP, INO and HX accumulation respectively. The number of experiments is 4 to EAMG and CFA animal groups. The results are expressed as mean ± SEM and are shown when they exceed the symbols in size. Statistical analysis of two-way ANOVA followed by Bonferroni's multiple comparison test, was performed.

The decrease in the extracellular concentration of ADP corresponds to its conversion sequentially into AMP, ADO, INO and hypoxanthine, as can be appreciated by the analysis of Figures 18 C, D, F e G. Following the extracellular ADP (30 µM) catabolism in hemidiaphragm preparations of both animal groups (Figure 19) there is a transient accumulation of AMP reaching approximately 5 µM during the first 10-15 min, which concentration decreased thereafter (Figures 19 B) leading to a progressive accumulation of adenine nucleosides (ADO, INO and hypoxanthine) towards the end of the incubation period (45 min) (see Figures 19 C, E and F). Interestingly, no differences were found in the accumulation pattern of IMP in the incubation fluid during the entire experimental period, between control and EAMG diaphragms (Figure 18 E and 19 D). This result indicates that ecto-AMP deaminase activity is not changed in myasthenic animals and, hence, adenosine formation deficits cannot be attributed to AMP deamination into IMP bypassing ADO generation by ecto-5'-nucleotidase (Magalhães-Cardoso et al., 2003).

The activities of ecto-ATPase and ecto-ADPase calculated by the ratios [ADP+AMP+IMP+nucleosides]/[ATP] per minute and [ADP+AMP+IMP+nucleosides]/[ADP] per minute using ATP (30 µM) and ADP (30 µM), respectively, as substrates are shown in Figure 20. We found no significant ($P>0.05$) differences in the extracellular catabolism of adenine nucleotides by these two ecto-enzymes in hemidiaphragms isolated from control and EAMG rats.

The results show here for the first time that ATP dephosphorylation into ADP and its subsequent hydrolysis into AMP are not significantly modified in the diaphragmatic neuromuscular junction of myasthenic rats compared to control littermates. Taking this into account and the results obtained previously by our group demonstrating that the activity of the ecto-5'-nucleotidase (CD73), which is the rate limiting enzyme for adenosine formation from the catabolism of adenine nucleotides at the rat neuromuscular junction (Magalhães-Cardoso et al., 2003), was also not affected in myasthenic animals (Oliveira et al., 2015), led us to conclude that the ecto-nucleotidase cascade is fairly conserved in myasthenics and, hence, should not be directly implicated in adenosine neuromodulation deficits detected in these animals (Neves, 2015, personal communication). Furthermore, alternative AMP deamination into IMP bypassing adenosine formation by ecto-5'-nucleotidase (Magalhães-Cardoso et al., 2003) may also not explain the deficit in adenosine formation in myasthenic rats because, under the present experimental conditions, the activity of AMP deaminase was irrelevant.

