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The role of adenosine A_{2A} receptors in the regulation of neuronal and immunological responses in rats with Experimental Autoimmune *Myasthenia gravis* (EAMG)

Sónia Isabel Nunes Guerra Gomes

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Dissertação de Mestrado em Bioquímica
Faculdade de Ciências da Universidade do Porto
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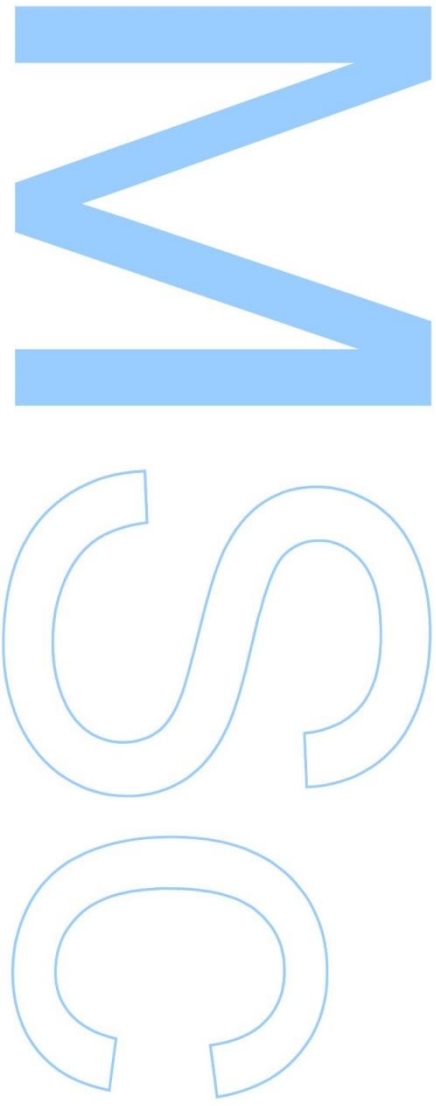
2013

Orientador
Doutora Fátima Ferreirinha, Técnica Superior, ICBAS/UP

Co-orientador
Prof. Doutora Laura Oliveira, Professor Auxiliar, ICBAS/UP
Professor Doutor Paulo Correia de Sá, Professor Catedrático, ICBAS/UP



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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.
O Presidente do Júri,

Porto, _____/_____/_____

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Abstract

Myasthenia gravis is a B-cell-mediated, T-cell dependent neuroimmunological disorder characterized by excessive muscle weakness and fatigue. Adenosine is a ubiquitous molecule acting as a potent modulator of both neuronal and immunological responses through the activation of A_{2A} adenosine receptors (Correia-de-Sá *et al.*, 1991; Csoka *et al.*, 2008). The activation of A_{2A} receptors on motor nerve terminals allows the recovery of neuromuscular transmission under fatigue conditions (Oliveira *et al.*, 2004). The nucleoside, via A_{2A} receptors, decreases cellular immunological responses by suppressing proliferation of effector $CD4^+CD25^-$ T cells and of activated $CD4^+CD25^+$ T cells; it also increases immunosuppressive properties of regulatory $CD4^+CD25^+FoxP3^+$ T cells populations (Csoka *et al.*, 2008; Ohta *et al.*, 2012). Taking this into consideration, disorders under the umbrella of neuroimmunology like *Myasthenia gravis* (MG) may benefit from therapeutic strategies targeting common molecular elements involved in both neuronal and immunological impairment. Recently, we demonstrated an impairment on A_{2A} receptors modulatory action of neuromuscular transmission in two models of *Myasthenia gravis*: (1) the toxicological (TIMG) (Noronha-Matos *et al.*, 2011) and (2) the experimental autoimmune (EAMG) models of MG (Almeida, 2012; Guerra-Gomes *et al.*, 2013). In parallel, Li and collaborators (2012) reported a reduced A_{2A} receptors expression by both T and B cells residing in spleen and lymph nodes following EAMG induction. In keeping with these concepts it is becoming increasingly appreciated that A_{2A} receptor pathway impairment maybe a common feature of neuronal and immunological dysfunction operating in MG. In order to understand the potential application of the pharmacological manipulation of A_{2A} receptors in therapeutic arsenal of MG, it is crucial to unravel the mechanisms associated to the reported A_{2A} receptor deficits. Considering the dynamics between effector $CD4^+CD25^-$ T cells, activated $CD4^+CD25^+$ T cells and regulatory $CD4^+CD25^+FoxP3^+$ T cells subtypes on the modulation of immune responses and the differential effects of A_{2A} receptor activation on these cell populations, it is of utmost relevance to assess A_{2A} receptors potential changes in all $CD4^+$ T cells populations. On the other hand, impairment of receptors sensitivity/efficiency may result from post-translational modifications of the receptor as well as changes in receptor density and distribution in myasthenic endplates. This led us to evaluate A_{2A} receptor density and distribution through cell compartments present on both motor endplates and $CD4^+$ T cell population from EAMG animals by immunofluorescence confocal microscopy and by flow cytometry, respectively. One of the reported mechanisms affording for A_{2A}

receptors neuroexcitatory and immunossupressor actions is the mobilization of extracellular Ca^{2+} through Ca_V1 channels (Oliveira *et al.*, 2004) and the amount of ADO production through T lymphocytes membrane bound ecto-5'-nucleotidase (CD73) enzyme (Mandapathil *et al.*, 2010), respectively. Taking this into consideration we sought to evaluate immunoreactivity against Ca_V1 channels on motor nerve endplates and to ecto-5'-nucleotidase (CD73) enzyme on $CD4^+$ T cells population through their differential cell compartments.

To this end, we took advantage of an animal model of experimental autoimmune *Myasthenia gravis* (EAMG) that has been developed by breaking of tolerance to a single T cell epitope of the self autoantigen induced by a single peptide corresponding to region 97-116 of the rat nAChR α subunit (Baggi *et al.*, 2004). *Wistar* rats were immunized with R97-116 peptide in CFA (Complete Freund's Adjuvant) on day 0; thirty days after, the animals were boosted with the same peptide in IFA. Control animals received CFA emulsion without the peptide; animals of the naive group were not submitted to any kind of treatment. The follow up of EAMG disease induction was assessed by monitoring the clinical scoring determined by the presence of tremor, hunched posture, muscle strength by grip strength test (BIOSEB, France) and fatigability. In agreement with other findings (Mu *et al.*, 2009; Wu *et al.*, 1997) the animals enrolled in the EAMG group exhibited two typical clinical phases: an acute/moderate and a progressive phase. None of the healthy animals, the naive and control group, presented detectable clinical signs. The EAMG animals were used during the progressive phase and presented signs of both immunological and neuronal imbalance. As already reported for human MG, the EAMG animals presented an increase in serum adenosine deaminase (ADA) activity (Chiba *et al.*, 1995) and a reduction of FoxP3 expression on T_{reg} cells (Balandina *et al.*, 2005; Zhang *et al.*, 2009). In parallel, an increased fatigue of diaphragm muscle contractions induced by indirect repetitive phrenic nerve stimulation and morphological changes of motor endplates was also confirmed on EAMG rats. These set of data indicates that this is a suitable model to study the immunopathophysiological mechanisms of MG.

The reduction of A_{2A} receptors density on EAMG $CD4^+$ T cells compartment were observed in both activated $CD4^+CD25^+$T cells and regulatory $CD4^+CD25^+FoxP3^+$ T cell populations, whereas only regulatory $CD4^+CD25^+FoxP3^+$ T cells population presented a decreased expression of CD73 density. The conjunction of these findings plus the observation of increased ADA activity strengthens the hypothesis that the adenosinergic pathway may be involved in MG pathogenesis. In fact, the event of specific recognition of nAChR epitopes expressed on antigenic presenting cells (APCs) by T cells may lead to an increased secretion of ADA (Zavialov *et al.*, 2010), which will

in turn decrease the amount of the endogenous ligand (ADO) for A_{2A} receptor on activated and regulatory T cells. This will promote activated $CD4^+CD25^+$T cells proliferation and impairment of regulatory $CD4^+CD25^+FoxP3^+$ T cells immunosuppressive properties (Ohta *et al.*, 2012). The decreased expression of FoxP3 transcription factor, which is under the control of A_{2A}R (Deaglio *et al.*, 2007), will consequently decrease CD73 expression reinforcing the paucity of endogenous ligand to activate A_{2A} receptors on regulatory $CD4^+CD25^+FoxP3^+$ T and activated $CD4^+CD25^+$T cells. The disruption of this adenosinergic loop on regulatory $CD4^+CD25^+FoxP3^+$ T cells may decrease the expression of A_{2A} receptors as an adaptive mechanism of regulatory $CD4^+CD25^+FoxP3^+$ T and activated $CD4^+CD25^+$T to a chronic reduction in ADO levels. The preliminary results obtained for A_{2A} receptor immunoreactivity on motor nerve terminals evaluated by confocal microscopy failed to show an evident modification on A_{2A} density. The nature of immunolabeling by confocal microscopy technique failed to evidence differences on the density of epitopes, meaning that no accurate statements could be drawn regarding the variations on A_{2A} receptor expression in EAMG animals. However, the immunofluorescence labeling for A_{2A} receptor and $Ca_v1.2$L suggests that they are present at motor endplates of EAMG animals and have a pre-synaptic localization indicating that A_{2A} receptors could constitute a potential pharmacological target to overcome tetanic failure operating in myasthenic conditions.

These results bring further insights about the role of adenosine pathway (via A_{2A} receptors activation) as a key regulator in the reestablishment of a proper dynamic plasticity of the neuroimmune system.

Keywords: adenosine, A_{2A} receptors, nAChR, experimental autoimmune *Myasthenia gravis* (EAMG), neuromuscular junction, $CD4^+$ T cells.

Resumo

A *Miastenia gravis* é uma doença neuroimunológica mediada por células B e dependente da acção de células T caracterizada por fraqueza muscular excessiva e fadiga. A adenosina é uma molécula ubiquitária que actua como um potente modulador das respostas neuronais e imunológicas, através da activação dos receptores A_{2A} (Correia-de-Sá *et al.*, 1991; Csoka *et al.*, 2008). A activação dos receptores A_{2A} nos terminais nervosos motores permite a recuperação da transmissão neuromuscular em condições de fadiga (Oliveira *et al.*, 2004), diminui as respostas celulares imunes através da supressão da proliferação de células T efectoras $CD4^+CD25^-$ e de células T activadas $CD4^+CD25^+$ e aumenta as propriedades imunossupressoras das populações de células T reguladoras $CD4^+CD25^+FoxP3^+$ (Csoka *et al.*, 2008; Ohta *et al.*, 2012). Tendo em conta estas evidências, doenças que abranjam o espectro da neuroimunologia, como a *Miastenia gravis* (MG), podem beneficiar de estratégias terapêuticas direccionadas para elementos moleculares comuns envolvidos no comprometimento neuronal e imunológico. Recentemente, o nosso grupo demonstrou a existência de comprometimento na função moduladora dos receptores A_{2A} na transmissão neuromuscular em dois modelos animais de *Miastenia gravis*: (1) o modelo toxicológico (TIMG) (Noronha-Matos *et al.*, 2011) e (2) o modelo experimental auto-imune (EAMG) de MG (Almeida, 2012; Guerra-Gomes *et al.*, 2013). Em paralelo, Li e colaboradores (2012) descreveram uma redução na expressão dos receptores A_{2A} nas células T e nas células B do baço e dos gânglios linfáticos após a indução de EAMG. Assim sendo, considera-se cada vez mais a hipótese de que o comprometimento da via dos receptores A_{2A} poderá ser um denominador comum nas disfunções neuronais e imunológicas existentes na MG. De forma a compreender a potencial aplicação da manipulação farmacológica dos receptores A_{2A} na MG, é necessário desvendar os mecanismos associados aos défices descritos para os receptores A_{2A}. Considerando a dinâmica entre as populações de células T efectoras $CD4^+CD25^-$, células T activadas $CD4^+CD25^+$ e células T reguladoras $CD4^+CD25^+FoxP3^+$ na modulação da resposta imune e os efeitos diferenciais da activação dos receptores A_{2A} nestas células, torna-se fundamental avaliar potenciais alterações nestes receptores em todas as populações de células T $CD4^+$. Por outro lado, a disfunção na sensibilidade/eficiência destes receptores pode resultar de modificações pós-translacionais do receptor, assim como de alterações na densidade do receptor e na sua distribuição nas placas motoras de animais miasténicos. Isto levou-nos a avaliar a densidade e distribuição dos receptores A_{2A} nos compartimentos

celulares presentes nas placas motoras e na população de células T $CD4^+$ de animais EAMG por imunofluorescência aplicada à microscopia confocal e citometria de fluxo, respectivamente. Um dos mecanismos descritos relata que as acções neuroexcitatórias e imunossupressoras dos receptores A_{2A} consistem quer na mobilização de Ca^{2+} através de canais Ca_V1 (Oliveira *et al.*, 2004) como também na quantidade de ADO produzida através da enzima ecto-5'-nucleotidase (CD73) que se encontra ligada à membrana dos linfócitos T (Mandapathil *et al.*, 2010), respectivamente. Tendo isto em consideração, procurou-se a avaliar a imunoreatividade dos canais Ca_V1 nas terminações nervosas motoras e da enzima ecto-5'-nucleotidase (CD73) em populações de células T $CD4^+$ através dos seus compartimentos celulares diferenciais.

Para esta finalidade, usamos um modelo animal experimental auto-imune de *Miastenia gravis* (EAMG) como ferramenta de estudo, que foi desenvolvido pela quebra de tolerância a um único epítopo da célula T do auto-antigénio induzido pela administração de um péptido correspondente à região 97-116 da subunidade α do receptor nicotínico de rato para a ACh (Baggi *et al*., 2004). Os ratos *Wistar* foram imunizados no dia 0 com o péptido R97-116 em CFA (Adjuvante completo de Freund); trinta dias após, os animais receberam um reforço com o mesmo péptido em IFA. Os animais controlo receberam apenas a emulsão de CFA sem o péptido; os animais do grupo naive não foram submetidos a qualquer tipo de tratamento. O acompanhamento da indução da doença em animais EAMG foi realizado através da monitorização do *clinical scoring* que era determinado pela presença de tremor, postura arqueada, força muscular demonstrada no *grip test* (BIOSEB, France) e fadiga. Em concordância com outras referências (Mu *et al.*, 2009; Wu *et al.*, 1997), os animais respeitantes ao grupo EAMG exibiram duas fases clínicas típicas: uma fase aguda/moderada e uma fase de progressão da doença. Nenhum dos animais saudáveis (naive e controlo) apresentou sinais clínicos detectáveis. Os animais EAMG foram utilizados durante a fase progressiva e apresentavam sinais de desequilíbrio, tanto imunológico como neuronal. Tal como já havia sido relatado para MG em humano, os animais EAMG apresentaram um aumento da actividade da adenosina deaminase (ADA) no soro (Chiba *et al.*, 1995) e uma redução de expressão nas células FoxP3 T_{reg} (Balandina *et al.*, 2005;. Zhang *et al*., 2009). Em paralelo, também se confirmou a existência de um aumento na fadiga muscular induzida por estimulação indirecta repetitiva do nervo frénico e alterações morfológicas das placas motoras em ratos EAMG. Este conjunto de dados indica que este é um modelo adequado para estudar os mecanismos de imunofisiopatológicos da MG.

A redução na densidade dos receptores A_{2A} no compartimento de células T $CD4^+$ foi observada em ambas as populações de células T activadas $CD4^+CD25^+$ e T reguladoras $CD4^+CD25^+FoxP3^+$ nos animais EAMG. Enquanto a análise para a densidade de CD73 revelou que apenas as células T reguladoras $CD4^+CD25^+FoxP3^+$ apresentaram uma expressão diminuída. A conjunção destes resultados e a observação de um aumento da actividade da ADA fortalece a hipótese de que a via adenosinérgica poderá estar envolvida na patogénese da MG. Na verdade, o evento de reconhecimento específico de epítopos dos nAChR expressos nas células apresentadoras de antigénios (APCs) pelas células T pode levar a um aumento da secreção de ADA (Zavialov *et al*., 2010) que, por sua vez, diminui a quantidade do ligando (ADO) disponível para o receptor A_{2A} nas células T reguladoras e T activadas. Isto irá promover a proliferação de células T activadas $CD4^+CD25^+$ e o comprometimento das propriedades imunossupressoras das células T reguladoras $CD4^+CD25^+FoxP3^+$ (Ohta *et al*., 2012). A diminuição da expressão do factor de transcrição FoxP3, que se encontra sob o controlo de receptores A_{2A}, (Deaglio *et al*., 2007), irá, consequentemente, agravar a expressão de CD73 reforçando a falta de ligando endógeno para os receptores A_{2A} presentes nas células T reguladoras $CD4^+CD25^+FoxP3^+$ e nas células T activadas $CD4^+CD25^+$. A disrupção desta via adenosinérgica nas células T reguladoras $CD4^+CD25^+FoxP3^+$ poderá promover uma diminuição na expressão dos receptores A_{2A} como um mecanismo adaptativo das células T reguladoras $CD4^+CD25^+FoxP3^+$ e T activadas $CD4^+CD25^+$ a uma redução crónica dos níveis de ADO. Os resultados preliminares obtidos para a imunoreactividade do receptor A_{2A} em terminações nervosas motoras avaliados por microscopia confocal não mostraram alterações evidentes na densidade de receptores A_{2A}. Porém, a natureza da técnica de imunomarcação por microscopia confocal não é a ideal para evidenciar ligeiras diferenças na densidade de epítopos, o que significa que devemos ter precauções nas inferências relativas às variações na expressão do receptor A_{2A} em animais EAMG. No entanto, a marcação por imunofluorescência para os receptores A_{2A} e para o $Ca_v1.2L$ sugere que estes estão presentes nas placas motoras de animais EAMG e têm uma localização pré-sináptica, o que indica que os receptores A_{2A} poderão constituir um potencial alvo farmacológico para superar a fadiga tetânica que ocorre em condições de *Miastenia gravis*.

Estes resultados abrem novas perspectivas para o estudo do papel da sinalização adenosinérgica (por via da activação de receptores A_{2A}) como um alvo-chave para o restabelecimento de uma plasticidade dinâmica adequada do sistema neuroimmune.

Palavras-chave: adenosina, receptores A_{2A}, nAChR, modelo experimental auto-imune de *Miastenia gravis* (EAMG), junção neuromuscular, células T $CD4^+$.

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List of figures and tables

Part of this work was presented as an oral presentation at the IJUP Congress 2013 (Sixth Meeting of Young Researchers of U. Porto – Porto), 13-15 February 2013:

Prevailing tonus of inhibitory A_1 receptors over facilitatory A_{2A} adenosine receptors on Experimental Autoimmune *Myasthenia Gravis* (EAMG). S. Guerra-Gomes, C. Costa, J. Leite, M.F. Ferreirinha, A. Correia, M. Vilanova, P. Correia-de-Sá & L. Oliveira.

