Role of adenosine A\textsubscript{2A} Receptors in Multiple Sclerosis: Neural Stem Cells as a potential target

Ana Marta Alonso Gomes\textsuperscript{1,2,3*}

Thesis to obtain the Master of Science degree in Biomedical Engineering

November 2018

Supervisors: Prof. Sara Xapelli\textsuperscript{2,3} and Prof. Margarida Diogo\textsuperscript{1,4}

\textsuperscript{1}Instituto Superior Técnico, University of Lisbon, Portugal; \textsuperscript{2}Instituto de Farmacologia e Neurociências, Faculdade de Medicina, Universidade de Lisboa, Lisboa, Portugal; \textsuperscript{3}Instituto de Medicina Molecular João Lobo Antunes (IMM – JLA), Faculdade de Medicina, Universidade de Lisboa, Lisboa, Portugal; \textsuperscript{4}Institute for Bioengineering and Biosciences, Instituto Superior Técnico, Universidade de Lisboa

\*Email: marta.alonso.gomes@tecnico.ulisboa.pt

Abstract: Multiple Sclerosis (MS) is a chronic neuroinflammatory autoimmune demyelinating disease of the central nervous system (CNS). MS pathogenesis begins with an exacerbated inflammatory response that deteriorates the myelin sheath that insulates neuronal axons. In the CNS, oligodendrocytes (OLGs) are the glial cells that produce the myelin sheath. Myelinating OLGs result from the differentiation of oligodendrocyte progenitor cells (OPCs) present in the brain parenchyma but also from neural stem cells (NSCs) of the subventricular zone (SVZ) neurogenic niche. Experimental Autoimmune Encephalomyelitis (EAE) is an animal model of MS sorely used in MS research. Previous studies have reported a spontaneous phenomenon of remyelination also seen in MS pathology, through the migration of OLGs to demyelinated areas. Furthermore, adenosine A\textsubscript{2A} receptors (A\textsubscript{2AR}) have been shown to have a protective role against inflammation in EAE, attenuating the phenotype of the disease. However, A\textsubscript{2AR} role in modulating adult oligodendrogenesis from NSCs was not studied. Thus, the aim of this project was to assess the role of A\textsubscript{2AR} in promoting OLGs differentiation and myelination under EAE pathogenesis. Female C57BL/6 mice were immunized with MOG\textsubscript{35-55} and injected with Pertussis toxin to induce the EAE model. EAE mice were administered in the lateral ventricle with vehicle or with A\textsubscript{2AR} agonist (CGS21680, 100 nM) for 6 days using micro-osmotic pumps. Behavioural tests were performed to evaluate EAE progression, along with cellular and molecular analyses to assess the role of A\textsubscript{2AR} agonist on inflammation, OLG differentiation and de- and remyelination. Low incidence of the EAE model limited the relevance of the results. Improvements on EAE induction protocol may allow further conclusions on A\textsubscript{2AR} relevance for regenerative therapies in MS.

Keywords: Multiple Sclerosis, EAE model, Adenosine A\textsubscript{2A} Receptors, Adult Oligodendrogenesis, Remyelination.

Introduction

Multiple Sclerosis (MS)

MS is a chronic neuroinflammatory autoimmune demyelinating disease of the central nervous system (CNS). MS has been entitiled as the main cause of multifocal areas of demyelination and inflammation, commonly known as sclerotic plaques, the pathological hallmark of MS\textsuperscript{5}. MS characteristic neuroinflammatory environment results from an intensified infiltration of T and B lymphocytes. This enhanced migration is due to an increase in the blood brain barrier (BBB) permeability.

Although the origin of MS pathology is not yet fully understood, its pathogenic agents and lesions have been thoroughly described and analysed. An exacerbated immune-mediated inflammatory response has been entitled as the main cause of multifocal areas of demyelination and inflammation.

MS pathophysiology can be expressed in four different forms: Relapsing Remitting MS (RRMS), Secondary Progressive MS (SPMS), Primary Progressive MS (PPMS), Progressive Relapsing MS (PRMS) The RRMS accounts for approximately 85% of MS cases, being characterized by acute attacks (relapses) that evolve during days to weeks, followed by partial or full recovery (remitting), with no neurological function deterioration\textsuperscript{2}.
plasma cells produce myelin-specific antigens that also participate in the insult against OLGs and myelin\(^6\).

Many studies have reported a spontaneous myelin repair in MS lesions, as a response to the demyelination phenomenon\(^7\). However, while this natural process is extremely relevant in early onsets of the disease or in acute lesions, in chronic conditions, as demyelination lesions accumulate and, consequently, disability and pathological impairments occur, remyelination becomes insufficient\(^8\). The main agents of this spontaneous remyelinating response are mature myelinating OLGs, derived from oligodendrocytes precursor cells (OPCs) proliferation and maturation\(^9\).