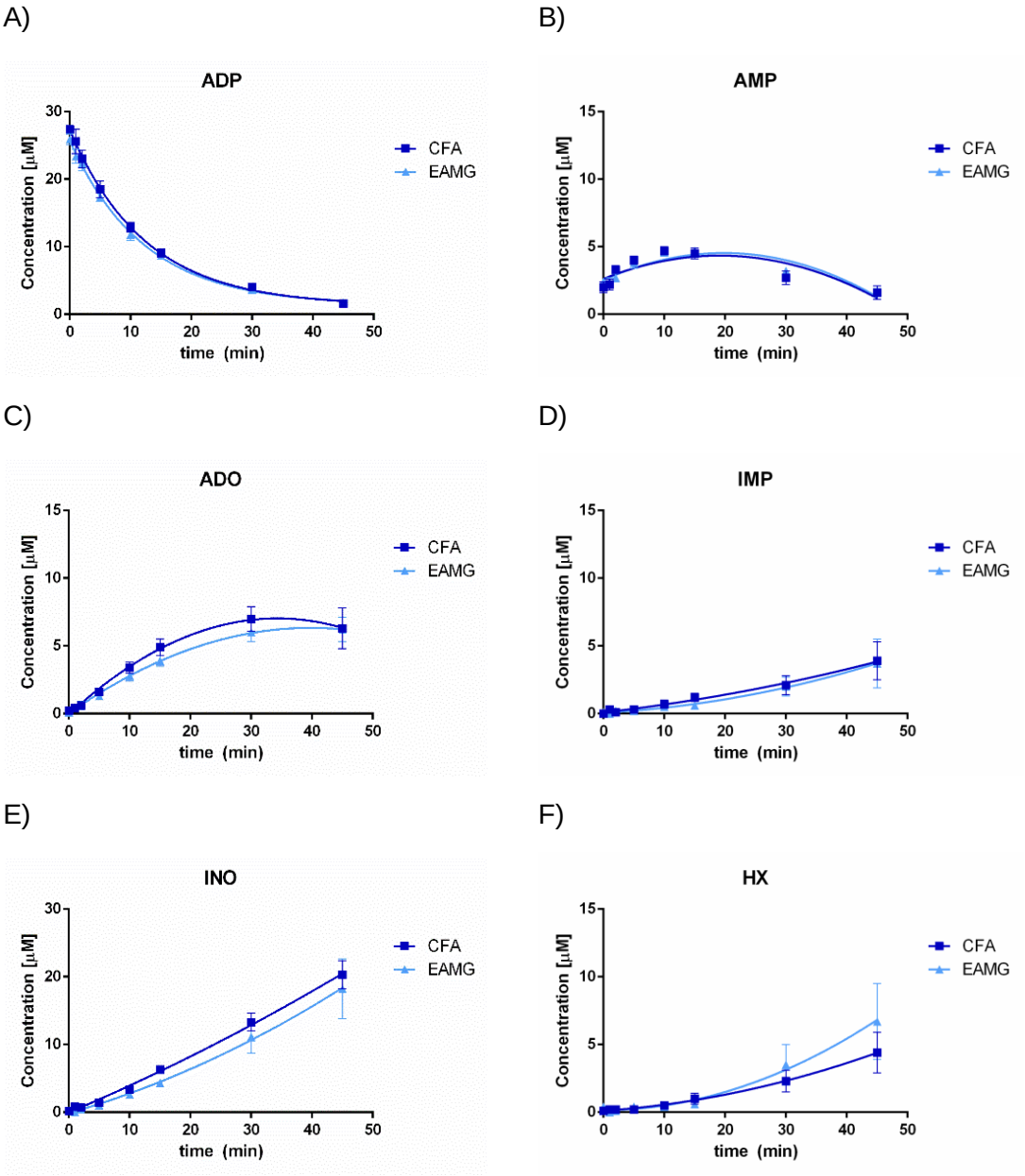

Figure 19. Time-course of extracellular ADP (30 µM) catabolism in phrenic nerve hemidiaphragm preparations from control (CFA) and EAMG animals. ADP (30 µM) was added at zero time and samples were collected at times 0, 1, 2, 5, 10, 15, 30, 45 and the abscissa represents the time scale. Quantification of AMP and all other metabolites was performed by HPLC analysis. (A) ,(B), (C), (D), (E) and (F) pictures depict the kinetics of extracellular ADP, AMP, ADO, IMP, INO and HX accumulation respectively. The number of experiments is 4 to EAMG and CFA animal groups in both ADP and ATP experiments. The results are expressed as mean ± SEM and are shown when they exceed the symbols in size. Statistical analysis of two-way ANOVA followed by Bonferroni's multiple comparison test, was performed.

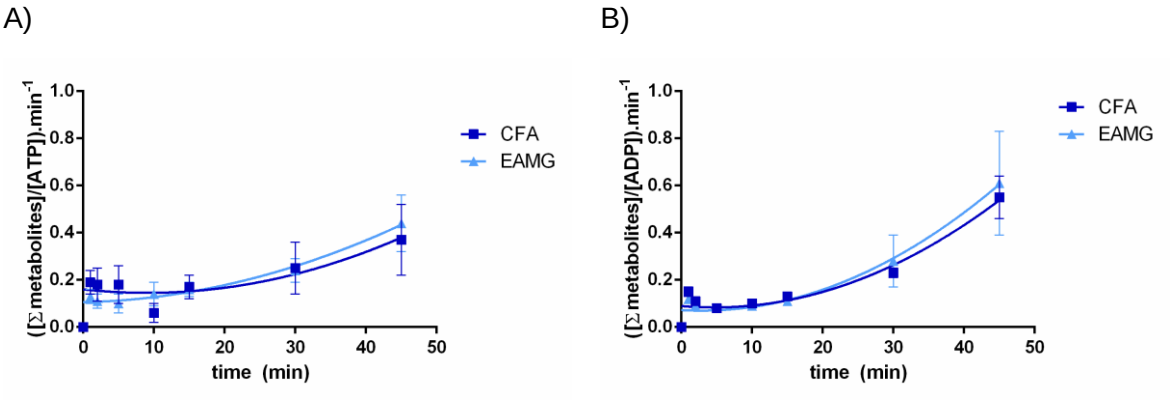

Figure 20. Evaluation of ecto-NTPDase activity when ATP (A) and ADP (B) were used as substrates of the ectoNTPDase cascade in the rat phrenic nerve hemidiaphragm preparations from control (CFA) and EAMG animals. (A) Shows the ratio quantification of [ADP+AMP+IMP+nucleosides]:[ATP] per min using ATP (30 µM) as substrate; and (B) shows the ratio quantification of [ADP+AMP+IMP+nucleosides]:[ADP] per min using ADP (30 µM) as substrate. The abscissa indicates the time scale. The ordinates show the ratio of concentration of metabolites per substrate per time point evaluated. The number of experiments is 4 to EAMG and CFA animal groups. The results are expressed as mean ± SEM and are shown when they exceed the symbols in size. *P<0,05 (two-way ANOVA followed by Bonferroni's multiple comparison test) represents significant differences as compared to CFA animals. .

In this context, our hypothesis to explain why endogenous adenosine accumulation at the myasthenic diaphragmatic neuromuscular junction does not accompany the increased amounts of ATP found after repetitive nerve activity is probably related (1) to the characteristic increase in ADA activity observed in myasthenics, (2) to excessive adenosine removal from the extracellular compartment by equilibrative nucleoside transporters, or (3) to deficits in retrograde adenosine release from paralyzed skeletal muscles of myasthenic animals caused by loss of nAChR or impairment of the oxidative metabolism of skeletal muscle fibers, or all of these mechanisms (Oliveira et al., 2015). Muscle paralysis with µ-conotoxin GIIIB, a toxin that blocks muscle-specific voltage-gated Na^+ channels without affecting neuronal function (Faria et al., 2003), decreased by more than 90% nerve-evoked adenosine outflow while marginally (~15%) affecting the release of ATP (Noronha-Matos et al., 2011). Moderate-to-severe MG patients have impaired oxidative metabolism and a noticeable shift to glycolytic metabolism during exercise, which yields to higher-end Pi/ATP ratio and reduced levels of synaptic adenosine levels (Ko et al., 2008).