Abbreviations

AC – Adenylate cyclase

ACh - Acetylcholine

Acetyl CoA- Acetyl coenzyme A

AChE - Acetylcholinesterase

AChR - Acetylcholine receptor

ADA - Adenosine deaminase

ADO - Adenosine

AMP - Adenosine 5'-monophosphate

APC – Antigen-presenting cell

ATP - Adenosine 5'-triphosphate

α-BTX - α-Bungarotoxin

BSA - Bovine Serum Albumin

Ca^{2+} - Calcium ion

cAMP- Cyclic adenosine monophosphate

CD4 - Cluster of differentiation 4

CD25 – Cluster of differentiation 25 (IL-2 receptor alpha-chain)

CD73 - Ecto-5'-nucleotidase

CGS21680C–2-[4-(2-p-carboxyethyl)phenylamino] -5'-N-ethylcarboxamido adenosine

CFA - Complete Freund's Adjuvant

ChAT – Choline Acetyltransferase

DAG - Diacylglycerol

EAMG - Experimental Autoimmune *Myasthenia gravis*

FBS - Fetal Bovine sérum

FoxP3 - Forkhead box P3

IFA - Incomplete Freund's Adjuvant

IgG - Immunoglobulin G

IL-2 - Interleukin 2

IL-6 - Interleukin 6

IMP - Inosine monophosphate

INO – Inosine

IP3 - inositol (1,4,5)-triphosphate

K^+ - Potassium ion

MACS - Magnetic affinity cell sorting

MEEP – Miniature endplate potential

MHC – Major histocompatibility complex

MG - *Myasthenia gravis*

MuSK - Muscle-specific receptor tyrosine kinase

Na^+ - Sodium ion

nAChR - Nicotinic acetylcholine receptors

NMJ - Neuromuscular Junction

PBS - Phosphate Buffered Saline

PKC - Protein kinase C

PLC - Phospholipase C

PLP - Periodate-Lysine-Paraformaldehyde fixative

PNF - Purine nucleoside phosphorylase

TCR – T cell receptor

TIMG - Toxin induced *Myasthenia gravis*

$TCD4^+$ - Effector T cells

$TCD4^+CD25^+$ - Activated T cells

$TCD4^+CD25^+FoxP3^+$ - Regulatory T cells

TMR-α-BTX - α-BTX peptide conjugated with tetramethyl rhodamine

VAChT - Vesicular ACh transporter

R97-116 - Single peptide corresponding to region 97-116 of the rat nAChR α subunit

SNARE - Soluble N-ethylmaleimide sensitive factor attachment receptor

SV - Synaptic vesicle

VSCC – Voltage-sensitive calcium channel

1. Introduction

1.1. *Myasthenia gravis*

Myasthenia gravis (MG) is a B-cell mediated, T-cell dependent chronic autoimmune disorder characterized by impairment of the neuromuscular transmission due to an autoimmune attack to acetylcholine receptors (AChRs) present in the postsynaptic membrane at the skeletal muscle. However, other non-AChR components of the neuromuscular junction (NMJ), such as the muscle-specific receptor tyrosine kinase (MuSK) may also be targeted (Engel *et al.*, 1977; Juel & Massey, 2007; Lindstrom *et al.*, 1976).

The antibodies block the binding site of the endogenous ligand of nAChR (nicotinic acetylcholine receptors) or even induce the loss of effective nAChRs expression by triggering a complement-mediated inflammatory destruction of the post-synaptic membrane of skeletal muscle cells at the motor endplate (Figure 1) (Tüzün *et al.*, 2003). Furthermore, the typical deep junctional folds are replaced by a relatively flat surface. The breakdown of self-tolerance in the thymus apparently leads to the development of anti-AChR autoantibodies (Baggi *et al.*, 2012; Melms *et al.*, 2006; Newsom-Davis *et al.*, 1981) with induction or activation of AChR-specific $CD4^+$ T helper cells and production of pro-inflammatory cytokines, consequently leading to the synthesis of high-affinity antibodies (Hoedemaekers *et al.*, 1997; Vincent *et al.*, 2003). Therefore, T cells play a pivotal role in MG since they lead the attack to the endplates by recognition of the antigen coupled to the major histocompatibility complex (MHC) class II molecules, promoting B cell production of anti-AChR antibodies by plasmocytes (Aricha *et al.*, 2006; Juel & Massey, 2007; Vincent *et al.*, 2003).

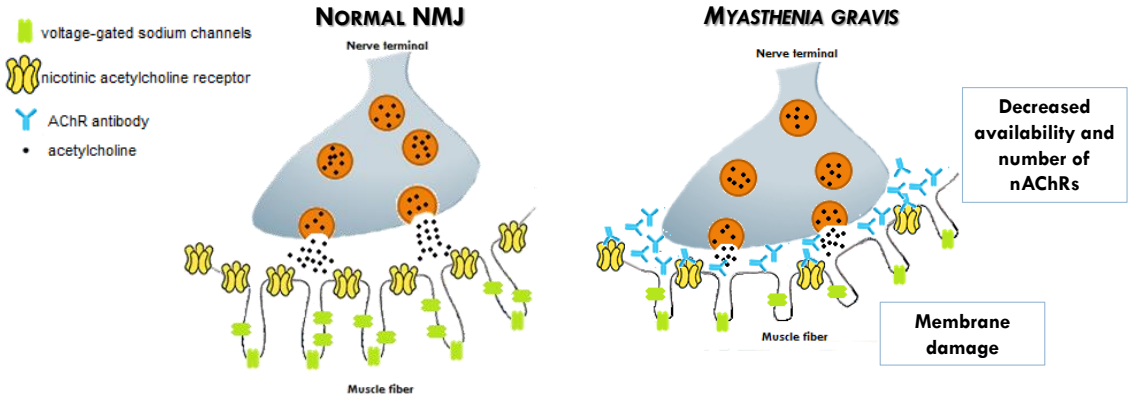


Figure 1 - Binding of the antibodies (anti-AChR) leading to a decreased availability and number of nAChR and destruction of muscular membrane (via activation of complement system) are the hallmarks of MG development. Adapted from Burden (2011).

Recent prevalence rates estimates that there are approximately one million MG patients worldwide (Gilhus *et al.*, 2011). Patients with MG present muscle weakness and fatigability, due to a neurotransmission impairment of signals from nerve to muscle. The elements of MG diagnosis include clinical history and examination findings of fluctuating and fatigable weakness. Signs of MG, typically involve ptosis (weakness of the upper or lower eyelid), diplopia (blurred vision), bulbar weakness (causing chewing difficulty) and, in extreme cases, respiratory muscle failure. The majority of seropositive patients have an abnormality of the thymus gland, either thymic hyperplasia (60%) or a thymoma (10%), although the exact relationship between the gland and MG largely remains obscure (Hirsch, 2007). Moreover, as previously referred, most of MG patients (85%) have IgG autoantibodies against binding sites on the AChR, while a minority of patients possess antibodies against MuSK and LRP4, other muscle endplate proteins (Cenacchi *et al.*, 2011; Gertel-Lapter *et al.*, 2013; Mossman *et al.*, 1986).

In normal situations the quantal content of neuromuscular transmission is higher than necessary to generate the action potential of the muscle fiber. However, in individuals with MG, the endplate potential generated by repetitive stimulation of the nerve gradually decreases to levels below the threshold of excitability of the muscle fiber (Kothari, 2004).

1.1.1. Therapeutic approach in *Myasthenia Gravis*

According to Gilhus *et al.* (2011), in MG the therapeutic opportunities can be defined according to the following clinical hallmarks:

1) MG is a well-defined autoimmune disease and consequently responds to immunosuppressive disease-modulators;

2) MG is due to an impairment of AChR stimulation in the postsynaptic skeletal muscle membrane and therefore responds to an increase in AChR activity;

3) MG is characterized by muscle weakness, thus should respond to therapeutics that increase muscle function and counteract this symptom.

Accordingly, neuromuscular function reestablishment could be achieved by two different approaches: symptomatic (acetylcholinesterase inhibitors) and the usage of immunosuppressive disease-modulating treatment (*eg* corticosteroids, azathioprine and monoclonal antibodies). Nowadays, crisis treatment involves the usage of acetylcholinesterase inhibitors (pyridostigmine, neostigmine and physostigmine). These drugs increase the availability of neurotransmitter by slowing down ACh hydrolysis, improving the contractile response. Thus, its action may lead to an increased margin of safety of neuromuscular transmission and consequently to activation of nicotinic and

muscarinic receptors present in motor nerve terminals (Faria *et al.*, 2003; Oliveira *et al.*, 2002). However, as they act on all known acetylcholinesterases its toxicity is associated with cholinergic crisis, which is very similar to MG crisis (Kothari, 2004; Romi *et al.*, 2005)

The second approach (immunosuppressive therapy) is increasingly common, being established in even earlier stages of disease progression. It acts at several levels of the immune system, inhibiting both humoral and cellular immunity and reducing the destruction of the postsynaptic membranes.

In clinically stable patients aged less than 60 years or with thymoma, thymectomy is established as part of the treatment. On the other hand, plasmapheresis can also be considered for removal of circulating antibodies or immunomodulation which consists in injecting intravenously immunoglobulins that bind to the anti-AChRs antibodies (Kothari, 2004; Romi *et al.*, 2005). At present, few attempts have been made to manipulate pharmacologically the pre-synaptic component of the neuromuscular junction. However, the application of the cardiotoxic potassium-blocking agent, 4-aminopyridine, proved to be able to improve neuromuscular transmission in MG (Lundh *et al.*, 1979).

Since these therapies have quite short-term benefits (Juel & Massey, 2007), it is critical to find new therapeutic strategies with less side effects.

1.2. Animal models for the study of *Myasthenia gravis*

Animal models became a pivotal tool to better understand the underlying mechanisms of several diseases. Accordingly, the experimental autoimmune myasthenia gravis (EAMG) model has been proven to be a valuable model to understand the immunological and molecular aspects of MG pathogenesis. In this model, a very common approach is to inject antibodies raised against nAChR into the animal and/or their immunization with nAChRs isolated from *Torpedo californica* (Aricha *et al.*, 2006).

Baggi and collaborators (2004) described the induction of EAMG in *Lewis* rats by the injection of a synthetic peptide corresponding to the region 97-116 of the rat AChR subunit in Complete Freund's Adjuvant (CFA) – a mixture of oils and water plus killed *Mycobacterium tuberculosis* strain, used to stimulate immune response. They found that breaking of tolerance to a single T cell epitope of the self autoantigen induces autoreactive T cells and specific antibodies to rat AChR.

The EAMG model consists in a practical instrument for testing the ability of possible treatment methods for MG and other antibody-mediated autoimmune diseases.

However, experimental MG differs from human disease in a few features (Table 1). Despite, myasthenic patients commonly present thymic alterations, suggesting a potential role of the thymus in the pathogenesis of the disease (Meinl *et al.*, 1991), induced animals develop EAMG after AChR-immunization and the auto-sensitization process seems to occur only in draining lymph nodes (Christadoss *et al.*, 2000), apparently without affecting the thymus, as in MG patients.

Table 1 – Similarities and differences between MG and EAMG. Adapted from Baggi *et al*., (2012).

	Similarities	Differences
Immunopathological features	Presence of anti-AChR antibodies in the serum Deposits of IgGs and C3 complement component at the NMJ Loss of muscle nAChRs MHC class II-restricted presentation of AChR epitopes Involvement of T helper cells in B-cell antibody production	Disease does not arise spontaneously in animals; need for induction factors Involvement of the thymus (present in MG, absent in EAMG) Thymic alterations are absent in EAMG; hypertrophy and thymomas are often present in MG patients Phagocytic cells detected in the acute phase of rat EAMG, are absent at the NMJ of human MG patients
Clinical manifestations	Muscle weakness, most prominent in the upper body Decreased response in the repetitive nerve stimulation test Reduction in the miniature end-plate potential amplitude Temporary improvement in muscle strength after anti-AChE treatment (Tensilon test) Increased sensitivity to curare administration	Absence of ocular signs Absence of relapse and remission periods

It is also possible to induce non-immunogenic MG in animals by administration of AChR-blocking toxins (e.g. α-Bungarotoxin) (Molenaar *et al.*, 1991), which is named *Toxin-Induced Myasthenia gravis* (TIMG). In this model, rats receive repeated injections of α-bungarotoxin (α-BTX) for 3 weeks (one subcutaneous injection of 3-5μg of α-BTX each 48h). α-BTX is well established as an irreversible antagonist of muscle nAChR containing α1 subunits. Plomp and colleagues (1992) have demonstrated that the number of functional nAChRs in rat hemidiaphragms was significantly reduced after 2-3 weeks of α-BTX treatment and an increase in the levels of neurotransmitter, without evidencing a structural damage of muscle membranes and/or changes in the endplate AChE activity (Van Kempen *et al.*, 1999). Given that in EAMG animals occurs a destruction of the postsynaptic membrane in muscle, it precludes the evaluation of neurotransmission features accurately. So, in this point of view, TIMG model is a better tool when we aim to understand the underlying molecular mechanisms behind the neuromuscular transmission deficit.

1.3. The neuromuscular junction: a specialized synapse

The NMJ is a specialized chemical synapse between the axon of a motor neuron and a somatic muscle fiber, which purpose consists on efficiently transmitting electrical impulses originated in motor neuron to the skeletal muscle via the chemical transmitter acetylcholine (ACh), which results in its contraction. In healthy conditions, the NMJ is an integral part of an impressively efficient biological amplification system, which converts minute nerve action potentials into muscle contraction (Bowman, 2006; Robitaille *et al.*, 1999; Rochon *et al.*, 2001; Ruff, 2003).

The development of advanced techniques (including electron microscopy and *in vitro* neurophysiologic studies) has considerably improved our knowledge of the microanatomy and physiology of the NMJ. The NMJ comprises portions of three distinct cells (tripartite synapse): the motor neuron (which accumulates mitochondria and synaptic vesicles (SVs)), the skeletal muscle fiber (corresponding to AChR-rich postsynaptic endplate) and the Schwann cell (that caps the motor nerve) (Figure 2). At mature NMJs it is possible to notice the emergence of secondary specializations that enhance neurotransmission and signal transduction. It involves the formation of active zones along the junctional surface, while organelles distribute themselves inside the nerve terminal asymmetrically to the synaptic cleft. Consequently, the formation of secondary clefts creates folds in the postsynaptic membrane where the nAChRs are clustered, while voltage-gated sodium channels concentrate in the depths of these folds (Hughes *et al.*, 2006).

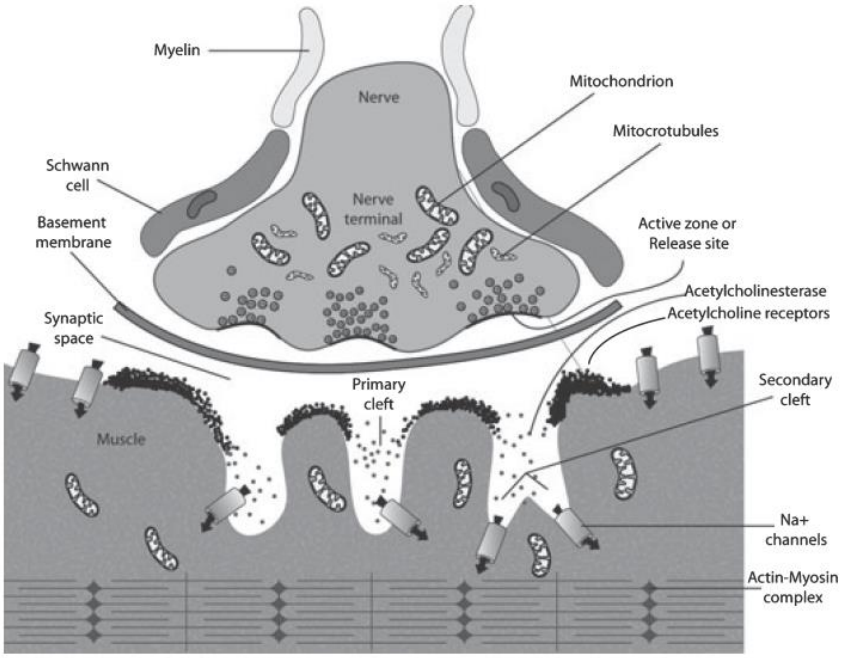


Figure 2 – Structure of the neuromuscular junction with prominence for its main components: the nerve terminal (presynaptic region), terminal Schwann cell and the muscle cell (specialized postsynaptic membrane). All three parts of the synapse contain organelles and molecules not found in extrasynaptic regions, or preferentially expressed when compared with extrasynaptic regions (Martyn *et al.*, 2009).

1.3.1. Presynaptic structure and function

The presynaptic region consists of the distal part of the motor neuron, corresponding to the distal and demyelinated part of the motor nerve axon (Fagerlund & Eriksson, 2009). The axon plays an essential role in this structure, since it innervates one or several muscle cells creating a cleft between its axon terminals (presynaptic part) and the sarcolemma of the muscle cell (postsynaptic part). The concept of motor unit arises from the combination of the terminal fibers from a motor axon with the muscle fibers. When a nerve impulse reaches an axon ending, it results in the release of a neurotransmitter – ACh – which will bind to nAChRs located on the postsynaptic surface. ACh is synthesized in the cytoplasm of nerve terminals, and results from the reaction between its two immediate precursors: choline and acetyl coenzyme A (acetyl CoA). This single step reaction is catalyzed by an enzyme called by choline acetyltransferase (ChAT). ChAT is produced in the cholinergic cell body and transported down the axon to the nerve endings. Synaptic vesicles (SVs) containing ACh molecules are located across from the ACh-rich synaptic folds and are aligned near release sites denominated active zones. The content of a single vesicle is referred to as a ‘quantum’ of the transmitter. The uptake of ACh into storage vesicle occurs through an energy-dependent pump that acidifies the vesicle. The acidified vesicle then uses a vesicular ACh transporter (VAChT) to exchange protons for ACh molecules.

When an action potential reaches the nerve terminal, the voltage-gated calcium (Ca^{2+}) channels are activated triggering the fusion of SVs with the presynaptic endplate membrane and the ACh is released into the synaptic cleft.

Thus, this presynaptic part of the NMJ is responsible for the efficiency of the neurotransmitter synthesis and transmitter incorporation.

1.3.2. Synaptic cleft structure and function

The synaptic cleft corresponds to a space of approximately 50nm that separates nerve terminal and specialized postsynaptic membrane. This structure is comprised of basal lamina, which contains several complex proteins (*e.g.* agrin) that have a key role in processes like integrity, formation, and clustering of the postsynaptic ACh receptors (Hirsch, 2007).