**Experimental autoimmune Encephalomyelitis (EAE) model**

Regarding animal models of MS, the Experimental autoimmune Encephalomyelitis (EAE) model is one of the most used animal models in MS research, for sharing many of the clinical and pathophysiological features of this condition\(^10\).

Active EAE induction form consists of animal immunization with emulsified myelin-related antigens. Myelin oligodendrocyte glycoprotein 35-55 (MOG\(_{35-55}\)) has become one of the most used peptides in active EAE induction, due to its high availability and producibility and for the pathological features of its induction closely mirroring the ones observed in human MS\(^11\). MOG\(_{35-55}\) antigen is prepared in an emulsion of Complete Freund’s adjuvant (CFA), a mineral oil-based adjuvant that increases the peripheral immune response. CFA is supplemented with Mycobacterium Tuberculosis (M. Tuberculosis), responsible for initiating the innate immune system response\(^12\). The expansion and differentiation of MOG-specific autoimmune cells is enhanced by a set of pertussis toxin (PTX) injections, which, by increasing BBB permeability, facilitates the entrance of autoimmune T cells into the CNS\(^13\).

Normally, C57BL/6 mice MOG\(_{35-55}\) induced exhibit a classic chronic EAE clinical course. EAE onset is 9 to 14 days after immunization, with peak of disease 3 to 5 days after onset for each mouse. Partial recovery is seen, but 25% of the induced animals will then show an increase in severity again\(^12\).

**Neural Stem cells (NSCs) and Neurogenesis overview**

NSCs are multipotent stem cells with the ability to self-renew and capable of differentiating into neurons, astrocytes or oligodendrocytes\(^14\). Neurogenesis is defined as the process of generating functional neurons from adult neural stem/precursor cells (NPCs). Nowadays, it is known that this process is not exclusive of embryonic and perinatal stages, but it is also seen in the adult mammalian brain\(^14\).

There are two main regions of the brain where NSCs are localized: the subventricular zone (SVZ) of the lateral ventricles and, at a lower magnitude, in the subgranular zone (SGZ) in the dentate gyrus of the hippocampus. In the SVZ, (NPCs) proliferate and migrate through the rostral medial stream (RMS), to the olfactory bulb, where they differentiate into mature interneurons; in the DG the maturation stages take place in the granular cell layer\(^15\).

**Oligodendrocytes (OLGs) and oligodendrogenesis overview**

Regarding OLGs, the myelinating cells of the CNS, they are the final product of OPCs maturation and differentiation, a mechanism termed oligodendrogenesis. During embryonic development, OPCs derived from SVZ NSCs maturation, migrate and populate the entire brain parenchyma and spinal cord, generating the entire OLGs population that ensures the myelination of the entire CNS during postnatal life\(^16\). By doing so, OLGs are fundamental glial cells responsible for axonal insulation required for a proper functioning of the nervous system. Any disturbance caused in this myelinating mechanism might be a trigger of neurodegenerative and demyelinating conditions as MS\(^17\).

After postnatal development, OPCs are still found in different structures of the adult brain parenchyma, making 2-9% of the CNS cell population\(^18\). These remaining OPCs, named adult OPCs, although maintained at a quiescent state of proliferation, are responsible for the maintenance of OLGs and consequent myelin production during adulthood. Moreover, evidence has been collected regarding continuous production of OPCs from SVZ-derived NSCs in the adult brain\(^19\).

**OLGs therapeutic potential in MS**

Many studies have demonstrated the capacity that SVZ-derived OPCs have to migrate to demyelinated lesions and, by differentiating into mature myelinating OLGs, enhance the remyelination response. Picard-RIera et al. have assessed this OPCs and OLGs migration to demyelinated areas in the EAE model\(^20\). This was the first study to show that the inflammatory and demyelinating pathological conditions of the EAE model induced the differentiation and generation of OLGs, not only in the SVZ but also in the olfactory bulb, which is usually the destiny of neurons originated from the SVZ. This newly formed OLGs migrate from the SVZ to injured CNS areas, especially demyelinated areas such as the corpus callosum (CC). In concordance to these findings, Nait-Oumesmar et al. have also observed the mobilization of SVZ-derived OPCs into MS lesions of human post-mortem brains\(^21\).

Nonetheless, given the multifocal nature of MS and the putative minor contribution of the endogenous SVZ cells to remyelination, as compared to the main effectors, the parenchymal OPCs, the efficiency of SVZ cells to promote repair in MS is still relatively modest\(^17\).

Thus, several research groups have been focusing on finding therapeutic approaches to enhance OPCs
proliferation and maturation into myelinating OLGs to support endogenous remyelination. Regarding so, G-protein coupled receptors (GPCRs) have been considered as potential mediators of the neuroinflammatory response.