Regarding the surplus ATP accumulation at the diaphragm neuromuscular junction of EAMG rats following repetitive phrenic nerve stimulation and considering that the extracellular catabolism of the nucleotide is not compromised in these animals, we should focus the debate on the cell type and underlying mechanisms responsible for increased ATP outflow. Besides the fact that ATP is co-stored and may be released synchronously with ACh from synaptic vesicles in a frequency-dependent manner (Magalhães-Cardoso et al., 2003; Smith, 1991), the nucleotide can also be originated

from stimulated skeletal myotubes in healthy animals, through pannexin-1 hemichannels, within a 15 s to 3 min time scale after a tetanus (Buvinic et al., 2009). Previous results from our group showed that diaphragm muscle paralysis affects only marginally the release of ATP (~15%) compared to adenosine outflow (>90%). Thus, one may speculate that the morphological changes occurring at motor endplates of myasthenic animals, which include a reduction in the total area of nAChR labeling per endplate and, hence, a decrease in the number of effective postsynaptic nAChR (Oliveira et al., 2015), are not sufficient to significantly decrease the amount of ATP release from stimulated skeletal muscle fibers of EAMG rats. Yet, this finding also does not explain augmentation of the nucleotide levels in the extracellular medium following a period of repetitive nerve stimulation. Our most probable hypothesis is to consider that extracellular ATP accumulated at the myasthenic neuromuscular junction may originate from stimulated pre-synaptic nerve terminals, although we have no evidences to discard facilitated ATP leakage from damaged cells during complement attack triggered by immune-mediated reactions against the post-synaptic nAChR, which consequences can be confirmed by the characteristic morphological alterations of myasthenic motor endplates.

Excluding vesicle exocytosis from nerve terminals, ATP can also be released to the extracellular compartment by non-lytic mechanisms, which include (1) nucleotide-permeable channels (connexin and pannexin hemichannels, maxi-anion channels, volume regulated anion channels or P2X7 receptor channels); (2) transport vesicles that deliver proteins to the cell membrane; and (3) membrane fusion with lysosomes (reviewed by Dahl and Muller, 2014; Penuela et al., 2013). Whether one or several of these mechanisms are responsible for surplus ATP outflow from myasthenic motor nerve terminals in addition to well-documented vesicle exocytosis, requires further investigations.

Among the plasma membrane-bound NTPDases (NTPDases 1, 2, 3 and 8), NTPDase1 (also named CD39, ATPDase, ecto- apyrase, ecto-ADPase) hydrolyzes ATP and ADP equally well, NTPDase2 is a preferential triphosphonucleosidase leading to transient ADP accumulation, whereas NTPDase3 (CD39L3, HB6) and NTPDase8 are functional intermediates between NTPDases 1 and 2 (reviewed by Zimmermann, 2001). Results from the enzymatic kinetic experiments of the extracellular ATP and ADP catabolism suggest the presence of an ecto-ATPase (E.C. 3.6.1.5), like NTPDase 1, with affinity for both NTP and NDP nucleotides, able to metabolize sequentially ATP into ADP and ADP into AMP at the rat phrenic nerve-hemidiaphragm both in control and EAMG animals. Characterization of the NTPDase subtype most involved in the extracellular

catabolism of ATP and ADP requires imaging studies and the use of selective enzyme inhibitors.

2. Pharmacological characterization of the P2R modulating ACh release from control and myasthenic motor nerve endings

In control rats, exogenously application of ATP (1 µM, for 15 min) increases [^{3}H]-ACh release from stimulated phrenic nerve terminals by about 60%. The facilitatory effect of ATP depends on its conversion into ADO and subsequent activation of pre-synaptic excitatory A_{2A} receptors (Neves, 2015, personal communication). This assumption was confirmed because pretreatment with ADA, the enzyme that inactivates endogenous adenosine into inosine, converted the facilitatory effect of ATP into an inhibitory action of about 30% compared to control conditions (Neves, 2015, personal communication). In accordance with findings reported by other groups (Giniatullin et al., 2015; Guarracino et al., 2016), these results suggest the presence of inhibitory P2Y receptors at rat motor nerve terminals. Therefore, we decided to re-evaluate the neuromodulatory role of ATP on the release of [^{3}H]-ACh from stimulated phrenic nerve terminals of control (CFA) and EAMG rats. To this end, we assessed (1) the effect of ATP (1 µM) using a shorter incubation period (only 3 min) in order to decrease the time available for ATP catabolism into ADO by ecto-nucleotidases (see the previous chapter), and (2) the effect of a non-hydrolysable ATP analogue, β,γ-imido ATP (100 µM), on evoked transmitter release.

Figure 21 shows that exogenously application of ATP (1 µM, for 3-min) decreased the release of [^{3}H]-ACh by 20±6% (S2/S1: 0.60±0.05, *n*=5) and 26±10% (S2/S1: 0.62±0.09, *n*=4) in EAMG and control rats, respectively, when the phrenic nerve trunk was stimulated electrically with 750 pulses delivered at 5-Hz frequency. The enzymatically-stable ATP analogue, β,γ-imido ATP (100 µM), failed to modify (P>0,05) S2/S1 ratios in EAMG (0.70 ± 0.06, *n*=6 vs. control 0.76 ± 0.03, *n*=4) and control (0.58 ± 0.13, *n*=5 vs. control 0.83 ± 0.08, *n*=4) animals, thus suggesting that the inhibitory effect of ATP on evoked [^{3}H]-ACh release may be mediated by prompt formation of ADP by E-NTPDases at diaphragm motor endplates of control and EAMG rats (data not shown). It is worth to note that 3 min is a time long enough to measure considerable amounts of ADP resulting from the extracellular catabolism of ATP in phrenic nerve-hemidiaphragm preparations from both control and EAMG rats (Figure 18), but within this timescale we were unable to detect the formation of adenosine.