The release of ACh into the synaptic cleft may be spontaneous or in response to a nerve impulse. After its diffusion, about 50% of the released ACh is either hydrolysed by acetylcholinesterase (AChE) or disperses out of the cleft before it reaches its target. AChE is synthesized in the neuronal cell body and distributed throughout the neuron by axoplasmic transport. This enzyme has a remarkable catalysis rate, one of the highest known in biology, ensuring a fast decrease of ACh levels after its release and, thus, preventing the desensitization of postsynaptic nAChRs. It is well known that drugs that inhibit AChE, such as pyridostigmine and edrophonium, prolong the duration of action of ACh on the postsynaptic compartment and are useful therapies for neuromuscular transmission disorders, as MG (Hirsch, 2007; Hughes *et al.*, 2006).

1.3.3. Postsynaptic membrane structure and function

The postsynaptic component involves a folded muscle membrane, located opposite to the presynaptic nerve terminal, into which nAChRs, present at an extremely high concentration (> 10000/μm^2), are clustered and fixed by cytoskeletal proteins, as dystroglycans (Fagerlund & Eriksson, 2009). Recent studies have reported that P2X2-receptor-mediated signaling could be related with abnormalities in neuromuscular junction structure and skeletal muscle function, having a pivotal role in the formation of postsynaptic structures (Ryten *et al.*, 2007).

The safety factor for the neuromuscular transmission is ensured by the high concentration of receptors, being that away from the endplate the density of nAChRs is one thousand times lower (Paton & Waud, 1967). The fact that this part of the muscle membrane is in close proximity with the perijunctional zone, where there is a high density of sodium channels, allows this region to be more capable of amplifying the

responses to depolarization and consequently to promote the transduction processes that lead to muscle contraction (Cohen-Cory, 2002).

The nAChRs belong to the superfamily of ligand-gated ion channels that includes $GABA_A$, glycine, and $5\text{-}HT_3$ serotonin receptors (Dani & Bertrand, 2007). The common architecture of these receptors includes five subunits surrounding a central pore, organized in a pentameric unit (α2βεδ), arranged in a barrel-like fashion which allows the formation of a transmembrane pore on the top of the postsynaptic folds. In the absence of ACh, the central pore remains impermeable to the flow of cations (Figure 3). On the other hand, activation of the nAChR by two molecules of ACh that will bind to the N-terminal domain of the interface between α/β and α/ε subunits leads to an influx of cations (i.e. Na^+ and Ca^{2+}) that will depolarize the cell membrane (Hughes *et al.*, 2006).

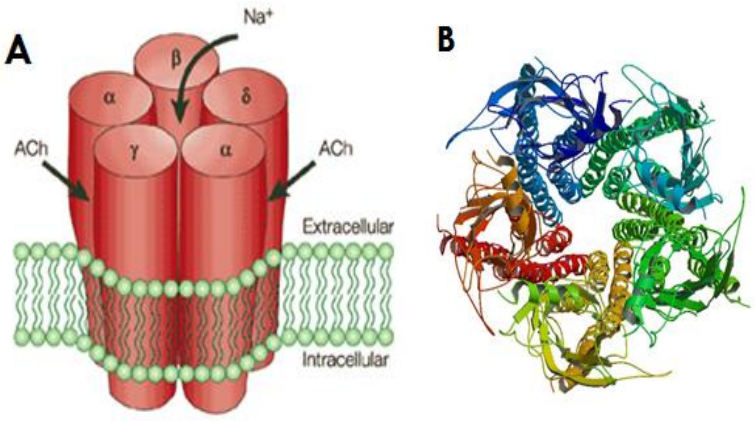


Figure 3 – Structure of the nicotinic acetylcholine receptors. ***(A)*** Schematic representation of the nACh receptor, showing that the five subunits in the muscle-type receptor join themselves to form a complex structure comprising 20 transmembrane domains that surround the axial cation-conducing channel. This is also possible to note the location of the two ACh-binding sites. Adapted from Karlin (2002). ***(B)*** Crystal structure of the nicotinic acetylcholine receptor (image obtained from Protein Data Bank (PDB) - PDB entry 2bg9).

1.4. Muscle fiber types: differences and similarities

In mammalian skeletal muscle exist different fiber types, classified as either fast-twitch or slow-twitch, and whose identity is dependent of the action of intrinsic myogenic control mechanisms that occur during embryogenesis and later controlled by neural and hormonal factors (Schiaffino & Reggiani, 2011). Specialization of skeletal muscle cells to several functional roles are related to their metabolic and contractile properties, being closely associated to which cells are grouped into separate fiber types (Rivero *et al.*, 1998).

The slow-twitch fibers (slow-oxidative), also called type I fibers, have an important role in aerobic type activities and therefore are used for endurance activities. These

fibers contain an increased number of mitochondria and myoglobin, and are capable of utilizing oxygen for the production of energy within the muscle, which makes them moderately resistant to fatigue.

Conversely, fast-twitch muscle fibers (type II) have less mitochondria and consequently develop force faster but have more difficulty in sustaining their activity than slow-twitch fibers (Prakash *et al.*, 1996). This fact makes them more suited for anaerobic activities such as weight training, sprinting, jumping and other intense type activities. Since these fibers are preferentially anaerobic, their system uses glucose as a prime energy source. Lactic acid is a sub-product of this reaction due to the continued dependence on glycolysis to produce adenosine 5'-triphosphate (ATP), resulting in a drop in the intracellular pH. This product accumulation in the muscle is responsible for fatigue and soreness, because as the pH drops, the ability of the muscle to produce ATP also diminishes. Type II fibers are further sub-classified as type IIa (or fast-oxidative glycolytic) or type IIb (or fast glycolytic) and also in IIx fibers (Prakash *et al.*, 1996) according to immunohistochemical techniques (Brooke & Kaiser, 1970; Rivero *et al.*, 1998).

Table 2 – Structural and functional properties of skeletal muscle fibers. Adapted from (Marieb & Hoehn, 2009).

	Slow oxidative fibers	**Fast oxidative fibers**	**Fast glycolytic fibers**
METABOLIC CHARACTERISTICS			
Speed of contraction	Slow	Fast	Fast
Myosin ATPase activity	Slow	Fast	Fast
Primary pathway for ATP synthesis	Aerobic	Aerobic (some anaerobic glycolysis)	Anaerobic glycolysis
Myoglobin content	High	High	Low
Glycogen stores	Low	Intermediate	High
Recruitment order	First	Second	Third
Rate of fatigue	Slow (fatigue-resistant)	Intermediate (moderately fatigue-resistant)	Fast (fatigable)

The neuromuscular transmission response can be closely dependent of the morphological differences present at the NMJ of different fiber types. Figure 4 reveals the histochemical study of samples from rat hemidiaphragm muscle by the succinate dehydrogenase (SDH) reaction. SDH is located in the inner mitochondrial membrane

and is involved in the Krebs cycle, being responsible for the oxidation of succinate to fumarate. The activity of SDH allows distinguishing type I and II muscle fibers by their relative oxidative potential. Thus it is possible to identify fibers with a higher (type I) or less (type II) amount of mitochondria by the color intensity.

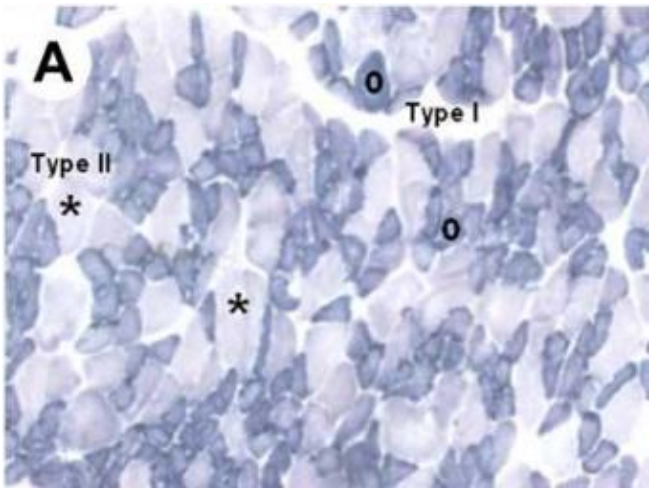


Figure 4 – Succinate dehydrogenase (SDH) staining of diaphragm muscle cross-section from a control rat. Asterisks point to large fibers which stained lightly with SDH (fast glycolytic type II fibers) and circles indicate smaller fibers stained darkly with SDH (slow oxidative type I fibers). Adapted from Correia-de-Sá *et al.* (2013).

An elegant study carried out by Prakash and collaborators (1996) showed that, in rat diaphragm muscle, type I fibers were innervated by the smallest axons and that their respective nerve terminals were small and less branched. In contrast, type IIa, IIx and IIb fibers were innervated by progressively larger axons and had a broader variability in nerve terminal morphology. Thus, there is a greater number of active zones at presynaptic terminals in type IIx and IIb fibers, and hence, the total number of synaptic vesicles released in response to a nerve action potential is superior at these fibers compared with type I and IIa fibers (Rowley *et al.*, 2007).

Prakash *et al* (1996) also observed that nerve terminals and endplates have an increased size and complexity from type I to type IIa, IIx and IIb muscle fibers (Figure 5).

Figure 5– Representative greyscale images of rat motor endplates. Absolute planar areas of endplates, progressively increase from type I, IIa, IIx to IIb fibers. These morphological differences may reflect differences in activation and in neuromuscular transmission (adapted from Prakash *et al*., 1996).

Furthermore, they observed a progressive decrease in the extent of overlap of pre- and postsynaptic elements of the NMJ on these fiber types. This is an important remark since a superior extent of overlap would imply a smaller diffusion path for ACh in type I fiber NMJs, and consequently will lead to a lower probability of neurotransmission failure. In opposition, a reduced extent of overlap in type IIa, IIx and IIb fibers at NMJ could significantly decreases the safety margin of the neuromuscular transmission (Wood & Slater, 2001), thus leading to its impairment.

Recently, our group provided evidences that myotoxic damage caused by a component of snake venom (Bothropstoxin-I) in hemidiaphragm preparations induce alterations in the area of type II fibers, whereas no changes were detected for type I fibers, suggesting a more resistant profile to myotoxic damage of slow oxidative fibers (Correia-de-Sá *et al.*, 2013). In agreement with this, higher incidence of neuromuscular transmission failure in type II fibers was reported (Gertler & Robbins, 1978; Johnson & Sieck, 1993).

1.5. Adenosine as a neuromodulator

Adenosine (ADO) is a ubiquitous molecule and an essential component of all living cells. This nucleoside is involved in key processes of the primary metabolism, especially the metabolism of nucleotides, nucleosides and amino acids that have sulfide groups and in the modulation of cellular metabolic state (*e.g.* transmethylation reactions and ammonia processing) (Cunha, 2001; Cunha, 2005; Stone, 1985). The first description that suggests that ADO and its precursor, adenosine triphosphate (ATP) could affect neuronal function has been advanced by Drury and Szent-Gyorgyi (1929). Later studies in the neuromuscular junction (Ginsborg & Hirst, 1972; Ribeiro & Walker, 1973) and cortical neurons (Phillis *et al.*, 1974) have shown that actually ADO plays a neuromodulatory role.

ATP is stored in synaptic vesicles and can also be released by nerve terminals during depolarization (Zimmermann, 1994). Previous studies using NMJs from different species reported that nerve stimulation triggers the release of ATP from the motor nerve terminal to the synaptic cleft (Magalhães-Cardoso *et al.*, 2003; Santos *et al.*, 2003). Most commonly, ATP co-released with ACh from motor nerve terminals is metabolized extracellularly via the ecto-nucleotidase pathway that sequentially catabolizes ATP into AMP and then into ADO through the action of an ecto-5'-nucleotidase (Magalhães-Cardoso *et al.*, 2003), which is feed-forwardly inhibited by ATP and/or ADP (Cunha *et al.*, 1996a). Interestingly, at the NMJ, AMP can be alternatively deaminated into the inactive metabolite, IMP through the action of 5´-

AMP-deaminase at the rat NMJ, thus bypassing adenosine formation (Magalhães-Cardoso *et al.*, 2003). Moreover, ADO can either be released as such, from activated nerve terminals, Schwann cells and activated muscle fibers (reviewed in Cunha, 2005).

Although there are no evidences of accumulation of ADO in synaptic vesicles or the release of this molecule as a quantum, the presence and accumulation of extracellular ADO in the synapses is related to the release of neurotransmitters and also with the frequency and intensity of neuronal firing (reviewed in Cunha, 2005). Cunha and co-authors (1996a) and Wieraszko & Seyfried (1989) demonstrated that ATP release is greater the higher the frequency of nerve stimulation and the contribution of ADO derived from ATP increases by enhancing frequency nerve stimulation. On the other hand, the contribution of ADO released through equilibrative nucleoside transporters is predominant at lower nerve stimulation frequencies (Correia-de-Sá *et al.*, 1996; Cunha *et al.*, 1996b). In basal conditions, the intracellular concentration of ADO is typically around 10-50 nM in the cell types where it was so far quantified. When intracellular levels of ADO exceed its extracellular concentration, for example under stressful situations where the exacerbation of intracellular ATP consumption exceeds its capacity of rephosphorylation, transport through equilibrative nucleoside transporters is reversed, *i.e.*, there is an increase in the extracellular ADO (Geiger & Fyda, 1991). Extracellular adenosine can be inactivated by cellular uptake through the equilibrative nucleoside transporters (Geiger & Fyda, 1991) or by deamination to inosine by adenosine deaminase (ADA) (Correia-de-Sa & Ribeiro, 1996).

The extracellular adenosine is able to act on metabotropic adenosine receptors located in the cell membrane of neighbouring cells (as well as of the cell that released adenosine). The activation of the different types of adenosine receptors can then modify cell metabolism according to the set-up of adenosine receptors and to the primary metabolism of each particular cell type (Cunha, 2005). Although ADO does not meet all the requirements to be considered a neurotransmitter, it is able to modulate the activity of the nervous system at a presynaptic level, exerting its action through its specific receptors (Correia-de-Sá *et al.*, 1996; Cunha, 2001).

1.6. Adenosine receptors

Adenosine is a neuromodulator with the ability to exert its physiological effects via cell surface receptors.

Purinergic receptors were first formally described in 1976 (Burnstock, 1976). Two years later these receptors were divided into two groups: specific receptors for

nucleosides (P1 receptors) and nucleotides (P2 receptors), which mediate the physiological effects of ADO and ATP, respectively (Figure 6) (Burnstock, 1978).

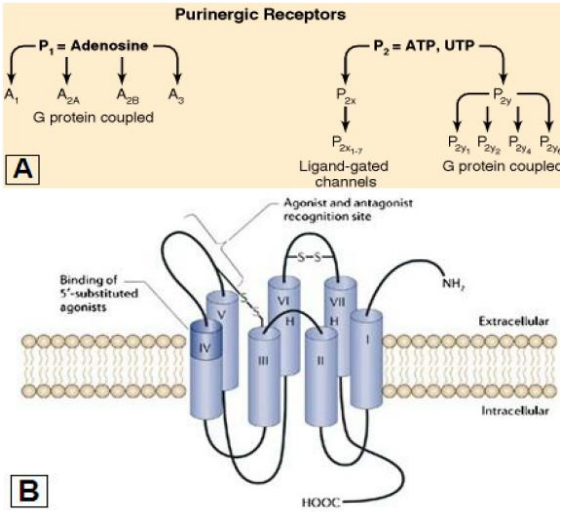


Figure 6 - The purinergic receptors family. ***(A)*** The purinergic receptors are divided into two major families: the P1, or adenosine, receptors and P2 receptors, which bind ATP and/or UTP. Adapted from http://www.ncbi.nlm.nih.gov/books/NBK27952/figure/A1251/?report=objectonly, accessed August 8, 2013. ***(B)*** Schematic representation of P1 adenosine receptors which are coupled to G-proteins that mediate responses through inhibition or activation of adenylate cyclase (AC). Adapted from Fields & Burnstock (2006).

ADO receptors have seven putative transmembrane (TM) domains and are coupled to heterotrimeric G proteins. There are four types of metabotropic receptors, denominated as A_1, A_{2A}, A_{2B} and A_3 receptors (Fredholm *et al.*, 2001). A_1 and A_3 receptors are coupled to $G_{i/o}$ inhibitory proteins, while A_{2A} and A_{2B} are coupled to G_s excitatory proteins (Linden, 2001; Ribeiro *et al.*, 2003). The binding of ADO to its receptor triggers a series of signal transduction mechanisms that are initiated by the receptor associated G proteins (Figure 7).

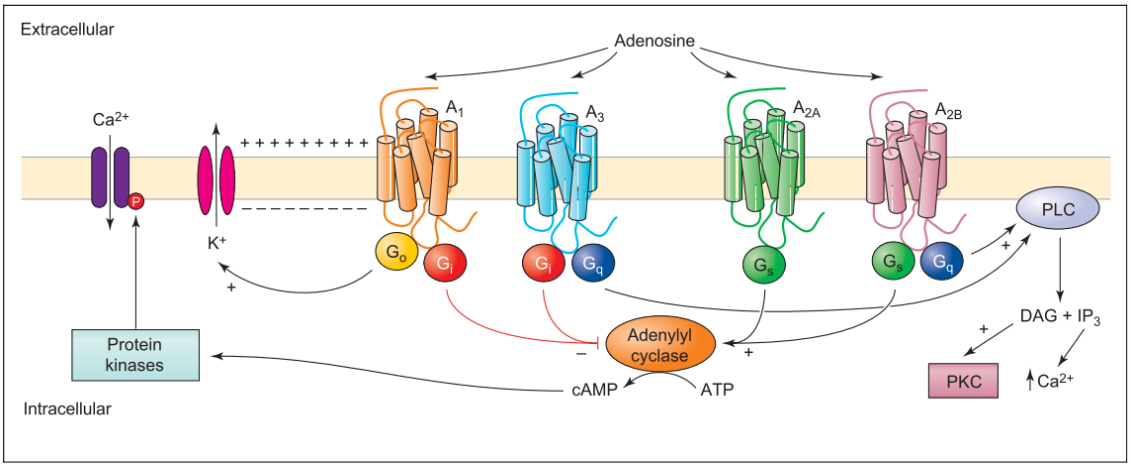


Figure 7 – Schematical representation of the signal transduction pathways associated with the activation of the human adenosine receptors. Abbreviations: ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; DAG, diacylglycerol; Gi, $G_{\alpha}i$

family of G proteins; Gs, G_{α}s family of G proteins; Go, G_{α}o family of G proteins; Gq, G_{α}q family of G proteins; IP3, inositol (1,4,5)-triphosphate; P, phosphate moiety; PKC, protein kinase C; PLC, phospholipase C. (Moro *et al.*, 2005).