**Adenosine A2A Receptors**

Adenosine is an endogenous purine nucleoside that modulates a wide range of physiological functions, with a relevant influence in cell homeostasis in the CNS. It has been shown to have a relevant role in sleep and arousal, cognition, memory, neuroprotection and inflammation. A2A receptors (A2AR) are high affinity receptors, which are activated by low levels of extracellular adenosine.

A2AR are expressed in different CNS regions and in different cell types of the CNS, including OPCs and OLGs, suggesting that these receptors might have a role in modulating neuron and glial communication. A2AR are also known for playing a relevant role in the modulation of the immune and inflammatory response.

. Moreover, recent studies have shown that A2A receptor has a critical role in the regulation of neuroinflammatory patterns, as the NF-κB signaling pathway, under MS and EAE pathological conditions. Along with its active participation in pathways that regulate cell differentiation and survival, the MAPK/ERK1/2 signaling pathway. Nonetheless, work performed in our group has assessed that A2ARs activation in SVZ neurospheres promoted oligodendrocyte differentiation and it also led to an increase of CNPase activity, an enzyme expressed by mature oligodendrocytes, which implies that differentiated OLGs were myelin producers.

Thus, in this experimental project I aimed at understanding the role of adenosine A2A receptors in promoting OLGs differentiation and myelination under MS conditions, using the EAE mouse model.

**Materials and Methods**

**Ethics Statement**

All experimental procedures performed in animals in the following study were carried out in conformity with the European Community legislation (86/609/EEC; Directive 2010/63/EU, 2012/707/EU). These procedures were approved by the Animal Ethics Committee of Instituto de Medicina Molecular (iMM), as well as by the Direção Geral de Alimentação e Veterinária (DGAV), the Portuguese competent authority for animal protection.

**EAE model induction**

Fifteen young C57BL/6 female mice (ten-weeks old) from Charles River (Barcelona, Spain) were used. Prior to any in vivo procedure, animals were housed in groups of five in individually ventilated cages (IVC) in the iMM rodent facility, in specific pathogen free (SPF) environmental conditions. All procedures were performed in SPF conditions.

Following micro-osmotic pump surgery implantation, animals were housed individually to avoid unnecessary post-surgical complications, easing recovery. Seven days following EAE induction, animals were moved to a virus antigen free (VAF) area.

EAE was induced in mice using a kit (Hooke Kit™ MOG35-55/CFA Emulsion PTX, Cat #EK-2110, Lot #0126), from Hooke Laboratories (Lawrence, MA, USA), according to manufacturer’s instructions. This set is composed of an antigen MOG35-55 rat emulsion in CFA with mouse heat killed M. Tuberculosis and PTX in glycerol buffer. On day 0 post-induction (p.i.), 100µg of MOG35-55/CFA emulsion were subcutaneously injected in both right and left side of the mouse’s ventral flank, making a total of 200µg of injected emulsion. A solution of PTX in phosphate-buffered saline (PBS) (NaCl 137mM, KCl 2.1mM, KH₂PO₄ 1.8mM and Na₂HPO₄ 2H₂O 10mM, pH 7.4) was prepared fresh and, approximately two hours after MOG emulsion injection, 100µL of PTX (120ng dose per animal), was intraperitoneally injected in the mice right flank. Approximately 24 hours later (day 1 p.i.) a second PTX intraperitoneal (i.p.) injection was administered.

The first and most visible signs of EAE development in mice are locomotor impairments, displayed as an ascending flaccid paralysis. EAE severity and disease onset was daily evaluated, starting on day 7 p.i., using the following clinical score (CS) scale: 0, healthy; 1, limp tail; 2, partial paralysis of the hind limbs; 3, complete paralysis of the hind limbs; 4, hind-limb paralysis and forelimb weakness; 5, moribund or deceased.

**BrdU Administration**

To assess NSCs proliferation and differentiation under EAE conditions, it was performed a (bromodeoxyuridine) BrdU administration protocol to identify SVZ-derived cells, ensuring that any labelled cells in other brain structures are originated from either the SVZ or the RMS. On day 2 p.i., BrdU (Sigma-Aldrich, MO, USA), dissolved in sterile 0.9% NaCl saline solution, was administered i.p. 7 times with 2 hours intervals (50mg of BrdU per Kg of mouse body weight).

**Micro-osmotic pump intracerebroventricular surgery**

EAE mice were implanted with micro-osmotic pumps containing a solution of the A2A receptor selective agonist CGS21680 (Tocris, Bristol, UK), a monocarboxylic acid and a dicarboxylic acid monoamine, derived from adenosine in artificial cerebrospinal fluid (aCSF) (NaCl 150mM, KCl 3mM, CaCl₂ 1.3mM, MgCl₂ 0.8mM, Na₂HPO₄ 0.8mM and NaH₂PO₄ 0.2mM).