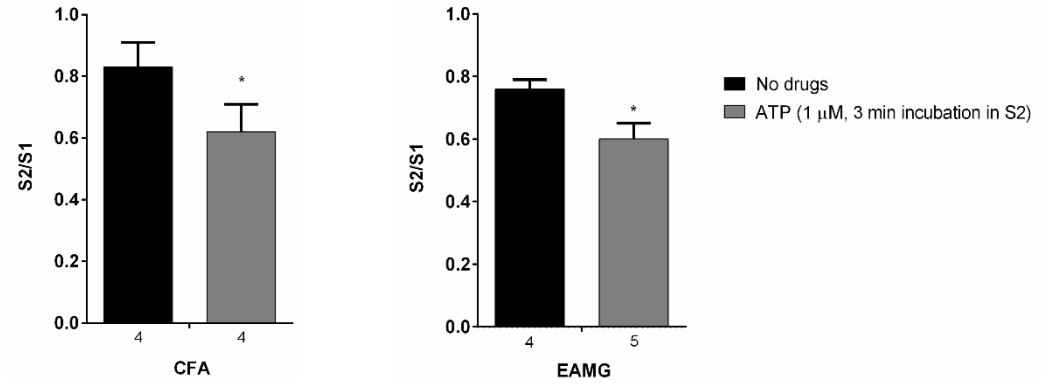

Figure 21. The effect of exogenously added ATP (1 μM) on evoked [^{3}H]-ACh release from phrenic nerve hemidiaphragm preparations from control (CFA) and EAMG animals [^{3}H]-ACh release was electrically evoked twice (S1 and S2) by 750 rectangular pulses applied with a 5Hz frequency. ATP (1 μM) was applied 3 min before S2. Ordinates represent the S2/S1 ratio and the dotted line represents the control value of the ratio for each animal group (absence of any drugs). The results are expressed as mean ± SEM and are shown when they exceed the symbols in size; the *n* number of experiments is shown in the graphs. * P < 0.05; * is the comparison with the S2/S1 ratio without any drugs (Student's t-test was performed to compare each condition in all animal groups).

Taking into account our hypothesis that short (3-min) periods of incubation with ATP is enough to generate considerable amounts of ADP that may mediate the inhibitory effects of the nucleotide on evoked transmitter release, then one must consider the involvement of ADP-sensitive P2Y purinoceptors of the $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ subtypes. As a matter of fact, the presence of inhibitory P2Y receptors has been suggested at neuromuscular junctions of frogs and mice. While in the frog, the inhibitory effect of ATP seems to be mainly mediated through the activation of the $P2Y_{12}$ receptor subtype (Giniatullin et al., 2015), in the mouse a role for the $P2Y_{13}$ receptor has been claimed (Guarracino et al., 2016).

Pre-incubation of phrenic nerve-hemidiaphragm preparations with a selective $P2Y_{13}$ receptor antagonist, MRS 2211 (10 μM) (Kim et al., 2005), attenuated (P<0.05) the inhibitory effect of ATP (1 μM, for 3 min) evoked [^{3}H]-ACh release, both in EAMG (to 0 ± 11%, S2/S1: 0.76 ± 0.08, *n*=4) and in control (to 2 ± 8%, 0.82 ± 0.07, *n*=10) rats (Figure 22). On its own, MRS 2211(10 μM) did not significantly change transmitter release in none of the animal groups (data not shown).

Selective blockade of the $P2Y_{12}$ receptor with AR-C 66096 (100 nM) (Humphries et al., 1994) significantly (P<0,05) potentiated the inhibitory effect of ATP (to 45±7%, *n*=4) in EAMG animals (Figure 23). This finding suggests that activation of the $P2Y_{12}$ receptor on motor nerve terminals of myasthenic animals is contributing to restrain the inhibitory action of ATP and/or ADP on evoked ACh release.

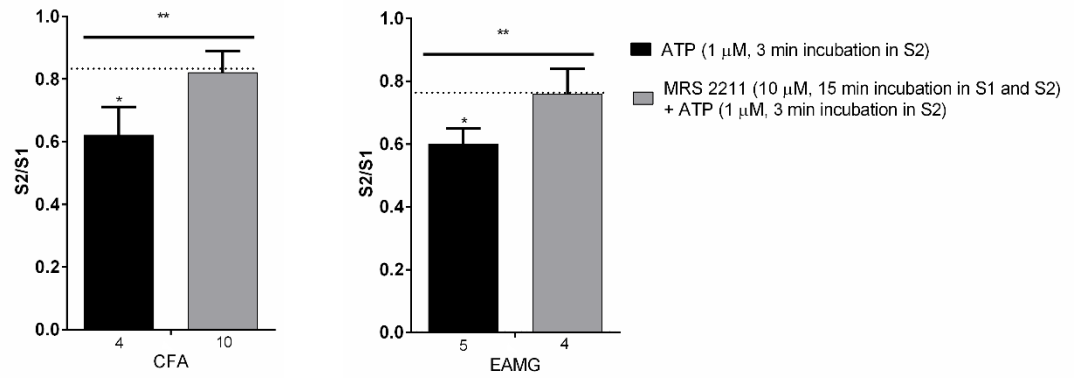

Figure 22. The effect of exogenously added ATP (1 μM) in the presence or absence of $P2Y_{13}R$ selective antagonist, MRS 2211 (10 μM) on evoked [^{3}H]-ACh from phrenic nerve hemidiaphragm preparations from control (CFA) and EAMG animals [^{3}H]-ACh release was electrically evoked twice (S1 and S2) by 750 rectangular pulses applied with a 5Hz frequency. ATP (1 μM) was applied 3 min before S2 and MRS 2211 (10 μM) was incubated 15 minutes before S1 and S2. Ordinates represent the S2/S1 ratio and the dotted line represents the control value of the ratio for each animal group (absence of any drugs). The results are expressed as mean ± SEM and are shown when they exceed the symbols in size; the *n* number of experiments is shown in the graphs. * $P < 0.05$; * is the comparison with the S2/S1 ratio without any drugs; ** is the comparison with the S2/S1 ratio of ATP (1 μM) incubated for 3 minutes before S2 (Student's t-test was performed to compare each condition in all animal groups).