The A_1 adenosine receptor is a well-characterized and widely distributed receptor that has an inhibitory action in most tissues. Its activation inhibits adenylate cyclase (AC) activity through the activation of pertussis toxin-sensitive G proteins, therefore leading to a cellular decrease of cAMP levels (Fredholm *et al.*, 2001; Linden, 1991). Moreover, when $G_{\alpha o}$ subunit dissociates occurs an increase of arachidonic acid and inositol triphosphate/diacylglycerol concentration as a result of phospholipase 2A and phospholipase C activation, respectively (Poulsen & Quinn, 1998). For instance, A_1 receptor function in cardiac muscle and neurons involve activation of pertussis toxin-sensitive K^+ channels and K_{ATP} channels and is responsible for inhibiting Q-, P- and N-type Ca^{2+} channels (Fredholm *et al.*, 2001; Jacobson & Gao, 2006).

A_{2A} receptors are coupled to stimulatory G proteins and can be found ubiquitously in the body, with a marked expression in the immune system and the striatopallidal system in the brain (Hasko *et al.*, 2008). Several studies reported the presence of A_{2A} receptors in the carotid body, T cells, human and porcine cardiovascular tissues, anglioblasts in the nerve fiber layer in dog retina, gastrointestinal tract and hippocampal nerve terminals evaluated by immunohistochemical techniques, reviewed by Fredholm *el al* (2002). This receptor activation mediates the activation of AC, leading to an increase of intracellular cAMP levels (Fredholm *et al.*, 2001), but other actions including mobilization of intracellular calcium have also been described (Fredholm *et al.*, 2002).

A_1 and A_{2A} receptors are responsible for the major effects exerted by ADO, namely at modulating synaptic transmission. Co-existence of both inhibitory A_1 and facilitatory A_{2A} receptors on the same nerve terminal was first described by neurochemical and electrophysiological methods at the rat NMJ (Correia-de-Sá *et al.*, 1991); this publication contained the first demonstration that ADO could facilitate the release of neurotransmitter via the activation of cyclic AMP-coupled A_{2A} receptors. At the NMJ, ADO plays a dual neuromodulatory role via the activation of presynaptic inhibitory A_1 and excitatory A_{2A} receptors, which activity is highly dependent on the nerve stimulation pattern (Correia-de-Sá *et al.*, 1996). Moreover, previous studies from our laboratory reported that endogenous ADO generated in TIMG motor endplates during repetitive nerve firing may be insufficient to preserve transmitter release via tonic activation of presynaptic facilitatory A_{2A} receptors (Noronha-Matos *et al.*, 2011).

1.7. Adenosine effects on acetylcholine release via A_1 and A_{2A} receptors

The modulatory pattern of neuromuscular transmission is adjusted to the stimulation conditions through the action of ADO, particularly when this nucleoside is build-up from the catabolism of released ATP (Magalhães-Cardoso *et al.*, 2003). The tonic inhibitory effect mediated by A_1 receptors is observed at low frequency stimuli. In contrast, tonic activation of A_{2A} receptors on motor nerve terminals is predominant during high stimulation frequencies, due to the accumulation of adenosine in the synaptic cleft which may overcome neuromuscular tetanic fade.

ADO by activating A_{2A} receptors is responsible for the fine-tuning control of Ca^{2+} influx through either P- or L-type channels (Figure 8) (Oliveira *et al.*, 2004). Previous studies from our laboratory reported that during short stimulation trains, $Ca_V2.1$ (P-type) Ca^{2+} channels clustered at active zones regulate nerve-evoked ACh release from adult mammalian motor nerve terminals. However, P-type channels function rapidly decline due to a Ca^{2+}-dependent inactivation system, thus contributing to a neurotransmission tetanic failure. Though, data indicated that tetanic depression of ACh release could be overcome during intermittent high-frequency bursts due to tonic activation of A_{2A} receptors operating additional Ca^{2+} recruitment via high-capacity/slow inactivating Ca_V1 L-type channels located away from the active zones. The predominance of inhibitory A_1/excitatory A_{2A} tonus is dependent on the stimulation pattern (train *versus* bursts), which also tightly regulates the amount of extracellular ADO formed from released ATP. In fact, the activity of the ecto-nucleotidase pathway appears to represent the rate-limiting step of formation of ATP-derived adenosine, since ATP is released synchronously with ACh. During 50 Hz-trains, ATP is able to reach high enough levels capable of inhibiting ecto-5'-nucleotidase (CD73). Interburst intervals favor recovery from enzymatic inhibition, as there is a delayed burst-like formation of ADO leading to high synaptic concentrations of the nucleoside similar to those required to promote the activation of facilitatory A_{2A} receptors. Altogether, these findings show that strong ADO facilitatory tonus operated by A_{2A} receptors on ACh release during tetanic stimulation, is closely related with time-dependent endogenous ADO formation from released adenine nucleotides and attenuation of the inhibitory action mediated by A_1 receptors. In parallel, there is a co-ordinated shift in Ca^{2+} channel dynamics operating ACh exocytosis, from the predominant fast desensitizing $Ca_V2.1$ (P-type) to the "facilitatory" Ca_V1 (L-type) channel during high frequency bursts, in a way completely reversed by blocking A_{2A} receptor activation. This mechanism represents a novel form of synaptic plasticity mediated by ADO at the rat motor endplate and offer new clinical prospects

by manipulating A_{2A} receptors activation in order to recruit spared voltage sensitive calcium channels (VSCCs) in order to overcome tetanic depression during intermittent neuronal firing (Oliveira *et al.*, 2004).

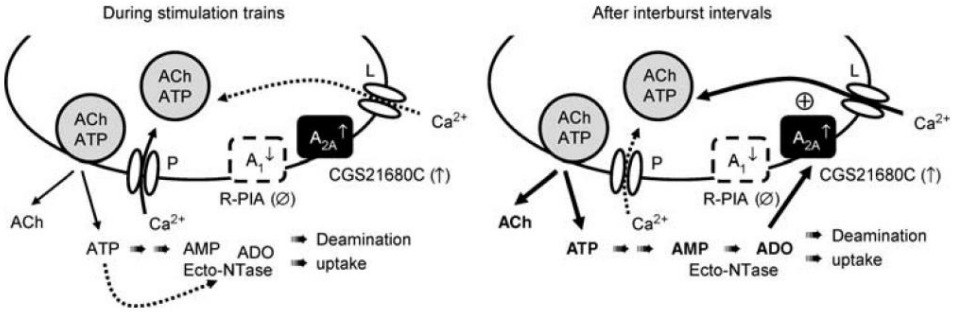


Figure 8 - Fine-tuning control of Ca^{2+} recruitment through P- and L-type VSCCs by endogenous adenosine generated during motoneuronal firing. Adapted from Oliveira *et al*., (2004).

Besides the neurotransmitter ACh, synaptic vesicles also contain other substances such as peptides, or ATP, being the entire contents released in the synaptic cleft. It is quite clear that there is an excess of released ACh into the synaptic cleft, around 10 times the necessary amount of ACh needed to achieve the postsynaptic ACh receptor threshold, which represents the high safety margin of neuromuscular transmission (Hirsch, 2007). Generally, one action potential results in the exocytosis of 50-300 SVs, still the release of a quantum is not always controlled. In the absence of an action potential, a small quantum of ACh is spontaneously released into the NMJ and cause very small depolarizations called miniature endplate potential (MEPP) at the end of the postsynaptic membrane.

Besides postsynaptic ACh binding to nAChRs, it can also exert its action at the pre-synaptic component regulating its own release through the activation of both muscarinic (M1 excitatory and M2 inhibitory) and nicotinic (containing α3β2 subunits) receptors (Oliveira *et al.*, 2002; Timóteo *et al.*, 2003). Moreover, functional studies show that several molecules, as adenosine (Burnstock, 2009; Ribeiro & Walker, 1975) and neuropeptides (Correia-de-Sá & Ribeiro, 1994; Correia-de-Sa *et al.*, 2001), can modulate ACh release.

1.8. Adenosine receptors in the control of the immune system

ADO is a nucleoside that is constitutively present at low levels in the extracellular space, but rapidly increases its concentration in conditions of ischemia, hypoxia, trauma and inflammation (Hasko *et al.*, 2008). A growing body of evidence confirms its important immunosuppressive role by acting through adenosine receptors, thus

regulating the immune system. Activation of T lymphocytes starts from the recognition of antigen by T cell antigen receptor and CD4 or CD8 co-receptors. After TCR stimulation, lymphocyte activation can result in T cell proliferation, cytokine secretion and cellular cytotoxicity (Gessi *et al.*, 2007).

Several studies indicate that A_{2A} receptors are the main purinergic actors in the regulation of lymphocytes response. In fact, A_{2A} receptors activation inhibits IL-2 secretion by naive $CD4^+$ T cells, leading to decreases in B cell proliferation (Naganuma *et al.*, 2006; Sevigny *et al.*, 2007), and partially restore the imbalance between Th1/Th2/Th17/T_{reg} cells (Li *et al.*, 2012). In this regard, A_{2A} receptors play an immunosuppressive action in $CD4^+$ T helper cells that are known to assist auto-reactive B cells to produce anti-AChR antibodies both in MG and EAMG model (Berrih-Aknin *et al.*, 2013; Conti-Fine *et al.*, 2008; Elson & Barker, 2000). Furthermore, it was found that the adenosine-A_{2A} receptor pathway mediates a negative feedback immunosuppressive mechanism that regulates properties of regulatory T cells (T_{reg}) (Ohta *et al.*, 2012). T_{reg} cells are produced in the thymus where they start expressing forkhead box P3 (FoxP3) transcription factor, which entitles these cells to act in the maintenance of immunological self-tolerance and in the regulation of immune responses by suppressing the proliferation and cytokine production of effector autoreactive T cells that arise *de novo* or escape thymic deletion (Becker *et al.*, 2006; Hasko & Cronstein, 2013; Miyara & Sakaguchi, 2007). Most endogenous $CD4^+$ T_{reg} cells constitutively express the CD25 molecule (IL-2 receptor α-chain) (Aricha *et al.*, 2008). The trigger to these cells activation is believed to be related with high-affinity interactions that occur between TCRs and class II MHC-peptide complexes in thymus, which “instruct” developing thymocytes to up-regulate FoxP3 and become T_{reg} cells (Kuczma *et al.*, 2009). In autoimmune disorders, such as MG, it has been reported decreased levels of FoxP3 expression, which is correlated with an impaired T_{reg} function (Balandina *et al.*, 2005; Zhang *et al.*, 2009). Interestingly, A_{2A} receptors stimulation was reported to up-regulate FoxP3 mRNA (Zarek *et al.*, 2008). T_{reg} cells also express high levels of CD39 and CD73 ecto-NTPDases on the surface of FoxP3 $T_{regs,}$ whose action leads to extracellular adenosine production from released adenine nucleotides; on its own, the nucleoside will exert its immunosuppressive action on $CD4^+CD25^-$ T cells by acting on A_{2A} receptors (Figure 9) (Hasko & Cronstein, 2013). Consequently, A_{2A} receptors exert its effects on many aspects of T-cell function as (Hasko *et al.*, 2008; Milne & Palmer, 2011):

a) Inhibiting T-cell activation and proliferation;
b) Inhibiting proinflammatory cytokine production;
c) Enhancing the production of anti-inflammatory cytokines;

d) Supressing proliferation of developing and mature Th1 and Th2;

e) Promoting the generation of T_{regs} by up-regulating FoxP3 expression.

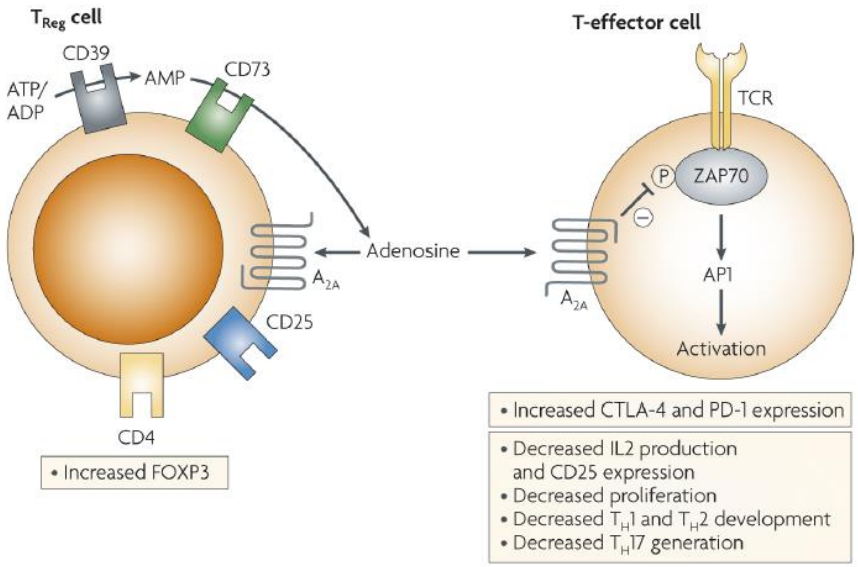


Figure 9 - Adenosine mediates immunosuppression and is generated by the ectonucleotidases CD39 and CD73 that are expressed on the surface of FoxP3 T_{regs}. This nucleoside binds to adenosine A_{2A} receptors: ***A)*** increases FoxP3 expression levels in these cells and ***B)*** inhibits T effector-cell proliferation and consequently B cell activation. Adapted from (Hasko *et al.*, 2008).

Thus, although the autoantibodies produced by B cells play a central role in the pathogenesis of MG, $CD4^+$ T cells (more specifically T_{regs}) and A_{2A} receptors are increasingly assumed as viable targets for future neuroimmune-orientated therapeutic strategies of autoimmune myasthenic disorders.

Aricha and collaborators (2008) described that T_{regs} cells transferred from healthy rat donors to myasthenic rats suppressed EAMG when treatment was initiated at the acute phase of disease. However, the same suppressive activity of T_{regs} was not observed for EAMG rats when compared to healthy donors, being an indicator of impairment in the T_{reg} cell compartment in EAMG. Moreover, a recent study from this group reported that rats treated with antibodies to IL-6, a regulator of T_{reg}/Th17 balance, suppress EAMG at acute and chronic phase of the disease (Aricha *et al.*, 2011). They also reported a reduction in the frequency of $CD4+CD25^{high}$FoxP3 cells in peripheral blood lymphocytes of myasthenic rats at an initial stage of the disease as compared to control littermates (Aricha *et al.*, 2008).

It was demonstrated that T cells from EAMG rats express less FoxP3 than healthy rats. It was also found that T_{reg} cells can improve disease manifestations in a

preventive regime. Regarding their use as a therapeutical approach, these cells failed to significantly alter the course of the disease (Nessi *et al.*, 2010).

The impairment of suppressive function by T_{reg} cells from EAMG rats was also reported by Gertel-Lapter *et al.* (2013), where they demonstrated that T_{regs} have a lower ability to suppress the proliferation of T effector (T_{eff}) and B cells in response to polyclonal activation in co-cultures of T_{reg}/T_{eff} cells, as compared with control littermates.

Another line of research proposed to investigate if A_{2A} receptors activation with an agonist (CGS21680) could modulate ongoing EAMG (Li *et al.*, 2012). The first finding was that A_{2A} receptor expression was reduced in both T and B cells from spleen and lymph nodes of myasthenic rats. Thus, we can conclude that the immunosuppressive effect mediated by A_{2A} receptors is compromised. Also, *in vitro* stimulation of A_{2A} receptors with a selective agonist inhibited the production of antibodies against nAChR and the proliferation of AChR-specific lymphocytes. This work further indicates that A_{2A} receptors stimulation is able of increasing T_{reg} cell levels and changed the profile of Th1, Th2 and Th17 cells. This therapeutic strategy proved to be similar to the previous approaches, in other words, a preventive treatment proved to be more efficient than a therapeutic one.

2. Aim

It is becoming increasingly appreciated that drugs targeted on adenosine pathways can exert beneficial effects in neuroimmune disorders.

In the presence of immune and neuronal challenges, the adenosine system acts as a sensor, which through dynamic action between ecto-enzymes and adenosine receptors, adapts neuronal and immunological responses to the stimulation conditions. In light of this, it is likely that in the context of diseases, induction of the mechanisms associated to adenosinergic pathway may be dysfunctional. Our main goal is to unravel the pathophysiological role of adenosinergic pathway, in particular A_{2A} receptors, on autoimmune *Myasthenia gravis*.

Unraveling of these mechanisms may give important insights through which pharmacological modulation of the adenosine pathway may have potential application in *Myasthenia gravis* therapeutic management.

Considering that the EAMG model shares the majority of the cardinal features of human *Myasthenia gravis* our studies were conducted in *Wistar* rats immunized by a single peptide corresponding to aminoacids 97-116 of the rat nAChR α subunit. Our model of breakdown of self-tolerance in *Wistar* rats, was screened for signs of immunological and neuronal imbalance by evaluating (1) serum ADA activity, (2) FoxP3 expression in $CD4^+CD25^+$T-cell populations (T_{reg}), (3) tetanic failure (fatigue) of diaphragm muscle contractions by indirect phrenic nerve stimulation with intermittent 50 Hz-bursts and (4) morphological alterations of motor nerve endplates.

Considering the proposed hypothesis we evaluated A_{2A} receptor density and distribution through cell compartments present on motor endplates and $CD4^+$ T cell population from EAMG animals by immunofluorescence confocal microscopy and flow cytometry, respectively. Regarding the mechanisms that accounts for A_{2A} receptors neuroexcitatory and immunossupressor actions like the mobilization of extracellular Ca^{2+} through Ca_V1 channels (Oliveira *et al.*, 2004) and the amount of ADO production through T lymphocytes membrane bound ecto-5'-nucleotidase (CD73) enzyme, respectively, we sought to evaluate immunoreactivity against Ca_V1 channels on motor nerve endplates and to ecto-5'-nucleotidase (CD73) enzyme on $CD4^+$ T cells population through their differential cell compartments.