On day 3 p.i., micro-osmotic pump icv implantation surgery (Alzet® Micro-osmotic pump Model 1004
combined with the Alzet® Brain Infusion Kit 3 (1-3mm, DURECT Corporation, Cupertino, CA, USA) was performed to the EAE-induced animals. Surgery was performed in the iMM rodent facility surgery room, in a SPF environment. The animal was anaesthetized, with isoflurane (Zoetis, NJ, USA) through inhalation, and kept under a deep anaesthesia stage throughout the whole procedure. Assisted by a stereotaxic apparatus (Stoelting, IL, USA), the desired coordinates for the intracerebroventricular (icv) cannula implantation, were marked in the cranium of the animal. Coordinates were referenced from the bregma point: -0.5mm anterior-posterior (AP), +1mm medial-lateral (ML) and +3mm dorsal-ventral (DV). This system locally administers in the lateral ventricle the agonist solution (CGS21680 in aCSF, 100 nM) or the vehicle solution for 26 days.

Animals were divided in groups of 5 animals: 1) control, a naïve control group that did not go through any experimental procedure; 2) EAE VEH, EAE mice with micro-osmotic pump filled with the vehicle solution, artificial cerebrospinal fluid (aCSF); 3) EAE CGS, EAE mice with micro-osmotic pump filled with the A2A receptor agonist, CGS21680, in aCSF.

**Behavioural tests**

Three different behavioural tests were performed to complement the CS analysis: pole test (PT), rotarod (RR) and open field (OF). The PT and RR were used to assess motor balance and coordination⁹, while the OF test was used to observe general motor activity and exploratory behaviour¹¹.

**Pole test**

Pole test (PT) was performed as previously described, with some minor alterations³⁰. The protocol was performed under red light and four trials were performed per day.

The system entailed a square base (15×15×1.5) with a rough-surfaced pole (height 50cm; diameter 2cm) on top of it. Each trial consisted of positioning the pole horizontally and the mouse was placed head-upward on its top. Immediately after, the pole was smoothly placed vertically, and the trial was concluded as the mouse descended the pole and touched the base with its four paws. Trials were excluded when the animal would go up the top of the pole. The time the animal took to orient downwards in a 180º movement, t-descend, in seconds (s); the time it took for the animal to reach the base of the pole, t-descend (s); and total experiment time (t-total), which was the sum of both t-orient and t-descend, were registered. An average of the four trials is accounted for all parameters.

**Rotarod**

Rotarod (RR) test was performed with a minimum 2 hours interval after PT trials were finished. The RR apparatus (Panlab, Harvard Apparatus, Barcelona, Spain) consists of a cylinder with 5 divisions, in which 5 different animals can stand there simultaneously. The system has an acceleration program, which linearly increases velocity from 4rpm to 40rpm, in approximately 300s. If the mouse fell before or at 7rpm, it would be placed back on the rod.

This test was performed under dim yellow light and 3 trials were performed per day, with a minimum 30 minutes interval between them. At the end of each trial, time of fall, t-fall (s), and maximum rotation (rpm) reached are registered for every animal. An average of 3 trials is accounted for both parameters.

**Open field**

Open field (OF) trial was performed under dim yellow light, the day after PT and RR tasks. The task consisted of placing the mouse in the center of a square wood arena (40×40×40cm), allowing the animal to freely explore it for 10 minutes. One trial was performed per day under dim yellow light. Travelled distance (m) and number of crossings from the peripheral area to the intermediate area and to the central area of the field were registered and analysed using the video tracking software Any-maze (Stoelting, Dublin, Ireland). Results from different behavioural test days were compared to assess EAE progression.

**Animal sacrifice and tissue processing**

EAE induced animals that presented CS above 1 were sacrificed at the peak, on day 23 p.i., two days before behavioural tests were finished. The remaining animals were kept until the end of the protocol, on day 28 p.i.. Animals were deeply anaesthetized with isoflurane, through inhalation and transcardially perfused with PBS. Brain left hemispheres were removed and preserved for tissue post-fixation in 4% paraformaldehyde (PFA) in PBS (pH 7.2) at 4°C, for 72 hours for immunohistochemistry analysis and Luxol fast blue staining. Brain right hemispheres were cryopreserved at -80°C after isolation of the brain areas of interest (SVZ, CC, striatum and cortex) for molecular analysis by western blotting.