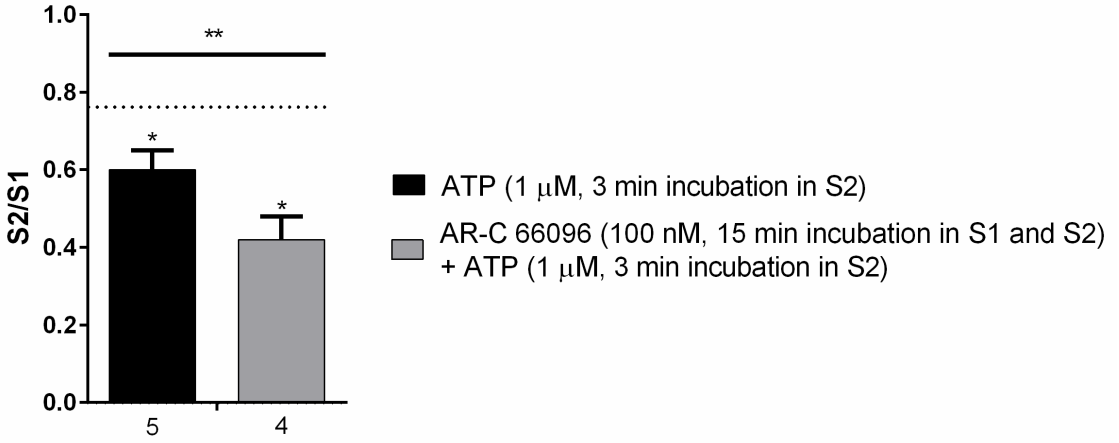

Figure 23. The effect of selective blockade of $P2Y_{12}R$ activity with the antagonist, AR-C 66096 (100 nM), on the inhibitory action of ATP (1 μM) on evoked [^{3}H]-ACh from EAMG rat motor nerve terminals. [^{3}H]-ACh release was electrically evoked twice (S1 and S2) by 750 rectangular pulses applied with a 5Hz frequency. ATP (1 μM) was applied 3 min before S2 and AR-C 66096 (100 nM) was incubated 15 minutes before S1 and S2. Ordinates represent the S2/S1 ratio and the dotted line represents the control value of the ratio for EAMG animal group (absence of any drugs). The results are expressed as mean ± SEM and are shown when they exceed the symbols in size; the *n* number of experiments is shown in the graphs. * $P < 0.05$; * is the comparison with the S2/S1 ratio without any drugs; ** is the comparison with the S2/S1 ratio of ATP (1 μM) incubated for 3 minutes before S2 (Student's t-test was performed to compare each condition in all animal groups).

Despite ADP-sensitive $P2Y_1R$ has not been documented on motor nerve terminals, some reports have shown that activation of this receptor subtype may decrease the release of ACh from stimulated cholinergic neurons in the rat myenteric

plexus (Duarte-Araújo et al., 2009). Selective blockade of the $P2Y_1$ receptor with MRS 2179 (300 nM) (von Kugelgen, 2006) fully prevented the inhibitory action of ATP (1 μM, for 3 min) on evoked [^{3}H]-ACh release from motor nerve terminals of EAMG rats (6 ± 10%, *n*=6, 0.81 ± 0.07, *n*=6). MRS 2179 (300 nM) was devoid of effect on ATP (1 μM, for 3 min)-induced inhibition of [^{3}H]-ACh release from stimulated phrenic nerve terminals of control animals (-22 ± 14%, *n*=5, 0.65 ± 0.12, *n*=5) (Figure 24).

Selective activation of the $P2Y_1$ receptor with MRS 2365 (30 nM) (Chhatriwala et al., 2004) inhibited evoked [^{3}H]-ACh release from phrenic nerve endings by 31 ± 4% (*n*=5) in EAMG rats and by 44 ± 6% (*n*=4) in control animals (Figure 24). These findings show that the selective $P2Y_1$ receptor agonist, MRS 2365 (30 nM), seems to be more potent than the natural compound, ATP (1 μM), in decreasing [^{3}H]-ACh release from stimulated motor nerve terminals.