3. Materials and Methods

3.1. Experimental Autoimmune *Myasthenia gravis* (EAMG) rat model induction and clinical assessment

The animals (*Wistar* rats) were handled as stated in the Portuguese Decree-Law nº113/2013 of 7th August concerning the protection of animals used for experimental procedures and for other scientific purposes. We also followed the rules of Federation for Laboratory Animal Science Associations (FELASA). *Wistar* rats were kept at a constant temperature (21ºC) and a regular light (06.30-19.30h) dark (19.30-06.30h) cycle, with food and water *ad libitum*. The rat induction was carried as follows (Baggi *et al.*, 2004): the animals (6-8 weeks) were anaesthetized and immunized subcutaneously at four sites (two hind footpads and shoulders) with 50 µg of R97-116 peptide – a synthetic peptide corresponding to a specific region on the α subunit of the rat nicotinic AChR – in 200 µl of CFA (Complete Freund's Adjuvant) on day 0 and were boosted on day 30 with the same peptide in 200 µl of IFA (Incomplete Freund's Adjuvant). On both immunizations PBS (phosphate-buffered saline) was also added, because it corresponds to the solution in which the peptide was reconstituted. CFA is an immunopotentiator consisting of an inactivated and dried antigen from *Mycobacterium tuberculosis*, emulsified in mineral oil, while IFA lacks *M. tuberculosis*. Control animals received CFA emulsion without the peptide and the naive group animals were not submitted to any sort of treatment. On the beginning of the induction protocol all control and naive animals presented the same age and similar weight as the animals conducted to the EAMG group. Each experimental animal was weighed and assessed for muscle strength by grip test at the beginning of the experiments and twice weekly until sacrifice 6 to 8 weeks later. Results are expressed as the mean of the evaluations for each animal at each time point.

3.2. EAMG clinical evaluation

Evaluation of disease manifestations in immunized and healthy (naive and control) rats was performed by assessing several parameters. Clinical scoring was based on the presence of tremor, hunched posture, muscle strength by grip strength test (BIOSEB, France), and fatigability after exercise (repetitive paw grips on the cage grid). Disease severity was expressed by assigning several degrees (Baggi *et al.*, 2004), as indicated in table 2.

Table 2 - Clinical score used to grade EAMG severity.

Clinical score	
Grade 0	Normal muscle strength and no muscle weakness
Grade 1	Normal at rest, but weak after exercise (chin on the floor; inability to raise head; hunched back)
Grade 2	Weakness at rest
Grade 3	Moribund, dehydrated and quadriplegic
Grade 4	Dead

3.3. Preparation and experimental conditions

The control, naive and EAMG animals were euthanized by decapitation and submitted to surgical isolation of the phrenic nerve hemidiaphragm. The experiments were performed using either left or right phrenic nerve-hemidiaphragm preparations (4-6mm width). Each muscle was superfused (5 $mlmin^{-1}$, 37°C, pH 7.4) with gassed (95% O_2-5% CO_2) Tyrode 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 37ºC (Correia-de-Sá *et al*., 1996).

3.4. Adenosine deaminase assay

The whole blood was collected from three different groups of rats after decapitation and was allowed to clot by leaving it undisturbed at 4ºC during approximately 6 hours. The portion of the sample that was separated from the clot was collected, and then centrifuged at 4,000 rpm for 20 minutes at 4ºC. The resulting supernatant is, from now on, designated as serum. Following centrifugation, the samples were separated in aliquots and stored at -20ºC until its analysis.

The determination of the catalytic activity of adenosine deaminase (ADA) in serum was carried out according to a kinetic colorimetric method based on hydrolytic action of ADA on adenosine to form inosine, which by the action of purine nucleoside phosphorylase (PNF), xanthine oxidase and peroxidase give rise to a colored compound whose formation may be monitored at 550 nm. The reaction is as follows:

$$Adenosine + H_2O \xrightarrow{ADA} Inosine + Ammoniac$$

$$Inosine + Pi \xrightarrow{PNF} Hipoxanthine + Ribose\,1-phosphate$$

$$Hipoxanthine + 2H_2O + 2O_2 \xrightarrow{\text{Xanthine oxidase}} Uric + 2H_2O_2$$

$$2H_2O_2 + EHSPT + 4-aminoantipyrine \xrightarrow{\text{Peroxidase}} Quinomeimine\,(550\,nm) + 4H_2O$$

While handling, the serum samples from all groups of animals were maintained at 2-8°C, which corresponds to the ideal temperature for preserving ADA stability. Hemolyzed samples were not considered in this determination. ADA activity was measured in a Cobas Mira S autoanalyser (Roche Diagnostics, Switzerland) at 37ºC, according to the method of Giusti (1974).

3.5. Myographic recordings

For tension response recordings, the innervated diaphragm strips were mounted in a 10-ml capacity isolated organ bath chamber. Tension responses were recorded isometrically at a resting tension of 50 mN with a force transducer and displayed on a Hugo-Sachs (Germany) recorder. These experimental conditions allowed a well-preserved contraction pattern for several hours after the initial stabilization period. Tetanic failure (fatigue) of diaphragm muscle contractions was induced by indirect nerve stimulation (intermittent 50 Hz-bursts).

3.6. Immunofluorescence staining and confocal microscopy observation

3.6.1. Tissue preparation

3.6.1.1. Tissue fixation and preservation

After dissection, muscle fragments were stretched to all directions, pinned onto Petri dishes coated with Sylgard® and incubated with Tyrode's solution continuously gassed without or with 0.1% collagenase (type I; Sigma Aldrich) for different periods of time (10, 20 and 30 min), in order to increase permeability of the antibodies at the NMJ. The collagenase solution was changed every 10 min and after the enzymatic treatment it was discharged and the hemidiaphragms were rinsed with Tyrode's solution. Tissues were then fixed in PLP solution (paraformaldehyde 2%, lysine 0.075 M, sodium

phosphate 0.037 M, sodium periodate 0.01 M) overnight at 4°C; washed 3 x 10 min with 0.1 M sodium phosphate solution; cryoprotected with cryoprotectant solution (anhydrous glycerol 20%, sodium phosphate 0,1 M) overnight at 4°C and stored in the cryoprotectant solution at -20ºC.

3.6.1.2. Tissue sectioning

At the time of sectioning, the selected tissue previously stored at -20°C was washed 10 min with 0.1 M sodium phosphate solution; embedded in a cryoprotective embedding medium (Thermo Scientific), and placed in a cryostat (Leica CM1950; Leica Microsysteems, Nussloch, Germany) at -25°C for 30 minutes to stabilize the temperature. Then, after the tissue is properly oriented, serial longitudinal sections of the muscle strips (45 µm) were cut.

3.6.1.3. Blocking and permeabilization

After sectioning, tissue fragments were incubated free floating, overnight at 4°C with a blocking buffer solution, consisting in foetal bovine serum 10%, bovine serum albumin 1%, Triton X-100 1% and 0,05% NaN_3 in PBS. Triton X-100, which is part of the composition of blocking buffer, increases membrane permeability thus facilitating the incubation of the tissues with antibodies (Ramos-Vara *et al.*, 2008). Incubation of the sections with blocking buffer is essential to prevent the formation of non-specific binding of the primary antibodies, thus reducing the probability of occurrence of nonspecific background labeling (Ramos-Vara, 2005). In order to evaluate the morphology of motor endplates, muscle strips were incubated with an α-BTX peptide conjugated with tetramethyl rhodamine (TMR-α-BTX diluted 1:1500; Molecular Probes) for 15 min; washed 2 x 10 min in PBS supplemented Triton X-100 0.3%, and 10 minutes in PBS; mounted on optical-quality glass slides using the antifading VectaShield medium (VectorLabs), and stored in the dark at 4ºC.

3.6.1.4. Antibody labeling

In this work the indirect immunofluorescence technique was performed in order to visualize the reaction that occurs between the antigen and antibody. This technique consists in the binding of the primary antibody to the antigen of interest, followed by the application of a secondary antibody coupled to a fluorophore that specifically recognizes the primary antibody (Ramos-Vara, 2005).

3.6.1.5. Incubation with primary antibodies

After blocking and permeabilization, samples were incubated with primary antibodies (see table 3) diluted in the incubation buffer (foetal bovine serum 5%, serum albumin 0.5%, Triton X-100 0.5% and 0,05% NaN_3 in PBS), at 4°C, for 48 h. For immunofluorescence double staining, antibodies were mixed before application to the samples.

Table 3– Primary and secondary antibodies used in immunohistochemistry experiments.

Antigen	Host	Code	Supplier	Dilution
Primary antibodies				
Adenosine receptor A_{2A}	Rabbit (rb)	A_{2A}R21-A	AlphaDiagnostics	1:75
$Ca_V1.2L$	Rabbit (rb)	ACC-003	Alomone Labs	1:100
Secondary antibodies				
Alexa Fluor 488 anti-rb IgG	Donkey	A-21206	Molecular probes	1:750

3.6.1.6. Incubation with secondary antibodies

After incubation, the sections were washed in PBS supplemented Triton X-100 0.3% (3 cycles of 10 min). Then, species-specific secondary antibodies (table 3) diluted in the incubation buffer previously referred, were applied to tissues samples overnight, at 4°C in the dark. All procedures from the time of incubation of these antibodies were performed in the dark to avoid excitation of fluorochromes. The secondary antibodies used must be chosen taking into account the host of the primary antibody, since they will specifically recognize its immunoglobulins. Another important aspect is the choice of fluorophores coupled to the secondary antibodies. In particular, while planning multiple label fluorescence staining protocols for laser scanning confocal fluorescence microscopy experiments, the judicious choice of probes is paramount in obtaining the best target signal while simultaneously minimizing bleed-through artifacts. After incubation with the secondary antibodies, the samples were incubated for 15 min at room temperature with the TMR-α-BTX peptide (1:1500; Molecular Probes) to provide nAChR detection and washed in 3 cycles of 10 min with PBS. Finally, tissue samples were mounted on optical-quality glass slides using the antifading VectaShield medium (VectorLabs), and stored in the dark at 4ºC. Non-specific binding was assessed by the

inclusion of negative controls by omission of primary antibodies from the reaction solution (Ramos-Vara *et al.*, 2008).

3.6.1.7. Confocal microscopy

Confocal microscopy allows acquiring images with an optimized and improved resolution, as compared to a conventional fluorescence microscope. The key feature of confocal microscopy is its ability to acquire in-focus images from selected depths which are acquired point-by-point and reconstructed with a computer, allowing three-dimensional reconstructions of topologically complex objects (Semwogerere & Weeks, 2005).

In this work, the glass slides were examined with a microscope confocal laser scanning Olympus Fluoview FV1000 (Tokyo, Japan) using the following lasers: a multi-line Argon, which emits in the range of 488nm and can therefore excite fluorophore Alexa Fluor 488 and a He-Ne laser, which emits at a wavelength of 543 nm and that will excite the TMR-α-BTX peptide. Tissue samples were subjected to a sequential scanning, and the images were acquired in multiple planes (Z-stack).

The images obtained were then processed using the software associated the confocal microscope, the FluoViewer FV10-ASW. Regarding the evaluation of the morphological component, the area (μm^2) of labeling by TMR-α-BTX was measured using the *Image J 1.46r* software (NIH, Bethesda, MD, USA).

3.7. Isolation and immunophenotypic characterization of $CD4^+$ T cells

3.7.1. Immunomagnetic positive selection of $CD4^+$ T cells

The ability to separate a heterogeneous cell population considering the cellular properties/characteristics is a relevant analytical tool to study functional and expression features of specialized cell types within complex biological systems. The characteristics that are considered to differentiate cell populations include DNA content, cellular pigment content, total protein content, intracellular pH, membrane organization and the key probe from a clinical point of view is the presence of specific surface markers (*e.g.* presence of CD4 and CD8 on lymphocytes) (Chalmers *et al.*, 1998).

According to the study which is pretended to be performed, a myriad of labels and “handles” can be chosen. The most specific labels include the ones that bind only to specific cell-associated molecules. In order to study the immune events associated with

the tripartite immunological synapse in MG, the need to purify $CD4^+$ T cells from heterogeneous cell population in lymph nodes arises.

Mononuclear cell suspensions from draining lymph nodes (popliteal and inguinal) were prepared by rubbing and pressing the lymph nodes between two microscope slides and subsequently filtered through pipettes with glass wool. After counting, the cells were magnetically labeled with CD4 microbeads (Miltenyi Biotech). Next, the cell suspension was loaded onto a MACS® LS column (Miltenyi Biotech) which was placed in the magnetic field of a MidiMACS separator (Miltenyi Biotech). The unlabeled cells run through and this cell fraction is depleted. $CD4^+$ cells stay attached to the column until the removal of the column from the magnetic field and immediately flush by firmly applying the plunger supplied by the manufacturer. This procedure is denominated positive immunomagnetic cell isolation (Figure 10). $CD4^+$ T cells total population were targeted for immunophenotypic characterization of the lymphocyte population by flow cytometry.

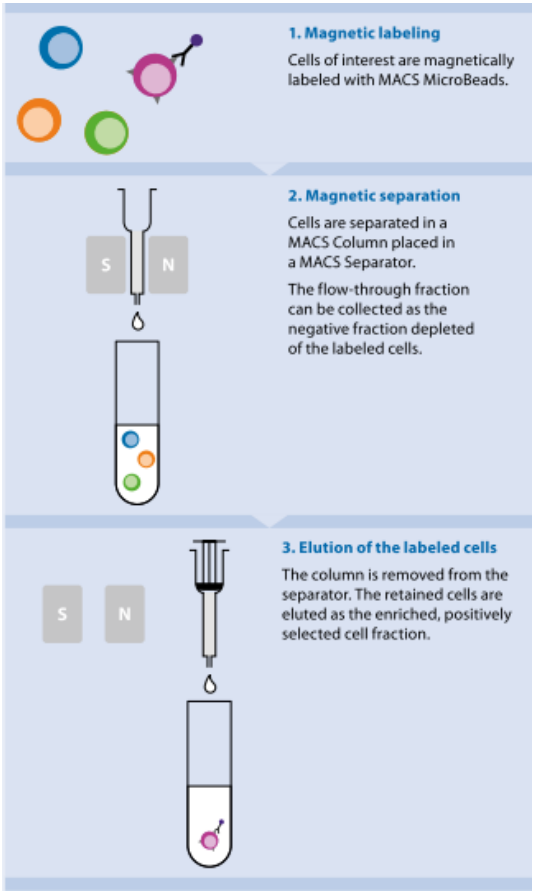


Figure 10 – Schematic representation of Immunomagnetic MACS® Cell Separation steps. Adapted from MACS® Technology Gold Standard in Cell Separation flyer (Miltenyi-Biotec, 2011).

3.7.2. Immunophenotypical characterization of CD4, CD25, FoxP3, A_{2A} receptors and CD73 markers on $CD4^+$ T cell subsets

After positive immunomagnetic cell isolation, $CD4^+$ T cells were re-suspended in FACS buffer (BSA 0.5% in PBS pH 7.2) at a cell concentration $1x10^6$ cells/ml, and then incubated with normal donkey serum (1:5) during 15 min on ice. Subsequently the samples were incubated with the following primary antibodies: FITC-conjugated anti-rat CD4 (1:100, eBioscience), PE-conjugated anti-rat CD25 (1:100; eBioscience), anti-canine A_{2A} adenosine receptor (1:50; Alpha Diagnostics) and anti-rat CD73 (1:750; kindly provided by Dr. Jean Sévigny) during 30 min at 4°C in the dark. Given that anti-A_{2A} receptor and anti-CD73 were not coupled to a fluorophore we applied an indirect immunofluorescent labeling with rabbit IgG (Fc specific)-Biotin antibody (1:750, Sigma Aldrich) following by the incubation with Streptavidin PE-Cy7 (1:100; eBioscience).

For analysis of FoxP3 expression, cells were incubated overnight with fixation/permeabilization working solution (eBioscience) at 4°C in the dark. Cells were washed twice with 150 µL of permeabilization buffer (PB). Then, antibody to FoxP3 conjugated with Cy5 (1:100, eBioscience) was supplemented to 10% rat serum previously added to each well, incubated at 4°C for 30 min in the dark. Stained cells were washed once with PB and resuspended in FACS buffer.

3.7.3. Flow cytometry analysis

The flow cytometer (Beckman Coulter Epics XL) used in this work is equipped with argon lamp that allows the evaluation of 6 basic parameters: size (FSC) and granularity (SSC), type 1 fluorescence (FL1), type 2 fluorescence (FL2) type 3 fluorescence (FL3) and type 4 fluorescence (FL4). FL1, FL2, FL3 and FL4 correspond to fluorescent signals emitted by the excitation of FITC, PE, PerCP-Cy5 and PE-Cy7, respectively. The identification of interest cell populations, as well as determining the percentage of these populations and sub-populations was performed by a computerized system and Cell Quest software (BD Bioscience). Initially it was selected a total population of lymphocytes, region R1, based on size and granularity parameters. Then, a region (R2) was drawn to select the $CD4^+$ T cell population and graphics of FL1 (CD4) vs FL2 (CD25) were constructed to identify the segregation of $CD4^+$ T cell population into three subpopulations: $CD4^+CD25^-$ (R3), $CD4^+CD25^+$ (R4) and $CD4^+CD25^+FoxP3^+$ (R5).

3.8. Presentation of data and statistical analysis

The data are expressed as mean±SEM from an *n* number of experiments. Statistical analysis of data was carried out using paired or unpaired Student's t-test or two-way

analysis of variance (ANOVA). *p* values <0.05 were considered to represent significant differences.

4. Results and Discussion

4.1. Clinical evaluation of Experimental Autoimmune *Myasthenia gravis* animal model (EAMG) induction by *Wistar* rats immunization with a synthetic peptide from the rat nAChR α subunit

Experimental autoimmune *Myasthenia gravis* (EAMG) is most frequently assessed by (1) monitoring clinical evaluation of muscle weakness with the grip strength test (BIOSEB, France), and by (2) observing the following signs: presence of tremor, hunched posture, fatigability, inability to raise the head and reduced mobility. As previously described in Material and Methods, the clinical diagnostic of EAMG manifestation is performed based on the scale presented in Table 2 (Baggi *et al.*, 2004). This procedure is crucial to follow the *in vivo* induction of EAMG disease and it is instrumental to select the animals with *Myasthenia gravis* for further studies. EAMG was induced in *Wistar* rats by immunization with R97-116 peptide from nAChR α subunit emulsified in CFA.

Figure 11A illustrates the onset and progression of the disease expressed in terms of mean clinical scoring for naive, control and EAMG rats.