**Cellular and Molecular analysis**

**Western Blot**

Western blot (WB) analysis was performed to quantify myelin proteins, as MBP and PLP to assess de and remyelination. NF-κB phosphorylated and total forms, along with its inhibitor IκBα forms and MAPK/ERK1/2 proteins were quantified to assess A2A agonist activation effect on oligodendrocytes remyelinating action and inflammation. SVZ, CC, striatal and cortical areas were selected for covering, not only the neurogenic niche of interest of our study, SVZ, but for being the most demyelinated areas under EAE pathogenesis.
Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% acrylamide/bisacrylamide gels and transferred onto PVDF (polyvinylidene difluoride) membranes. Membranes were blocked and incubated with primary antibodies against MBP (Cell Signalling Technology, Danvers, MA, USA), PLP (Cell Signalling Technology), TNFα (Santa Cruz Biotechnology, Dallas, TX, USA), pNFκB (Abcam, Cambridge, UK), tIkBa (Abcam), pIkBa (Cell Signalling Technology), IERK1/2 (Cell Signalling Technology), PERK1/2 (Cell Signalling Technology). Mouse anti-vinculin antibody (Sigma, St. Louis, MO, USA) was used as loading control. Proteins were revealed with Clarity™ Western ECL Substrate (Bio-Rad Laboratories), using ChemiDoc™ XRS+ imaging system with Image Lab™ software (Bio-Rad Laboratories, Hercules, CA, USA). WB images were processed and analysed using ImageJ software (NIH, Bethesda, MD, USA). Results are expressed as protein levels, normalized to the percentage of control (100%).

**Free-floating Immunohistochemistry (IHC)**

To assess brain demyelination, particularly in the CC, it was performed on left hemisphere slices an immunohistochemistry against anti-MBP rabbit (1:200) primary antibody (AB5320, Merck, Darmstadt, Germany) in 3% blocking solution (6% bovine serum albumin (BSA) and 0.2% Triton X-100 in PBS). Representative images of the CC were acquired using the Zeiss Axiovert 200 Inverted Microscope (Carl Zeiss, Corp., Oberkochen, Germany), with a 5× resolution. For MBP staining, CC portions were manually delimited, using ZEN 2.5 lite software (Carl Zeiss, Corp., Oberkochen, Germany), and the area of the delimited region was determined. Results are presented normalized to the percentage of control (100%).

**Luxol fast blue staining**

To evaluate demyelinated areas in the white matter throughout EAE development, Luxol fast blue (LFB) protocol was performed on left hemisphere slices. LFB is a classical histological method that allows a distinction between myelinated and demyelinating regions, by assigning a strong blue colour to myelin. Luxol staining is usually counterstained with eosin staining to distinguish myelinated from demyelinated regions.

**Statistical analysis**

Statistical analysis was performed using the software Graphpad Prism 6 (Graphpad, La Jolla, CA, USA). Collected data is presented as the mean ± standard error of the mean (SEM) for each experimental animal group. Ordinary one-way analyses of variance (ANOVA) followed by Bonferroni’s multiple comparisons test were used to evaluate the significance of differences between means of two or more conditions, considering p<0.05 to represent statistically significant differences.

**Results**

**EAE Clinical Scores**

EAE-induction was analysed by examining daily the physical condition of the animals since the beginning of the in vivo protocol. Regarding CS analysis, it is possible to observe that EAE incidence was low (40% in EAE VEH). In fact, on average the CS per group does not overpass a CS of 1 (fig. 1-A). In comparison, the EAE onset was similar between EAE VEH and EAE CGS (fig. 1-A). In the EAE VEH group, only 2 individuals presented a CS peak of 2-3 at 20-21 days p.i., following which both animals showed partial recovery (fig. 1-B). Concerning the EAE CGS group, only two animals developed clinical signs, starting both at day 19 p.i. (fig. 1-C).

**Locomotor and exploratory activity analysis**

Since the behavioural analysis with all animals was inconclusive, only individuals with CS>1 from EAE CGS and EAE VEH groups were analysed (n=2). Although a tendency is visible, these results are not significant due to low experimental n (EAE VEH CS>1, n=1; EAE CGS CS>1, n=2).

**EAE VEH and EAE CGS animals showed impaired PT performance on day 21 p.i.**

The most relevant result concerns a tendency of t descent to increase from day 17 to day 21 p.i. in both EAE CGS and EAE VEH groups (EAE VEH: day 17 p.i. – 2.12 s; day 21 p.i. – 10.04 s; EAE CGS: day 17 p.i. – 4.50 s; day 21 p.i. – 7.38 s, n=2, fig.1-D), as well as an increase in t ascend in EAE VEH group (day 17 p.i. – 5.25 s; day 21 p.i. – 10.0 s; n=2, fig.1-E). These changes are in accordance with the CS development observed in both groups. In fact, motor impairment worsened with EAE progression, while the locomotor capacity of the animals to efficiently complete the task was disturbed. Moreover, on day 21 p.i., both EAE VEH and EAE CGS groups show a higher t descend than the CTRL group (CTRL: 1.75 ± 0.27 s; EAE VEH: 10.04 s; EAE CGS: 7.38 s; n=5, n=2, fig.1-D).