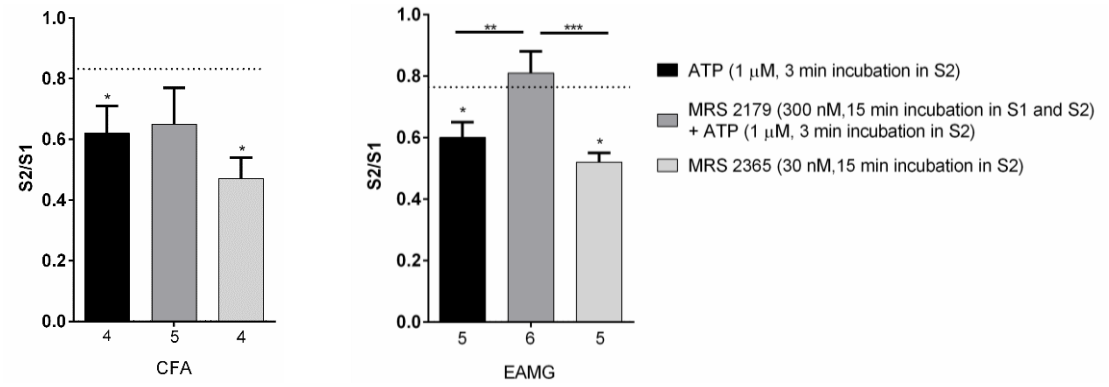

Figure 24. Evaluation of P2Y1 receptor contribution on the inhibitory action of ATP (1 μM) on evoked [^{3}H]-ACh release from innervated hemidiaphragm preparations of control (CFA) and EAMG rats. [^{3}H]-ACh release was electrically evoked twice (S1 and S2) by 750 rectangular pulses applied with a 5Hz frequency. ATP (1 μM) and the selective P2Y1R agonist, MRS 2365 (30 nM) was applied 3 min and 15 min before S2, respectively. $P2Y_1$R antagonist, MRS 2179 (300 nM) was incubated 15 minutes before S1 and S2. Ordinates represent the S2/S1 ratio and the dotted line represents the control value of the ratio for each animal group (absence of any drugs). The results are expressed as mean ± SEM and are shown when they exceed the symbols in size; the *n* number of experiments is shown in the graphs. * P < 0.05; * is the comparison with the S2/S1 ratio without any drugs; ** is the comparison with the S2/S1 ratio of ATP (1 μM) incubated for 3 minutes before S2; *** is the comparison with the S2/S1 ratio of ATP (1 μM) incubated for 3 minutes before S2 in the presence of $P2Y_1$R antagonist, MRS 2179 (300 nM) incubated for 15 minutes before S1 and S2 (Student's t-test was performed to compare each condition in all animal groups).

Previous reports have documented the involvement of inhibitory P2Y receptors in the modulation of ACh release from motor nerve terminal (Giniatullin et al., 2015; Guarracino et al., 2016), yet the functional impact of these receptors in neuromuscular transmission in myasthenic animals was never investigated. Here, we show that the $P2Y_1$ receptor modulates negatively the release of ACh from stimulated motor nerve terminals

from both control and myasthenic animals. Coupling of the $P2Y_1$ receptor to $G_{q/11}$ leading to the activation of PLC and Ca^{2+} mobilization from IP_3-sensitive intracellular stores is not supposed to decrease transmitter release from motor nerve terminals (Oliveira and Correia-de-Sá, 2005; 2006). It has been demonstrated that (1) the endogenous $P2Y_1$ receptor is capable of shifting its coupling to inhibitory $G_{i/o}$ protein under certain pathological conditions (Marcet et al., 2004), and that (2) the $P2Y_1$ receptor can decrease the release of neurotransmitters through a non-canonical inhibitory coupling to adenylyl cyclase via $G_{i/o}$ proteins (Filippov et al., 2000; Quintas et al., 2009).

Despite the fact $P2Y_1$ receptors are present in both control and EAMG animals the activation of $P2Y_1$ receptors by ATP/ADP was only observed in myasthenic conditions. Considering that (1) ecto-nucleotidases activity is similar between CFA and EAMG animals and (2) $P2Y_1$R selective agonist exert effect on both group of animals, whereas, (3) ATP only acted on PY_1R in EAMG animals we may assume that a redistribution of ecto-NTPDases close to the vicinity of $P2Y_1$R could occur in EAMG conditions.

Alterations in the coupling between E-NTPDase subtypes and $P2Y_1$ receptors due to morphological modifications of the myasthenic motor endplate may be enough to change the location where enzymes deliver their metabolic products (e.g. ADP) which are required to activate a certain receptor subtype (e.g. $P2Y_1$), while these modifications do not have major repercussions on the enzymatic activity of the whole tissue. This hypothesis was inspired in the close relationship postulated for ecto-5'-nucleotidase and A_{2A} receptors at the rat neuromuscular junction (Cunha et al., 1996) and other excitatory synapses at the peripheral (e.g. myenteric plexus) (Duarte-Araújo et al., 2009) and the central (e.g. striatum, hippocampus) (Augusto et al., 2013; Barros-Barbosa et al., 2016; Cunha et al., 1996) nervous system. The tight association between ecto-5'-nucleotidase (CD73) and the A_{2A} receptor has been elegantly and definitively documented by co-immunoprecipitation and proximity ligation assays in the striatum (Augusto et al., 2013) and it may explain why A_{2A} receptors are activated preferentially by adenosine resulting from the extracellular catabolism of released ATP (Cunha et al., 1996; Duarte-Araújo et al., 2009). Correlation between changes in purinergic signaling and tissue redistribution of ecto-NTPDases was already observed in inflammatory conditions by other authors (Vieira et al., 2014).

The presence of $P2Y_{13}$ receptors has been demonstrated by immunofluorescence confocal microscopy at the mouse neuromuscular junction; these receptors exert a presynaptic inhibitory role on ACh release (Guarracino et al., 2016). Using a selective $P2Y_{13}$ receptor antagonist, MRS 2211 (Kim et al., 2005), we could block the inhibitory action of ATP on evoked transmitter release, both in control and in EAMG

phrenic nerve-hemidiaphragm preparations. In myasthenic animals the inhibitory action of exogenously applied ATP and/or ADP seems to be dependent on simultaneous activation of both $P2Y_1$ and $P2Y_{13}$ receptors because the selective blockade of $P2Y_1$ or $P2Y_{13}$ receptors totally prevented the inhibitory action of ATP/ADP. It is well established that individual G protein coupled receptors (GPCRs) can modulate the signals initiated by another GPCR to potentiate or inhibit the activity of these receptors. A common form of cross-talk is for two receptors to produce a greater than additive change in the level of second messengers (Abbracchio et al., 2006). Considering that $P2Y_{13}$ and $P2Y_1$ receptors use the same intracellular signaling, the co-activation of both $G_{i/o}$-coupled receptors will probably be necessary to induce a synergic inhibition adenylyl cyclase and a consequent decrease in intracellular cAMP levels (Abbracchio et al., 2006). So far, no interactions between $P2Y_1$ and $P2Y_{13}$ receptors were reported. However, a dependence on $P2Y_1$ receptors co-activation was already reported for other receptors, the CXCR2 chemokine receptor (Werry et al., 2002).