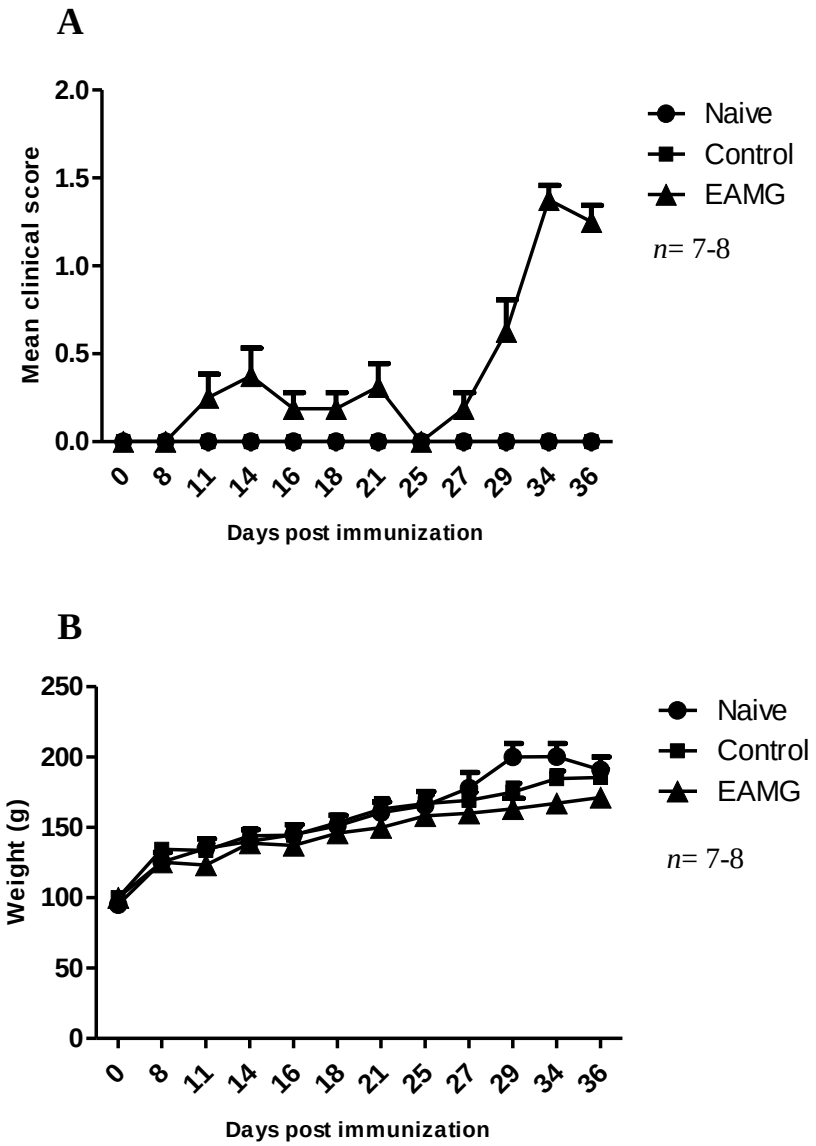


Figure 11 – Mean clinical score assessment and variations in body weight in naive, control and EAMG rats. ***A)*** Clinical manifestations of EAMG were only evident in R97-116 immunized rats (▲; received the R97-116 peptide corresponding to a specific region on the α subunit of the rat nicotinic AChR made up in a solution containing CFA), while the naive (●; not submitted to any kind of treatment) and control animals (■; only received the CFA emulsion without the R97-116 peptide) did not show signs of muscle weakness (maximal score: 4 points). ***B)*** The weight variation over time shows no differences in the growth curves from three groups of animals. The presented data result from the clinical evaluation and body weight assessment of seven animals from naive and eight animals from control and EAMG groups.

As can be seen in figure 11A, *Wistar* rats immunized with R97-116 peptide presented clinical signs of EAMG. The graph shows two distinct episodes of clinical disease: (1) an acute/moderately phase and (2) a progressive phase. The immunized *Wistar* rats exhibited a moderate EAMG score (0.25±0.13, *n*=8) starting on day 11, which subsided 21 days after immunization. The EAMG clinical signs relapsed 29 days after the first immunization (0.625±0.18, *n*=8) and increased thereafter. Interestingly, no changes in the average weight were observed in EAMG rats as compared to control

and naive animals (Figure 11B); these findings indicate that muscle weakness observed in EAMG animals did not preclude food intake. CFA-treated animals showed no clinical manifestations of EAMG. These results are in agreement with the findings reported by other groups (Baggi *et al.*, 2004; Mu *et al.*, 2009) in *Lewis* rats. Thus, our data indicate that clinical manifestations of EAMG can also be successfully induced in *Wistar* rats by immunization with a single peptide of the self AChR.

4.2. Evaluation of immunological and neuronal imbalance features commonly associated with *Myasthenia gravis*

ADA is involved in the development and maintenance of the immune system (Chechik *et al.*, 1981; Chechik & Sengupta, 1981; Van der Weyden & Kelley, 1976). Measurements of serum ADA activity have been used to assess T cell dysfunction; exacerbation of serum ADA activity is positively correlated with the clinical score of myasthenic patients (Chiba *et al.*, 1995). Therefore, measurement of total serum ADA activity may be an important biochemical marker to assess the gravity of MG (Chiba *et al.*, 1995). In light of this, the total serum ADA activity was assessed in EAMG *Wistar* animals.

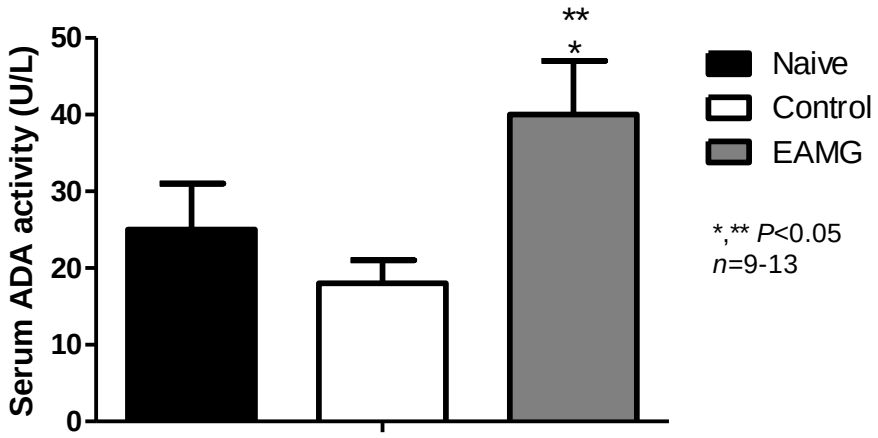


Figure 12 – Serum adenosine deaminase (ADA) activity in naive, control and EAMG rats. Results are mean ± SEM of 9 to 13 experiments. *,** P< 0.05 (one-way ANOVA following Tukey's multiple comparisons test).

Our results indicate that serum ADA activity in EAMG animals was significantly higher (40±7 U/L) as compared with control (18±3 U/L) and naive (25±6 U/L) littermates (Figure 12).

Interestingly, our results are in agreement with previous reports referring that increased serum ADA activity may be a hallmark of MG pathophysiology, since ADA influences proliferation and differentiation of lymphocytes, specially T-lymphocytes (Shore *et al.*, 1981). In addition, we also showed that inoculation of R97-116 peptide into *Wistar* rats induces a dysfunction on cellular immunity. This effect seems to be specific, since exacerbation of total serum ADA activity was observed only in EAMG animals, whereas the adjuvant used for the immunization protocol, CFA, failed to modify ADA activity.

Considering that ADA hydrolysis adenosine into its inactive metabolite, inosine, and that adenosine is an important immunosuppressive nucleoside by inhibiting T-cell activation and by stimulating T_{reg} suppressive function via A_{2A} adenosine receptors activation (Csoka *et al.*, 2008), the levels of serum adenosine maybe crucial to determine the magnitude of immunological responses mediated by effector T cells ($TCD4^+$), activated T cells ($TCD4^+CD25^+$) and regulatory T cells ($TCD4^+CD25^+FoxP3^+$). So, this led us to evaluate T cells subset frequencies in draining lymph nodes from EAMG *Wistar* rats and control littermates by flow cytometry. To this end, the volume of $TCD4^+CD25^-$, $TCD4^+CD25^+$ and $TCD4^+CD25^+FoxP3^+$ reservoir in the cell suspension obtained from draining popliteal and inguinal lymph nodes were analysed. We chose to study lymph nodes for our molecular analysis because they correspond to the major site of regulatory $CD4^+CD25^+FoxP3^+$ T cells accumulation which allows this cell subtype to exert its major immunosuppressive activity on both effector and activated T cells (Samy *et al.*, 2005).

After the extraction of popliteal and inguinal lymph nodes it was possible to observe a marked difference in the morphology of naive and control lymph nodes as compared to EAMG rats. Namely, it is quite apparent that EAMG animals have swollen and enlarged lymph nodes, being indicative of the occurrence of an active immune response (Figure 13). Moreover, secondary lymphoid organs correspond to areas within lymphoid tissue where B-cells interact with helper T-cells to produce antibodies, so it is expected to observe an increase in the size of lymph nodes in EAMG animals due to increased mobilization of immunological cells towards these compartments. In fact, cell suspensions obtained from draining popliteal and inguinal lymph nodes gave significantly higher yield in EAMG animals ($1.4x10^8 \pm 0.2$; mean cell number±SE) as compared to naive ($1.2x10^7 \pm 0.2$) and control ($2.5x10^7 \pm 0.4$) animals. These results are indicative that EAMG animals present lymphoid hyperplasia which usually occurs in autoimmunity conditions.

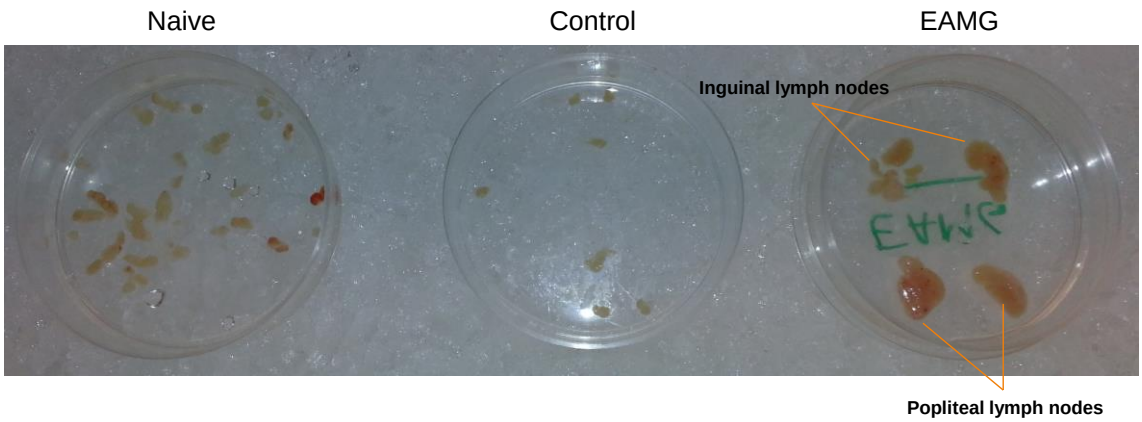


Figure 13 – Illustrative representation of inguinal and popliteal lymph nodes morphology from naive, control and EAMG animals.

The percentage of $TCD4^{+}CD25^{-}$ and $TCD4^{+}CD25^{+}$ cells subsets among the $TCD4^{+}$ population observed in EAMG animals were similar to healthy animals (control and naive) (Figure 14A). However, the analysis of FoxP3 expressing cells among $TCD4^{+}CD25^{+}$ population by flow cytometry in healthy and EAMG rats (Figure 14B) demonstrate that EAMG animals have an altered expression profile of FoxP3 (42±5%, *n*=8) as compared to both control (57±4%,*n*=9) and naive (56±2%,*n*=26) animals. Our results are in agreement with previous studies reporting altered expression of FoxP3 with concomitant T_{reg} functional defects in patients affected with common autoimmune disorders, including adult T cell leukemia (Karube *et al.*, 2004), psoriasis (Sugiyama *et al.*, 2005) and MG (Zhang *et al.*, 2009). Moreover, our data demonstrate that EAMG induced in *Wistar* rats by immunization with the synthetic peptide corresponding to the region α97-116 of the rat AChR α subunit presents the same pivotal sign of immunological imbalance, as that observed for the *Lewis* rats counterpart (Nessi *et al.*, 2010) and human MG (Balandina *et al.*, 2005; Zhang *et al.*, 2009).

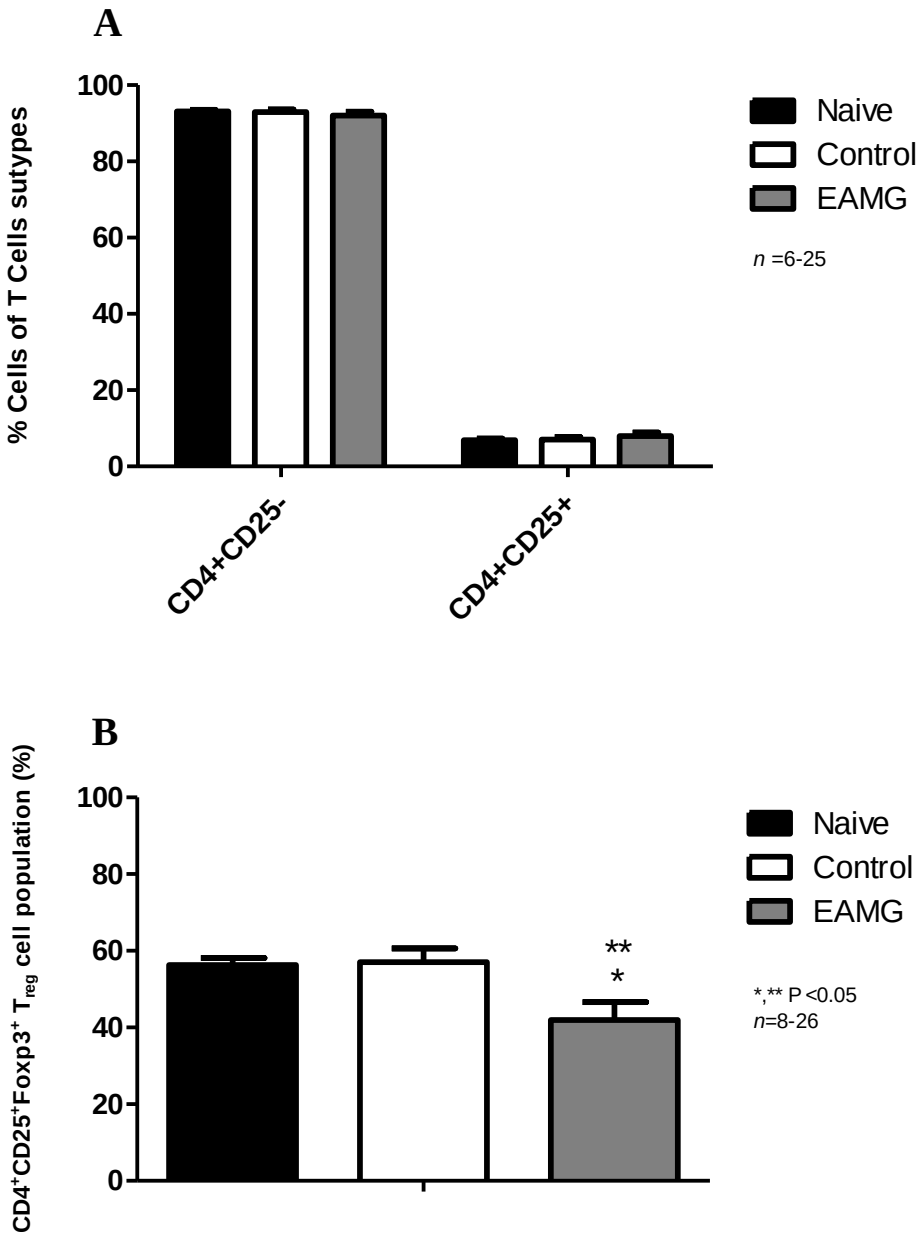


Figure 14 – Evaluation of the percentage of T cells subsets was performed by flow cytometry for surface levels of CD4, CD25 and the intracellular levels of FoxP3. Cell suspension from all groups of animals were isolated from lymph nodes obtained 6-8 weeks after the first immunization with R97-116 (EAMG), control (CFA) and from age matched naive (not subjected to any kind of treatment) rats. ***A)*** Percentage of $TCD4^{+}CD25^{-}$ and $TCD4^{+}CD25^{+}$ cells subsets among the $TCD4^{+}$ population is not altered in all groups of animals. ***B)*** Occurrence of regulatory $CD4^{+}CD25^{+}FoxP3^{+}$ T cells is diminished in myasthenic rats. Data are expressed as the mean±SD of *n*=8-26 rats representative of 6-8 independent experiments. *,** P< 0.05 (one-way ANOVA following Tukey's multiple comparisons test).

Reduced muscle strength during repetitive nerve stimulation reflects the immunological imbalance operating in *Myasthenia gravis*. To assess the contractile properties of the respiratory neuromuscular system in EAMG rats, phrenic nerve hemidiaphragm preparations from EAMG animals were used for myographic recordings under fatigue conditions produced by high-frequency (50 Hz) intermittent

(17 pulses per sec, during 3 minutes) nerve stimulation. Figure 15 shows that the muscle fatigue was more intense in EAMG animals than in both naive and control littermates. The results support the hypothesis that immunization of *Wistar* rats with R97-116 peptide is able of generating animals with a symptomatic myasthenic profile exhibiting immunological impairment, similar to that occurring in MG patients.

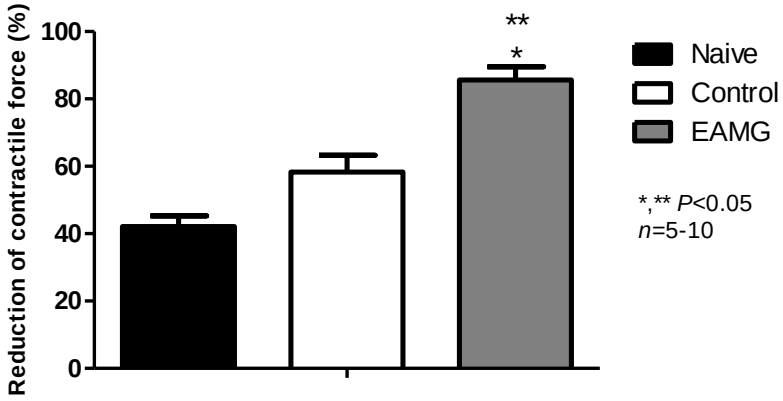


Figure 15 – Percentage of muscle strength reduction evaluated in isolated hemidiaphragm preparations from myasthenic and control animals at the end of the period of intermittent nerve stimulation (3 min, 50 Hz, 17 pulses). Results are mean ± SEM of 5 to 10 experiments. *,** P< 0.05 (one-way ANOVA following Tukey's multiple comparisons test).

Taking into account that neuromuscular transmission failure results from a B mediated antibody attack orchestrated by T cells leading to postsynaptic muscle nAChR clusters disorganization, we decided to look for morphological alterations (Figure 16) of motor nerve endplates from EAMG animals by immunofluorescence confocal microscopy. Previous studies performed in our laboratory demonstrated that immunofluorescence labeling of postsynaptic nAChR was instrumental to evaluate morphological changes on motor nerve terminals from myasthenic animals induced with an antagonist to nAChR (TIMG) (Noronha-Matos *et al.*, 2011).