**EAE VEH and EAE CGS animals showed impaired RR performance on day 21 p.i.**

RR results show a tendency in both EAE VEH and EAE CGS groups for a decrease in t fast (EAE VEH: day 17 p.i. – 110.2 s; day 21 p.i. – 53.5 s; EAE CGS: day 17 p.i. – 77.2 s; day 21 p.i. – 44.7 s; n=2, fig.1-F) and maximum rotation (EAE VEH: day 17 p.i. – 17.0 rpm; day 21 p.i. – 10.3 rpm; EAE CGS: day 17 p.i. – 13.2 rpm; day 21 p.i. – 9.2 rpm; n=2, fig.1-F).
This time point coincides with the peak of CS in the EAE VEH group and progression of CS in the EAE CGS group. In fact, the capacity of the animals to stand longer on the RR declined, as tfall and max. rotation mean values decreased. Nonetheless, when comparing EAE VEH with EAE CGS no significant differences were observed.

**EAE CGS and EAE VEH animals showed impaired locomotor and exploratory activity on day 18 and 22 p.i.**

Regarding OF analysis from day 15 to day 22 p.i., a considerable tendency of decrease in the travelled distance in both EAE VEH and EAE CGS groups was seen (EAE VEH: day 15 p.i. – 30.51 m; day 18 p.i. – 13.67 m; day 22 p.i. – 9.620 m; EAE CGS: day 15 p.i. – 29.47 m; day 18 p.i. – 17.96 m; day 22 p.i. – 11.65 m; n=2, fig.1-G). This decrease, although less striking, is also seen in the nr. of crossings (EAE VEH: day 15 p.i. – 201.5; day 18 p.i. – 121.5; day 22 p.i. – 60.5; EAE CGS: day 15 p.i. – 202.0; day 18 p.i. – 165; day 22 p.i. – 105.0; n=2, fig.1-H). Altogether, these data suggest that a higher CS has influence in the exploratory and motor activity of the animals, being particularly relevant that day 18 p.i. coincides with the appearance of EAE phenotype on EAE VEH group, which corresponds to a decrease in travelled distance and nr. of crossings. Nonetheless, this decrease was also observed in EAE CGS animals, that at this time point still did not display EAE phenotype.
**Cellular and Molecular analysis**

**EAE VEH and EAE CGS animals showed no changes in NF-κB signaling pathway**

Regarding pNF-κB levels, observing striatal samples, no significant changes in pNF-κB protein levels were seen between groups, despite a slight increase in EAE CGS animals with CS>1 when compared with animals with CS=0 (EAE CGS: CS=0: 67.30 %; CS>1: 100.0 %; n=2, n=1, respectively, fig. 2-A). Considering CC samples, although variations are not prominent, a tendency to an increase in EAE VEH animals with CS>1 comparatively to EAE VEH animals with CS=0 (EAE VEH: CS=0: 68.67 %; CS>1: 129.5 %; n=3, n=2, respectively, fig. 2-B). In addition, in CC samples, EAE CGS animals with CS=0 also presented higher NF-κB protein levels when comparing with EAE CGS animals with CS>1 (EAE CGS: CS=0: 121.5 %; CS>1: 85.90 %; n=2, fig. 2-B).

**EAE CGS animals showed no changes in the MAPK/ERK(1/2) signaling pathway**

No major changes were seen in pERK protein levels. However, a tendency for an increase in EAE CGS animals with CS>1 comparatively to EAE CGS animals with CS=0 is observed in cortical (EAE CGS: CS=0: 50.70 %; CS>1: 137.9 %; n=1, n=2, respectively; fig. 2-C) and striatal samples (EAE CGS: CS=0: 50.50 %; CS>1: 73.00 %; n=2; fig. 2-D) pERK protein levels in EAE VEH groups are very similar to CTRL groups in all brain areas studied.

**MBP expression in the CC remained unaltered in EAE VEH and EAE CGS animals with CS>1**

To assess demyelination in the CC, an IHC for MBP was performed in samples from one animal of each condition: CTRL, EAE VEH and EAE CGS with CS=0, EAE VEH and EAE CGS with CS=2.2-3. In sum, no significant differences were observed between conditions (CTRL: 100.0 ± 10.48 %; EAE VEH: CS=0: 105.9 ± 17.44 %; CS>1: 85.40 ± 12.83 %; EAE CGS: CS=0: 68.75 ± 4.784; CS>1: 91.20 ± 12.76 %; n=1; fig. 3-B).
Demyelination was not observed in EAE VEH and EAE CGS animals

Regarding LFB assay, no significant differences were observed between the same conditions analysed in IHC MBP assay. Peripheral cortical regions display a more rose tone, when compared with ventral regions of the cortex, and the CC maintains an intense blue colour, in all experimental conditions. Blue colour staining appears to be lighter in both EAE VEH and EAE CGS animals with CS>1 than in CTRL or EAE slices from animals with CS=0 slices, although colour distribution remains quite similar (fig. 3C,D,E). Taken together, EAE model pathophysiology did not seem to cause any apparent effects on myelin levels in the brain.