Data from the present study also show that activation of the $P2Y_{12}$ receptor at diaphragmatic motor endplates of myasthenic rats partially counteract the inhibitory effect of ATP or, more exactly, its more potent derivative, ADP. This was assumed because selective blockade of the $P2Y_{12}$ receptor with AR-C66096 (Humphries et al., 1994) significantly potentiated the inhibitory effect of ATP on evoked ACh release without affecting the transmitter release when applied alone. These results contrast with previous observations by Giniatullin and col. (2015) showing that activation of the $P2Y_{12}$ receptor mediates the inhibitory action of ATP on synaptic transmission at frog motor endplates.

The $P2Y_{12}$ receptor subtype is generally coupled to $G_{i/o}$ proteins leading to decreases in intracellular levels of cyclic AMP. Although we did not investigated directly $G_{i/o}$ protein coupling of both $P2Y_1$, $P2Y_{13}$ and $P2Y_{12}$ receptors, previous results from our group indicate that inhibition of adenylyl cyclase activity by adenosine A_1 and muscarinic M_2 receptors (Correia-de-Sá et al., 1991; Oliveira et al., 2002) are linked to inhibition of ACh release from stimulated motor nerve terminals of the rat diaphragm.

The mechanism(s) underlying the crosstalk between $P2Y_1$ and $P2Y_{12}$ receptors requires further elucidation, but one can speculate about several possibilities which include physical oligomerization of the two receptors and/or an interaction at the second messenger system level. As a matter of fact, this is not the first time such explanation has been put forward. The formation of functional heterodimers of human $P2Y_1$-$P2Y_{12}$ receptors has been demonstrated and may explain the inhibitory action of $P2Y_1R$ and $P2Y_{12}R$ on two pore potassium ion channels (K_{2P}) following incubation with ADP. In HEK293 cells expressing only the human variant of the $P2Y_{12}$ receptor, the ADP effect was prevented by the application of both AR-C69913MX (a $P2Y_{12}$ receptor antagonist,

1μM) and MRS 2179 (10 μM, a $P2Y_1$ receptor antagonist), thus indicating that the inhibitory effect is shared between activation of the recombinant $hP2Y_{12}$ and the endogenous $P2Y_1$ receptors. When $hP2Y_1$ and $hP2Y_{12}$ receptors were co-expressed in the same cells, the antagonism of both receptors prevents the action of ADP, whereas application of pertussis toxin (PTX) did not. When both receptors are co-expressed, the pharmacological profile follows the same tendency as when only the $hP2Y_{12}$ receptor is expressed, but the transduction pathway is no longer dependent of the $G_{i/o}$ coupling (Shrestha et al., 2010). Besides $P2Y_1$-$P2Y_{12}$ coupling there are reports of heterodimer formation of $P2Y_1R$ with $P2Y_{11}R$, which controls ligand selectivity and internalization of $P2Y_{11}R$ (Ecke et al., 2008), and of adenosine A_1 receptors with $P2Y_1R$ leading to interference with the intracellular $G_{i/o}$ coupling of A_1R (Yoshioka et al., 2001). There are currently evidences that $P2Y_1R$ and $P2Y_{12}R$ form functional heterodimers. Fluorescence Resonance Energy Transfer (FRET) experiments were conducted in human tsA201 cells transfected with $hP2Y_1$-eYFP and $hP2Y_{12}$-eCFP cDNA showing that recombinant $P2Y_{12}R$ heterodimerise with the native $P2Y_1R$ (Naughton et al., 2014). It has long been known that the formation of oligomers is a key to the expression, trafficking and functional activity of GPCR, since they link different transduction pathways and integrate receptor functions giving GPCR novel signaling and pharmacological properties (reviewed by Milligan, 2013).

Despite the preliminary data from our work, we observed that the ADP-sensitive P2Y receptors, $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors modulate neuromuscular transmission at myasthenic NMJ. These results give new insights regarding the neuromodulatory players that are probably contributing to tetanic depression operating in myasthenic rats. So, we consider that an elucidation on the dynamic interplay between ADP sensitive P2Y receptors could greatly improve our knowledge on the pathophysiology as well as in the identification of new therapeutic targets.

Chapter V

Conclusions and future work

In this study, our aim was to provide a better understanding of the neuromodulatory role of ATP and its metabolite, ADP, on synaptic transmission in MG.

Data show here for the first time that ADP-sensitive P2Y receptors ($P2Y_1$, $P2Y_{12}$, $P2Y_{13}$ receptors) play relevant roles in the purinergic modulation of neuromuscular transmission in myasthenic animals. ADP resulting from the extracellular catabolism of ATP by ecto-NTPDases decreases evoked ACh release from motor nerve terminals through activation of inhibitory $P2Y_1$ and $P2Y_{13}$ receptors. ADP-induced inhibition of ACh release is partially restrained by synchronous activation of $P2Y_{12}$ receptors. In this context, blockade of inhibitory $P2Y_1$ and $P2Y_{13}$ receptors and/or selective inhibition of ecto-NTPDase1 activity, thus decreasing ADP formation from the extracellular catabolism of released ATP while keeping adenosine formation unrestrained and thereby A_{2A} receptor-mediated facilitation of ACh release may be therapeutically useful to increase the safety margin of the neuromuscular transmission in myasthenic animals. On the other hand, one may hypothesize that increasing the $P2Y_{12}$ receptor tonus could be beneficial in myasthenics, taking into consideration that this receptor partially restrains the inhibitory actions of both $P2Y_1$ and $P2Y_{13}$ receptors.