Figure 16 is composed of representative images of type I and II muscle fibers from both healthy (naive and control) and myasthenic (EAMG) rats obtained by labeling postsynaptic nAChR with TMR-α-BTX (red).

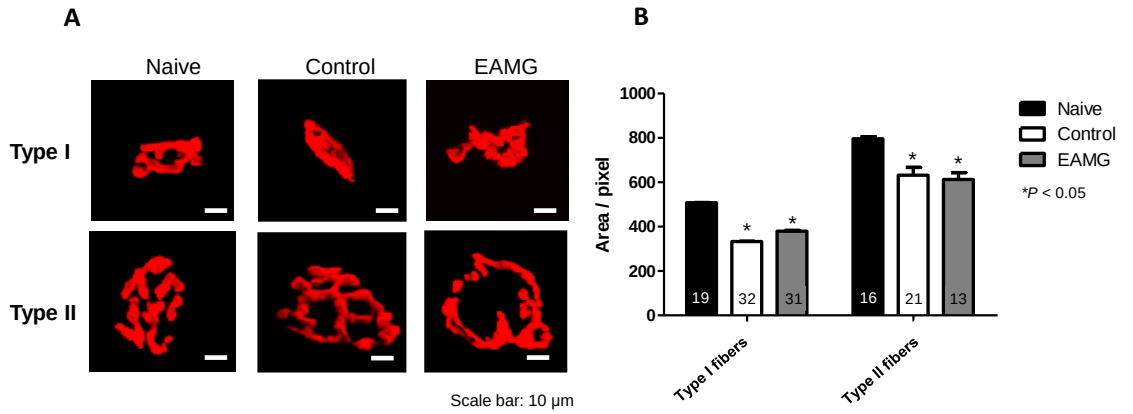


Figure 16 – Analysis of the morphology of motor endplates (*Wistar* rats) loaded with TMR-α-BTX from naive, control and myasthenic rats. ***A)*** Illustration of the differences for both slow (type I) and fast (type II) muscle fibers from healthy (naive and control) and myasthenic (EAMG) rats. ***B)*** Representation of the area (pixel) of muscle endplates (types I and II) from all groups of animals. Each column represents pooled data from two animals of each group; the number of motor endplates is shown at the bottom of each column. *P< 0.05 (Student's t-test).

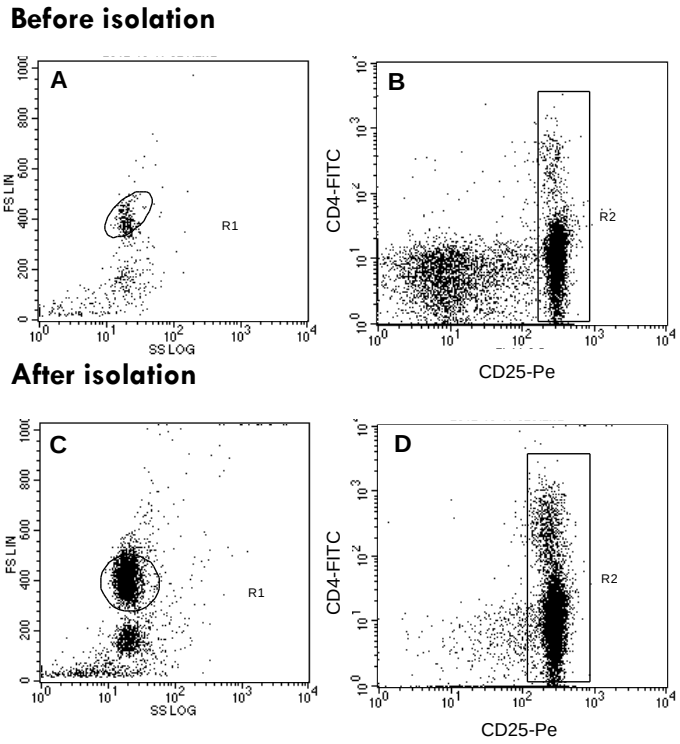
We used the description of Prakash and co-workers (1996) to distinguish the morphological characteristics of different skeletal muscle fibers co-existing in hemidiaphragm preparations. Planar area quantification of motor endplates, using the Image J software to select and measure areas based on color, showed a significant reduction in the area of endplate acetylcholine receptor clusters for both type I and II diaphragm muscle fibers from myasthenic animals, as compared to naive rats. These changes are in agreement with a work by Noronha-Matos (2011), reporting structural changes of motor endplates, more specifically reductions in motor endplate areas in TIMG animals. In fact, the existence of morphological disarrangements for EAMG motor endplates, is in agreement with previous reports indicating that muscle weakness is a consequence of cellular mediated attack of antibodies to nAChRs located postsynaptically leading to increased AChR endocytosis and destruction of NMJ mediated by complement system activation (Juel & Massey, 2007). Regarding histological analysis of samples from patients with MG, it also supports these evidences since motor endplates also present considerable variation in size and morphology (Macdermot, 1960).

The overall set of data suggests that immunization with a single peptide fragment, which sequence is homologous to a region of α subunit of the rat nicotinic AChR in *Wistar* rats, shares clinical, pathological and physiological features of human MG as closely as possible, thus this model might be extensively used to unravel the pathogenic mechanisms and explore therapeutic approaches for MG (Tuzun *et al.*, 2012).

4.3. A_{2A} receptors and CD73 expression is altered in $CD4^+$ T cells subsets in lymph nodes of EAMG rats

A_{2A} receptors are arising as important negative regulators of T-cell function and its importance is being recognized in the immunopathogenesis of MG (Li *et al.*, 2012). ADO by activating A_{2A} receptors plays a dual role on T cells; its action inhibits T-cell receptor (TCR)-mediated signaling, which consequently leads to a decrease in IL-2 production and CD25 expression, decreasing T effector cell proliferation (Csoka *et al.*, 2008; Hasko *et al.*, 2008; Milne & Palmer, 2011). On the other hand, activation of A_{2A} receptors is pivotal at regulating FoxP3 expression in T_{reg} cells (Zarek *et al.*, 2008). Li and collaborators (2012) have recently reported a reduction on A_{2A} receptors expression by both T cells and B cells residing in lymph nodes of EAMG animals. However, the differential modification of A_{2A} receptors expression in subtypes of T cells has not been described till date. In order to evaluate A_{2A} receptors differential expression on $TCD4^+CD25^-$, $TCD4^+CD25^+$ and $TCD4^+CD25^+FoxP3^+$ cells populations, the cell suspension obtained from inguinal and popliteal lymph nodes was enriched in the cells of interest, $CD4^+$ T cells total population, by positive immunomagnetic cell isolation.

Figure 17 corresponds to representative forward scatter (FSC) vs. side scatter (SSC) plots and scatter plot of two-color fluorescence obtained for the cell suspension before and after $CD4^+$ T cells isolation. The population of T cells was identified on the FSC vs. SSC scatter plot based on their size and granularity and gated (R1) to determine the percentage of T cells on the cell suspension (Figure 17A e 17C). The positive immunomagnetic cell sorting revealed to be very effective in isolating $CD4^+$ T cells. The percentage of these cells initially corresponded to 28.10±0.05% (*n*=4) to naive, 34.78±3.46% (*n*=4) to control and 38.25±5.48% (*n*=4) for myasthenic animals, and after isolation this procedure afforded an enrichment of these cells to 85.08±0.78% (*n*=4) in naive, 81.93±2.37% (*n*=4) for control and 79.8±2.96% (*n*=4) for EAMG rats.



CD4⁺ T cells		
Naive	Control	EAMG
28.10± 0.05%	34.78± 3.46%	38.25± 5.48%

CD4⁺ T cells		
Naive	Control	EAMG
85.08± 0,78%	81.93± 2.37%	79.84± 2.96%

n= 4-17

Figure 17 – Flow cytometry profile of $CD4^+$ lymphocytes population before and after positive immunomagnetic cell sorting. ***A)*** and ***C)*** show T cells profile using forward scatter (FSC) vs. side scatter (SSC) plot, before and after isolation, respectively. ***B)*** and ***D)*** are two color fluorescent dot plots representative of $CD4^+$ T lymphocytes labeled with mAbs specific for molecules (CD4 and CD25) expressed on lymphocyte subsets, before and after the isolation procedure, respectively.

Although no differences were found in the density levels of A_{2A} receptor on the $CD4^+CD25^-$ T cell population a reduced expression of these receptors on $CD4^+CD25^+$ cells and on T_{regs} cells was observed in EAMG animals as compared to healthy (naive and control) animals (Figure 18). These observations are in agreement with recent studies demonstrating a reduction of A_{2A} receptors expression at the total pool of $CD4^+$ T cells population obtained from lymph nodes of myasthenic animals (Li *et al.*, 2012). In agreement with these results, it has been reported that reduced expression levels of A_{2A} receptors in myasthenic rats compromise T_{regs} immunosuppressive activity, since activation of these receptors is responsible for up-regulating FoxP3 expression in these cells (Zarek *et al.*, 2008). Moreover, A_{2A} receptors play an important role in the Th1/Th2 paradigm by decreasing the proliferation and IL-2 production of effector T cells. Indeed, Nessi and collaborators (2010) put the hypothesis that the incapacity of T_{reg} cells to revert ongoing EAMG may be due to inadequate control of T_{effs} leading to B cell activation and differentiation into AChR Ab-secreting plasma cells. This incapacity might be associated with the reduction in the expression levels of A_{2A} receptors, since activation of these receptors lead to apoptosis of effector T cells.

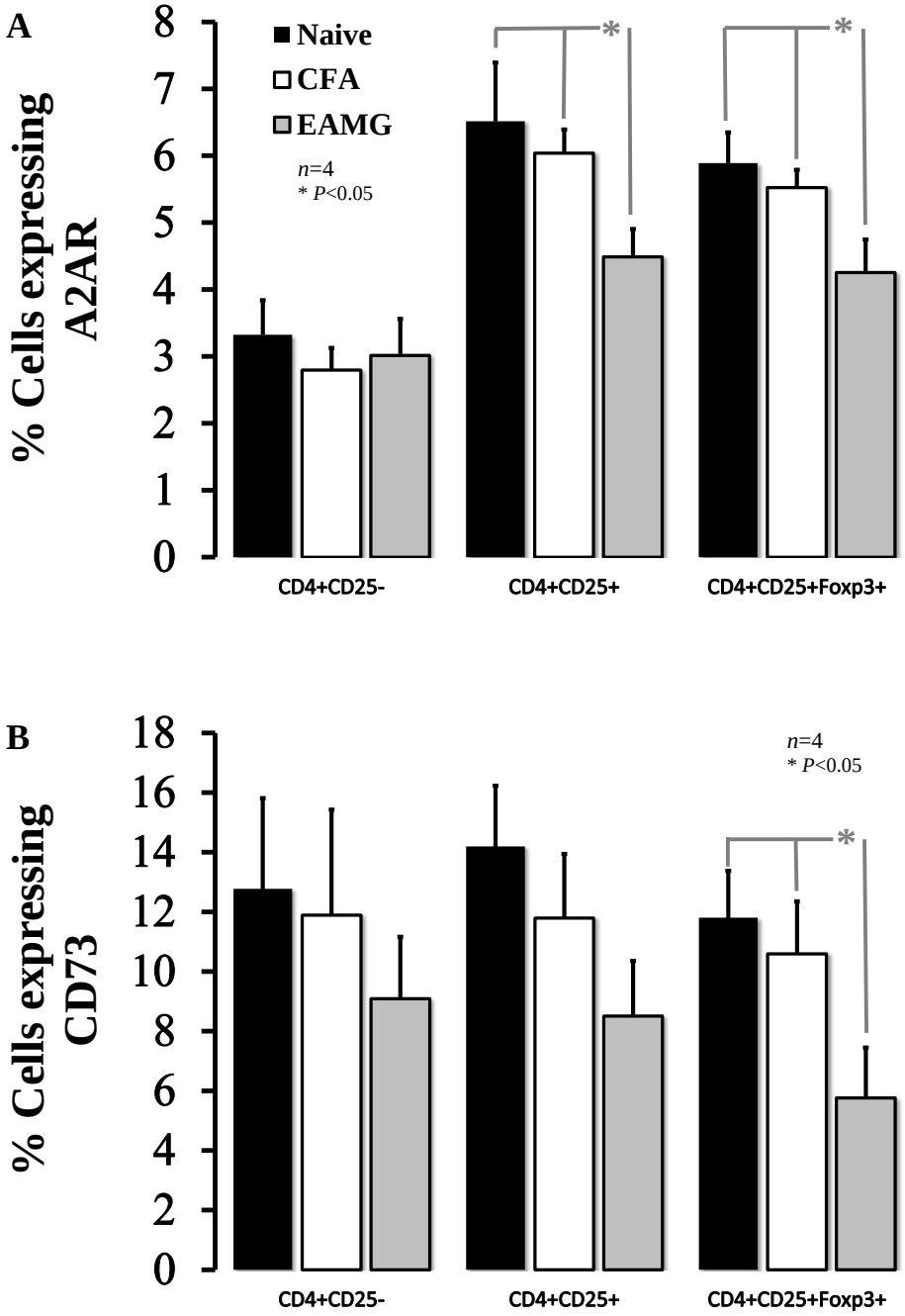


Figure 18 – Graphic representation of A_{2A} receptor ***(A)*** and CD73 ***(B)*** expression on $CD4^+$ T cells subtypes from naive, control and EAMG rats analysed by flow cytometry. Data are expressed as the mean±SD of *n*=4 independent experiments. *P< 0.05 (Student's t-test).

On the other hand, ecto-5'-nucleotidase (CD73) is an enzyme responsible for adenosine formation from the catabolism of adenine nucleotides, which has a critical role in the functional activation of T cells. Some studies reported alterations in CD73 activity on several diseases, such as lymphoproliferative disorders and systemic lupus erythematosus (Gessi *et al.*, 2007). According to Vivekanandhan and collaborators

(2005) abnormal expression levels of these enzymes may be associated with an autoimmune pathology. The production of adenosine represents one of the immunosuppressive mechanisms mobilized by T_{reg} cells and expression of CD73 is under the control of FoxP3 transcription factor (Zarek *et al.*, 2008). Taking this into consideration and the previously observed decreased expression of FoxP3 in the regulatory $CD4^+CD25^+FoxP3^+$ T cells population from EAMG animals we decided to evaluate the expression of CD73 in all $CD4^+$ T cells populations.

Figure 18 shows that CD73 expression profile is altered in T_{reg} cells from myasthenic rats as compared with healthy littermates. No differences were observed for other T cell populations tested. Firstly, this may have functional repercussions since CD39/CD73 pathway is responsible for increasing the production of ADO by T regulatory cells, which, in turn will exert an immunosuppressive action on $CD4^+CD25^-$ T activated cells by acting on A_{2A} receptors (Mandapathil *et al.*, 2010). Secondly, abnormal levels observed for CD73 expression in EAMG rats may significantly compromise the regulatory loop of ADO accumulation in the close vicinity of regulatory $CD4^+CD25^+FoxP3^+$ T cells since a decreased tonic A_{2A} receptors activation on regulatory $CD4^+CD25^+FoxP3^+$ T cells may impair the up-regulation of FoxP3 mRNA (Zarek *et al.*, 2008) which will in turn decrease CD73 expression (Deaglio *et al.*, 2007).

4.4. Adenosine A_{2A} receptors are present on presynaptic cellular components of motor endplates but its function is impaired at EAMG motor nerve terminals

Previous findings showed that activation of A_{2A} receptors at the rat neuromuscular junction is responsible for mediating the neurofacilitatory actions of ADO (Correia-de-Sá *et al.*, 1991). We demonstrated that tonic activation of A_{2A} receptors on motor nerve terminals may overcome neuromuscular tetanic fade during high frequencies of nerve stimulation (Oliveira *et al.*, 2004), which might be of clinical interest to overcome neuromuscular transmission deficits observed in MG. Our group has previously reported that endogenous ADO generated in TIMG motor endplates during repetitive nerve firing may be insufficient to preserve transmitter release via tonic activation of presynaptic facilitatory A_{2A} receptors (Noronha-Matos *et al.*, 2011). Additionally, functional studies performed at Laboratory of Pharmacology and Neurobiology from ICBAS-UP demonstrated that motor endplates from EAMG rats have impaired A_{2A} receptors activity, since the application of a enzymatically stable A_{2A} receptor agonist,

CGS21680C, failed to modify evoked [^{3}H]-ACh release (Almeida, 2012; Guerra-Gomes *et al.*, 2013).

All together, these evidences leave open the question whether impairment of A_{2A} adenosine receptors may result from post-translational modifications of the receptor protein structure, or to changes in receptor expression and distribution in EAMG motor endplates. In order to answer this question, we evaluated A_{2A} receptor immunoreactivity among cell compartments of rat motor endplates from naive, control and EAMG littermates by immunofluorescence confocal microscopy.

The NMJ structure is quite intricate due to the presence of intramuscular connective tissue components, which totally conceal the surface of NMJ (Desaki & Uehara, 1981). In fact, there are not many studies reporting immunofluorescence experiments of adenosine receptors in the NMJ and our group demonstrated that the characteristic morphology of NMJ hampers the accessibility of antibodies to the motor endplate (Viegas, 2011). So, we proposed to perform an epitope retrieval method to increase the sensibility of the immunohistochemical detection of epitopes for A_{2A} receptors. Thereby, hemidiaphragm strips from naive animals were enzymatically digested with 0.1% type IA collagenase with different incubation times (10, 20 and 30 minutes), so that we might determine the best experimental conditions to enable the detection of the proteins of interest.

Figure 19 revealed that the longest incubation time (30 minutes) appears to increase the accessibility of antibodies to the endplate, since immunoreactivity for A_{2A} receptor was significantly ameliorated in these conditions. Immunofluorescence experiments also show that after 30 minutes of treatment, besides improving the labeling for A_{2A} receptor in the motor endplate, also increased the staining intensity at nerve terminals. Furthermore, in contrast to collagenase-treated muscle sections for 10 and 20 minutes, where immunofluorescence labeling is rather faint, the majority of the motor endplates which were positive for TMR-α-BTX labeling muscle type nicotinic receptors also exhibit significant immunoreactivity against adenosine A_{2A} receptors located at the presynaptic component.