Discussion

The first step of our work intended to efficiently induce the EAE model on female C57BL/6 ten-weeks-old mice, using a commercialized induction kit by Hooke Laboratories, composed of a MOG35-55 rat emulsion in CFA with M. tuberculosis and PTX. However, incidence was only of 40% in EAE VEH, which reduced the significance of the results. EAE onset expression is highly dependent on a variety of aspects, from species, age and gender of the animals to housing conditions, stress and diet.

Animal stress might be one of the major impact factors for the low incidence and severity of the EAE model. On the day following the induction protocol, animals were subjected to a very demanding BrdU protocol, which, in turn, was immediately followed by the micro-osmotic pump implantation surgery. These procedures require an intensive handling and restrain of the animals, which may increase the distress of the animals, causing the delay observed on EAE onset and its low severity. Moreover, the fact that animals were individually housed to allow a full post-surgery recovery may also be a stress factor due to lack of social contact, as mice are a social interactive species. Concurrently, housing acclimatization was also altered on day 7 p.i., with animals being moved from an SPF facility to a VAF area. This change was due to previous assessments on how environment may influence EAE pathogenic autoimmune response, where it was observed that animals induced in a SPF environment displayed a typical course of EAE onset when compared with animals induced in conventional housing or in a germ-free environment. However, animals had to be changed on day 7 p.i. so that behavioural tests could be performed.

For a proper initiation of the autoimmune response against MOG35-55 antigen, PTX is required to be functional so as to increase BBB permeability, facilitating pathogenic T cells migration, thus exacerbating the inflammatory response. PTX dosage was adjusted to optimize its potency, accordingly to the Hooke Kit's PTX dose adjustment methodology, considering stress augmentation due to the osmotic pump implantation and BrdU administration. Moreover, PTX was administered i.p. to reduce procedure stress. Yet, PTX dosage may still not be the most adequate which may interfere with MOG35-55 immunization.
activation in the CNS. In fact, although many studies have confirmed that PTX is essential for MOG induction in C57BL/6 mice,12,13 Yin et al. have presented data observing that PTX has a protective effect in EAE, by reducing lymphocyte infiltration, decreasing EAE clinical signs.25 Ultimately, PTX malfunctioning or inactivation could have annihilated its role in the model induction. Moreover, due to the pump implantation procedure, MOG injection site was performed not dorsally but ventrally which is accessible to the animal who involuntarily can rupture the emulsion site, causing emulsion leakage, thus, tarnishing the antigen administration.

Importantly, the low incidence in EAE CGS group and lower CS might reflect a putative protective role of A2aR agonist. Several studies have reported that CGS21680 has an ambiguous role in the inflammatory response under EAE conditions, by both enhancing migration of inflammatory lymphocytes into the CNS and diminishing the expression of pro-inflammatory cytokines, thus attenuating EAE expression. Additionally, in previous work performed in our lab, it was observed that A2aR activation promoted OPCs differentiation and maturation into myelinating OLGs from SVZ NSCs. Therefore, we expected that CGS icv administration would have had some effect on EAE phenotype expression. However, no significant differences are observed when comparing EAE VEH group clinical course with EAE CGS group. Due to the low EAE model incidence and low experimental n, it was not possible to conclusively assess the role of the A2aR agonist in the EAE clinical course. Additionally, the CGS21680 concentration may not have been enough to cause any major impacts in the lateral ventricle environment, especially, under EAE neuroinflammatory conditions.

EAE progression monitoring was complemented with a battery of behavioural tests, which included OF, PT and RR. Overall, in all 3 behavioural assays, it was observable that EAE animals with higher CS presented motor and balance impairments thus having a negative impact on their performance in the test. However, results did not provide any conclusions on the influence that CGS21680 administration might have had in motor and exploratory abilities, as results from the EAE CGS CS>1 group did not achieve significant variations when compared with the EAE VEH CS>1 group. Once again, this lack of significance and conclusiveness in behavioural results is due to a low incidence obtained in the model, leading to a low experimental n in both groups.

Immunohistochemical and histological assays did not provide any additional information regarding myelin levels in different conditions. Regarding CC myelination, immunohistochemistry for MBP staining and LFB results are quite coherent. In both tests, in every condition, this area is presented as highly myelinated, with no relevant CC area reduction between conditions in IHC and with an intense blue colour in LFB, indicative of high myelin levels. Regarding LFB assay, the intense blue colour visible in the CC area could be a sign of an intense remyelinating response, as this is one of the most affected areas by demyelination. Picard-Riera et al. have shown that OPCs migrate from the SVZ to the restoring myelin levels.