Data obtained in this study, together with previous findings from our group indicating that the adenosine neuromodulatory control of synaptic transmission in myasthenic motor endplates is deficient compared to the control situation (Oliveira et al., 2015), strengthens our assumption that purinergic signaling plays a chief role in neuromuscular transmission and it is significantly impaired in patients with MG. The mechanisms accounting for purinergic signaling deficits in myasthenic patients may result from decreased endogenous ADO accumulation in myasthenic motor endplates, which results in insufficient levels to activate presynaptic facilitatory $A_{2A}R$ required to resist tetanic depression of neuromuscular transmission, a characteristic feature of the myasthenic neuromuscular deficits. In addition, the increased levels of ATP released during neuronal firing may be diverted to ADP formation and, thereby, to excessive activation of ADP–sensitive inhibitory $P2Y_1$ and $P2Y_{13}$ receptors, also contributing to decrease the safety margin of the neuromuscular transmission in myasthenic animals.

Elucidation of this complex scenario regulating the neuromuscular transmission in myasthenic patients requires further investigations. Understanding the mechanisms underlying the disparity between high ATP levels cohabitating with reduced accumulation of ADO (which is not very common) will certainly contribute to clarify the disequilibrium between the effects of these two purines. Another very important issue remaining to be fully elucidated concerns the crosstalk between inhibitory $P2Y_1R/P2Y_{13}R$ and excitatory $A_{2A}R$ activation, as well as the extent of the role played by the $P2Y_{12}R$ to control inhibition of ACh release triggered by $P2Y_1R/P2Y_{13}R$ in myasthenics. These

efforts will certainly contribute to our knowledge about the pathophysiology of MG and to discover novel therapeutic targets to manage the neuromuscular transmission deficits afflicting patients with MG.

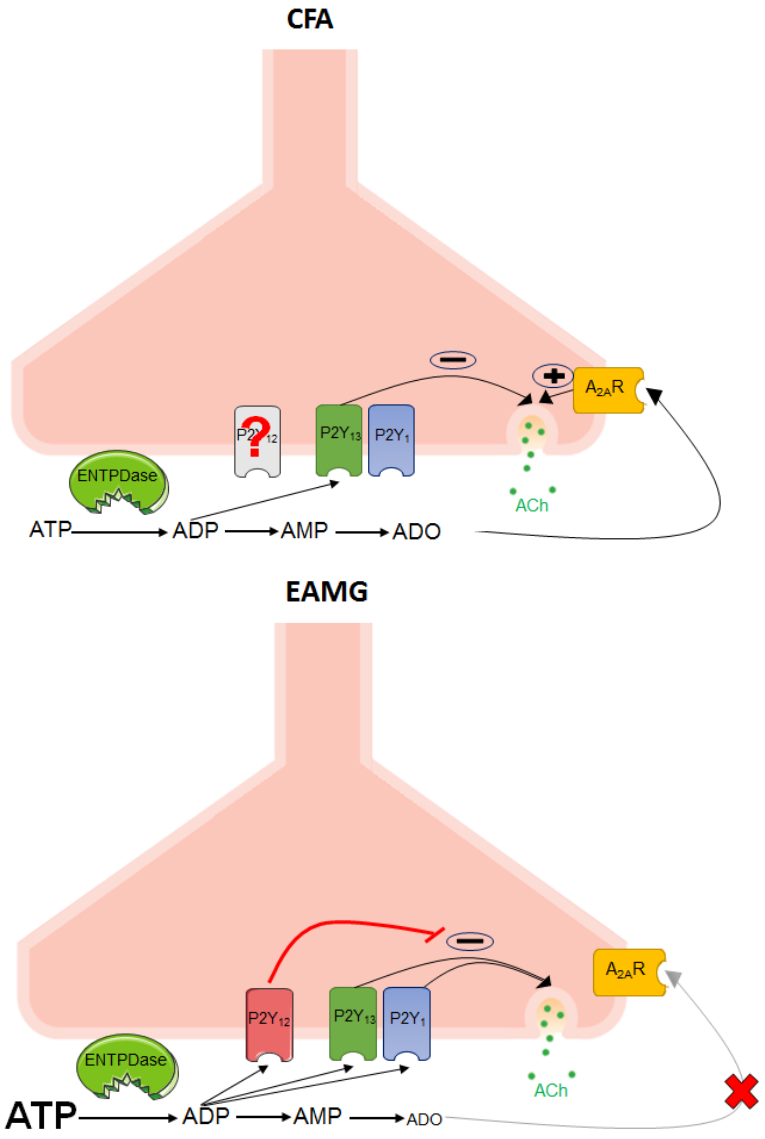

Figure 25. Purinergic unbalance in the control of evoked neurotransmitter release at motor endplates of myasthenic rats (EAMG) and healthy controls (CFA). At the NMJ of myasthenic animals, there is an apparent paradox between increased accumulation of ATP and decreased accumulation of adenosine, which is not due to changes in the ectonucleotidase cascade upstream adenosine formation. ATP (after its extracellular catabolism into ADP) exerts a predominant inhibitory control of ACh release in both control and myasthenic rats, via the activation of presynaptic $P2Y_{13}R$. The inhibitory effect of ATP/ADP can also be mediated by the $P2Y_1R$ in myasthenic rats. The dominant inhibitory action of ATP/ADP in myasthenics can be observed despite this effect is partially restrained by co-activation of the $P2Y_{12}R$. At the myasthenic motor endplate, the extracellular ADO accumulation is severely impaired leading to deficient activation of excitatory A_{2A} receptors. This situation, contributes definitively to unbalance ATP/ADP mediated inhibitory effects towards the decrease of evoked transmitter release and to neuromuscular deficits.

Chapter VI

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