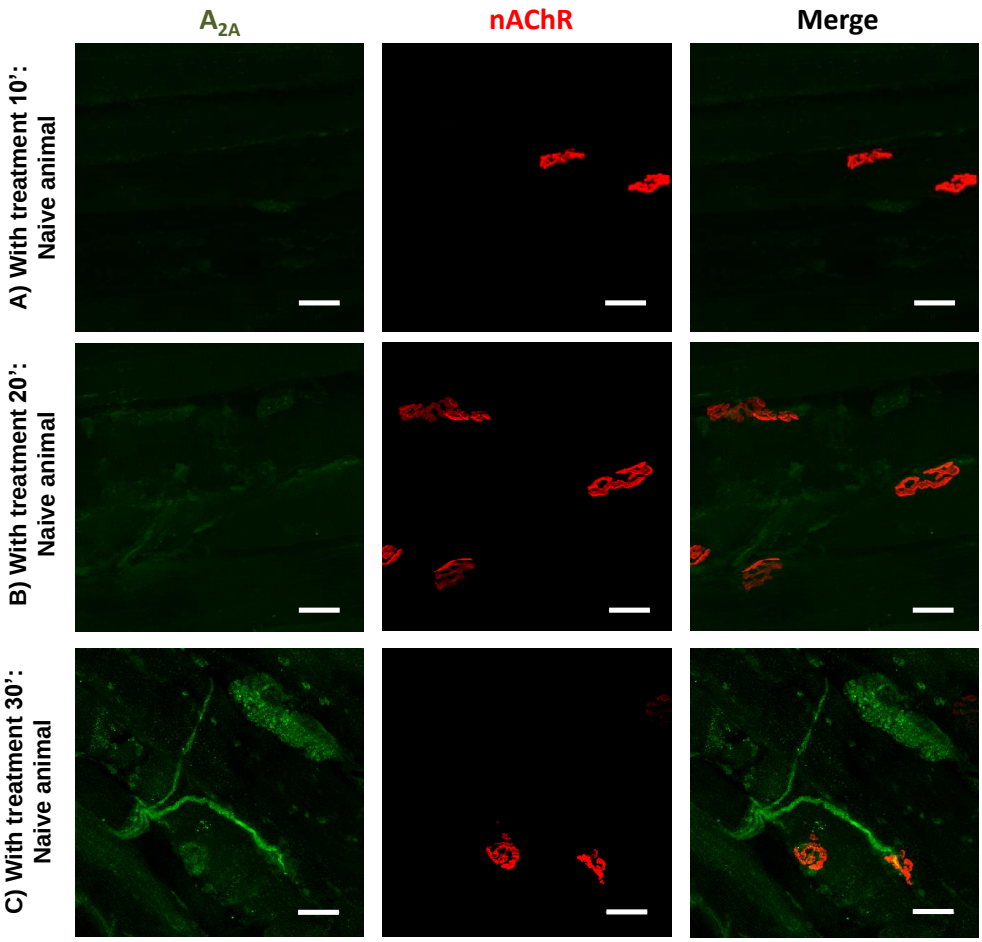


Figure 19 - Confocal immunofluorescence analysis of A_{2A} adenosine receptors in rat hemidiaphragm muscle from naive rats. *A)* NMJs with 10', *B)* 20' and *C)* 30' treatment with collagenase (0,1%) double stained with TMR-α-BTX and A_{2A} adenosine receptors, respectively. Scale bar: 30 µm

Taking into account these observations, we analysed the immunoreactivity of A_{2A} receptor in hemidiaphragm sections from naive, control and EAMG rats treated with collagenase during 30 minutes (Figure 20). Since functional studies showed that the activity of adenosine A_{2A} receptors was impaired in myasthenic animals, we aimed at evaluating if the expression of these receptors was altered in EAMG motor nerve terminals. Our first finding was that skeletal muscle fragments from EAMG rats probably would not require such a long enzymatic digestion time as naive animals. This may be due to the fact that these animals have a more fragile tissue as compared to healthy littermates, since its muscle membrane is damaged due to the attack mediated by the complement system.

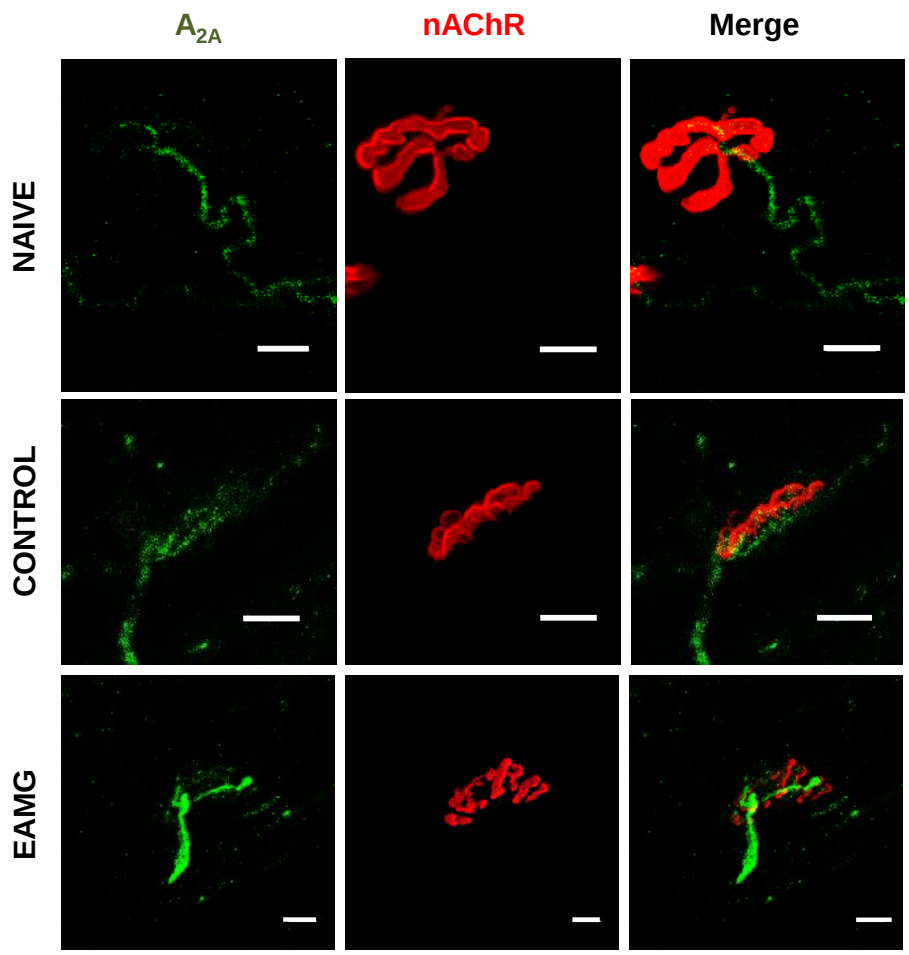


Figure 20 - Confocal immunofluorescence analysis (top and side views) of A_{2A} adenosine receptors in rat hemidiaphragm muscle from naive, control and EAMG rats. Hemidiaphragm sections were treated with collagenase (0.1%) for 30 min and double labeled with A_{2A} receptor *(green)* and TMR-α-BTX *(red)*, respectively. Scale bar: 10µm.

Careful comparison of the immunoreactivity for A_{2A} receptor in healthy (naive and control) and myasthenic (EAMG) rats is consistent with a presynaptic localization of these receptors. In fact, using as a reference the fluorescent labeling of nAChR clusters with TMR-α-BTX (motor endplate region), A_{2A} receptors appear to be distributed predominantly in regions appearing to ‘‘interlink’’ synaptic buttons. Despite the fact that we observed no dramatic changes in the A_{2A} receptor immunolabeling of motor nerve terminals from EAMG animals as compared to healthy animals, variations on A_{2A} receptors expression cannot be rule out. We also found no evidence to suggest localization of these receptors in skeletal muscle fibers. Since, the staining pattern observed for A_{2A} adenosine receptors was not restricted to synaptic buttons, we cannot rule out at this stage the presence of A_{2A} receptors on nerve axons and perisynaptic Schwann Cells.

Despite we observed no evident changes in the A_{2A} receptor immunolabeling of motor nerve terminals in all groups of animals, its function is impaired. Previous evidences indicates that at the NMJ, A_{2A} receptors stimulate the activity of the adenylcyclase/AMPc transduction system, recruiting calcium through intracellular calcium reservoirs and through the activation of Ca_V1 (L-type) channels (Correia-de-Sá *et al.*, 2000). Moreover, our group also reported that A_{2A} receptors operating Ca^{2+} influx via "quiescent" high-capacity / slow-inactivating Ca_V1 (L-type) channels may contribute to overcome tetanic depression during neuronal firing in normal rats (Oliveira *et al.*, 2004). So, we put the hypothesis that the functional impairment observed for these receptors could be related to changes in the effector mechanism.

Immunofluorescence staining for $Ca_v1.2L$ (Figure 21) reveals that these channels have a presynaptic localization, still exhibit a more marked staining in nerves than in the motor endplate. Preliminary results seem to indicate that there are no differences in $Ca_v1.2L$ immunoreactivity in all groups of animals, so it would be interesting to further explore the inherent signalling pathway of A_{2A} receptors.

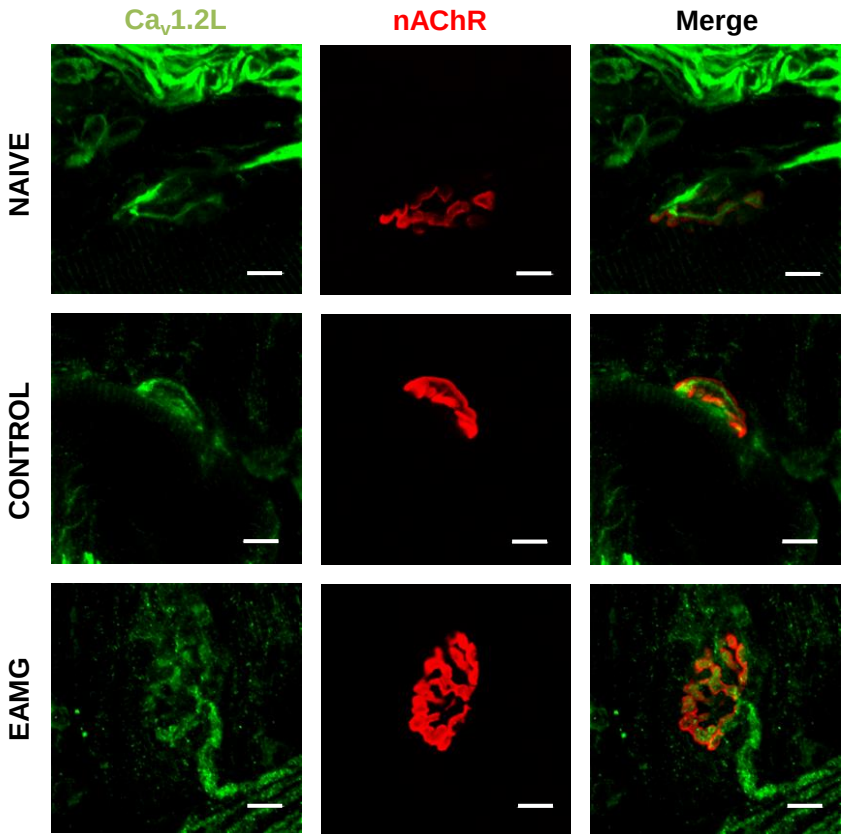


Figure 21 – Confocal immunofluorescence analysis (top and side views) of $Ca_v1.2L$ channels in rat hemidiaphragm muscle from naive, control and EAMG rats. Hemidiaphragm sections were treated with collagenase (0.1%) for 30 min and double labeled with $Ca_v1.2L$ *(green)* and TMR-α-BTX *(red)*, respectively. Scale bar: 10µm.

5. Conclusions and future perspectives

Adenosine is an extracellular signaling nucleoside that has a dynamic role in the regulation of neuromodulatory (Correia-de-Sá *et al.*, 1991; Oliveira *et al.*, 2004) and immunosuppressive (Hasko *et al.*, 2008; Koshiba *et al.*, 1999; Lukashev *et al.*, 2003) processes, via the activation of excitatory A_{2A} receptors. The study of adenosine-receptor-based therapies, namely acting on A_{2A} adenosine receptors, urges in order to provide new insights into the investigation of treatment strategies.

In this sense, our study is pioneering as we proposed to study the mechanisms associated with A_{2A} receptors and their net contribution in the immune and neuronal dysfunction operating in MG. In fact, our results point to an impairment of A_{2A} adenosine receptors at both levels: neuronal and immunological.

Regarding the immune component, we show here that there is a down regulation of A_{2A} receptors on subsets of activated $CD4^+CD25^+$ and regulatory $CD4^+CD25^+FoxP3^+$ T cells, thus compromising adenosine immunosuppressive effect. Li and co-workers (2012) have already reported a reduced A_{2A} receptor expression by $CD4^+$ T cells isolated from spleen and lymph nodes of EAMG rats. However, considering that the immunological synapse is a dynamic system, where all $CD4^+$ T cells populations interact with each other, it is crucial to evaluate these receptors on these populations. The observed down regulation in $CD4^+CD25^+$ and T_{reg} cells certainly compromises A_{2A} receptors ability to stimulate immunosuppressive activity of T_{reg} (Ohta *et al.*, 2012) and to inhibit the proliferation of effector and activated T cells (Hasko & Cronstein, 2013). Moreover, previous studies have already reported that A_{2A} receptors stimulation is responsible for the up-regulation of the key transcription factor controlling T regulatory cells, FoxP3 (Zarek *et al.*, 2008). These observations are consistent with our results showing that there is a decrease of FoxP3 expression in EAMG rats, since it is regulated by A_{2A} receptors. In the light of these results, we also observed that CD73 expression was compromised in T_{reg} cells of myasthenic rats. This might be due to the fact of FoxP3 be a transcription factor for the expression of CD73. Moreover, a decrease of the pool of A_{2A} receptors might be a consequence of CD73 conditional expression.

Our study also provides further evidences of immunological imbalance in EAMG model, by measuring serum ADA activity. In fact, increased serum ADA activity observed in EAMG animals is a proof that cellular immunity is being stimulated (al-Shammary, 1997). Interestingly, this immunological sign as well as the reduction in the

expression levels of CD73 might influence the endogenous levels of adenosine, which in turn compromises the function of A_{2A} receptors.

All together, the results obtained so far allow us to hypothesize that initiation of the autoimmune process is triggered by a lymphocyte hyperactivity, which leads to increased serum ADA levels, with a consequent increase in its activity. The exacerbated activity of this enzyme results in decreased levels of endogenous adenosine, thus compromising the immunosuppressive activity of A_{2A} receptors. Therefore, A_{2A} mediated effects, as the suppression of $CD4^+CD25^+$ lymphocyte population and the stimulation of immunosuppressive activity of T_{regs} are impaired. It is important to note that at this stage of the disease, the impairment of A_{2A} receptor function is only associated with low levels of ligand in the extracellular environment. Consequently, deficits in the A_{2A} receptor activation lead to a dysfunction of T_{reg} cells, since these receptors influence the expression of FoxP3 and CD73. At this stage, we may speculate that the existent dysfunction in two key steps for the accumulation of ADO (ADA and CD73) might be crucial since it will decisively trigger the onset of the disease. Together, these events might influence the reduction in the pool of A_{2A} receptors in a later stage of the disease. Likewise, the mechanism by which T_{reg} exerts its immunosuppressive activity is suppressed. In the context of this hypothesis is possible to understand the results obtained by Li and collaborators (2012), reporting that a preventive treatment, *i.e.* intervention at an early stage of the disease with CGS21680, was effective in down-modulating MG manifestations. Contrariwise, therapeutic treatment fails to ameliorate ongoing EAMG.

Concerning the neuronal component, despite we observed that the A_{2A}-receptor-mediated facilitation is compromised in EAMG rats (Almeida, 2012), immunofluorescence experiments did not allow a clear view of this phenomenon. However, caution must be taken with the use of immunofluorescence confocal microscopy as a technique to analyze variation in expression levels of the receptors, since it is semi-quantitative. Though, these results by confirming a presynaptic localization of A_{2A} receptors, as well as the integrity of Ca_V1 expression (effector mechanism) in EAMG rats, reinforces the hypothesis that a therapeutic intervention on A_{2A} receptors might be pivotal in the recovery of muscle weakness observed in MG. In short, it is necessary to conduct further studies to understand the mechanisms involved on A_{2A} function conditioning, as well as understand how to recover it in myasthenic conditions.

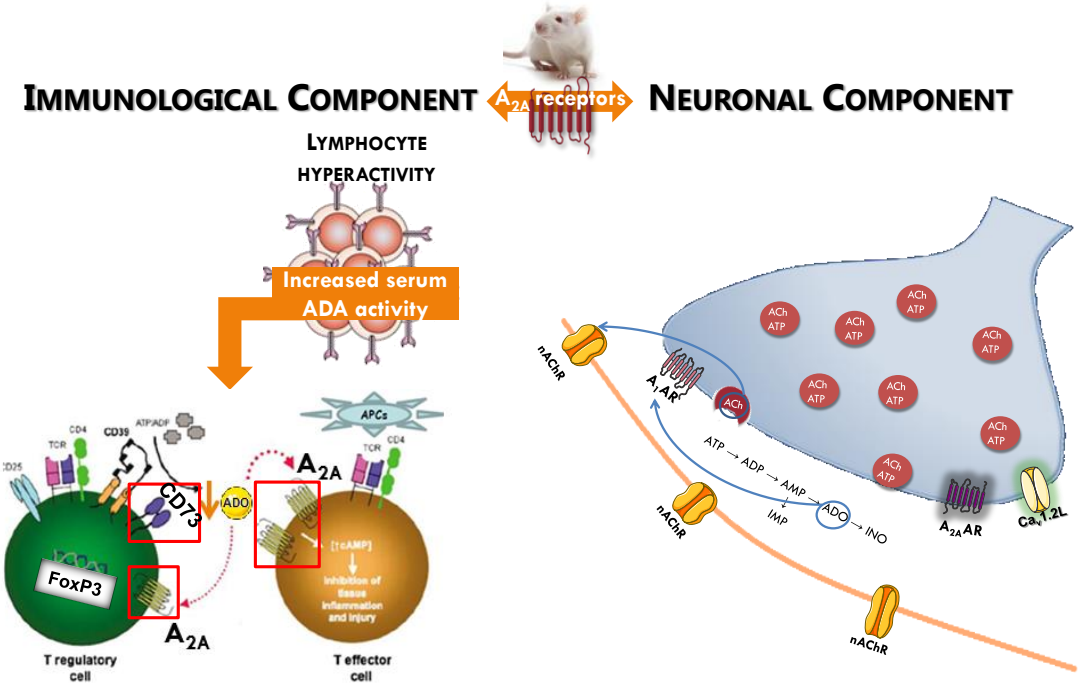


Figure 22 - Adapted scheme showing the importance of A_{2A} receptors on neuroimmune system: implications for the immunological and neuronal synapse.

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