Regarding NF-κB signaling pathway activation, results were also not very informative. NF-κB activation has been described to enhance the inflammatory response under MS or EAE pathogenesis. It has also been described that, in spinal cord injured animals, the inhibition of NF-κB transduction in astrocytes promoted oligodendrogenesis in this inflammatory environment. Furthermore, A2aR role has an inhibitory role in the activation of NF-κB, suppressing inflammation. However, in the four brain areas analysed it was not possible to observe any changes in this pathway. Variations between conditions are not prominent, despite a slight increase observed pNF-κB protein levels in cortical samples of EAE CGS CS>1 animals. This increase might be associated with EAE pathogenic inflammation. Regarding pIκB protein levels, it was not possible to establish a correlation with pNF-κB protein levels, besides a similar tendency in striatum and SVZ samples. Hence, sample size should be increased to better assess these hypotheses.

In regard to ERK1/2 pathway activation, Morello et al. have shown that A2aR activate this signaling pathway. In addition, Maricich et al. have demonstrated that this signaling is activated as OPCs differentiate into mature myelinating OLGs. Moreover, in MS and EAE conditions, although inflammatory T cells and macrophages expressed MAPK/ERK phosphorylation, this pathway activation has not been described to cause a major impact in the diseases clinical course. Regarding pERK1/2 protein levels no changes were observed between EAE CGS and EAE VEH. However, in cortical and CC samples an increase in pERK1/2 was seen in EAE CGS CS>1, possibly related with mature myelinating OLGs activity in response to demyelination. Contrarily, in both SVZ and striatum, this tendency is not observed. Thus, once again, sample size and low EAE incidence were not sufficient to make any conclusions on the role that CGS21680 might have in the activation of the ERK1/2 pathway under EAE pathological conditions.

Taken together, the low incidence and severity of the EAE model were the main limiting factors of this work. Consequently, a small sample size of EAE CGS and EAE VEH conditions did not allow a conclusive analysis of the A2aR role in EAE phenotype ablation or its effects in OLGs remyelinating activity.

**Conclusions**

The aim of this project was to evaluate the role of A2aR in modulating the production of OLGs, thus inducing myelination under EAE pathogenic conditions.
Overall, EAE model induction was not successful, with only an incidence of 40% in EAE VEH, which constrained the significance of the results. EAE animals showed impaired performance in behavioral tests as EAE phenotype was progressing. However, no relevant differences were observed in EAE CGS when compared with EAE VEH animals. Moreover, no tendency for changes was observed at a molecular level, regarding either the NF-kB or the MAPK/ERK(1/2) pathway. Regarding demyelination, both LFB and MBP immunohistochemical staining showed that myelin amounts in the EAE brain, for both EAE VEH and EAE CGS animals, were similar to CTRL animals. The major drawback of this work was the low incidence of the EAE model induction that, consequently, reduced sample size of animals expressing EAE phenotype, limiting statistical analysis assessments and EAE phenotype correlations with behavioural performances or results from molecular assays. Moreover, EAE severity was rather mild, never surpassing a CS of 2-3, which may have hindered EAE pathological symptoms intensity, namely inflammation and demyelination.

In the future, some troubleshooting approaches should be performed as an attempt to optimize EAE model induction. For instance, MOG35-55 injection site could be performed on the lower dorsal flank to avoid emulsion leakage. Furthermore, it would be of further interest to perform a permeability assay of the BBB, to evaluate PTX ability to disrupt the BBB and adjust its optimal dose regarding our experimental protocol. Moreover, to assess whether animal stress induced by the intense in vivo protocol, i.e., the BrdU administration protocol, the osmotic pump implantation surgery, it would be appropriate to immunize the animals and avoid any stress-inducing procedures, leaving the animals at rest, for, at least, two weeks. Albeit improvements on the EAE induction protocol, our future work will be focused on increasing sample size in order to perform supplementary molecular and cellular analysis, that could sustain our hypothesis. BrdU staining should be analysed to compare oligodendrogenesis and cell proliferation under A2AR activation in EAE conditions, combined IHC assays of OLGs lineage markers, as Olig2, GalC, or NG2, to thoroughly assess oligodendrogenesis in the brain. Furthermore, additional A2AR signaling pathways could be assessed to further evaluate the role of this receptor in MS. For instance, assessing JNK/MAPK signaling pathway activation along with phospho-JNK expression in OLGs would be an exciting approach to assess CGS21680 protective role in neuroinflammation, particularly in OLGs, as described by Genovese and colleagues. Altogether, these assays could provide useful data to optimize A2AR concentration. Moreover, LFB assays on spinal cord samples would complement our studies on demyelination and endogenous remyelination phenomena in the CNS.

In sum, further assays are required to corroborate the hypothesis presented in this work, in order to unequivocally consider A2AR a promising approach to reinforce oligodendrogenesis under demyelinating and neuroinflammatory conditions towards the development of regenerative therapies in MS.

References


