Aus der Klinik für Neurologie
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# Adenosine 2a receptor signaling in autoimmune neuroinflammation

Dissertation

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*“I do not know what I may appear to the world, but to myself I seem to have been only like a boy playing on the sea-shore, and diverting myself in now and then finding a smoother pebble or a prettier shell than ordinary, whilst the great ocean of truth lay all undiscovered before me.”*

Sir Isaac Newton

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Natalizumab restores aberrant miRNA expression profile in multiple sclerosis and reveals a critical role for miR-20b.

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**Abstract**

Background & Hypothesis: Adenosine is known to play an anti-inflammatory role in the immune system. Previous studies implicate that ligation of this purine nucleoside to the adenosine 2a receptor (A2aR) unfolds the key mechanism for its immunomodulatory impact. However, possible immune-related effects in the central nervous system (CNS) remain unclear, particularly in autoimmune neuro-inflammation.

Methods: With my work I clinically and histologically investigated the effect of A2aR signaling in the experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis (MS) in mice. Using genetic deletion and both pharmacological activation and inactivation of the A2aR, I analysed the functional motor impairment and the extent of tissue alterations in brain and spinal cord. These tissue alterations were investigated using immunohistochemistry, the key tool for visualizing morphological changes on cellular level. This technique is capable of showing immune cell infiltration, demyelination and the extent of tissue destruction. In order to confirm the myelin-related damage patterns, I also performed Oil-Red-O lipid staining. In addition to EAE, I used lipopolysaccharide (LPS)-based experiments which allowed an investigation of microglia without contamination of peripheral macrophages.

Results: In line with the well-investigated anti-inflammatory effect of A2aR, mice lacking the A2aR ($A2aR^{-/-}$) developed exacerbated disease scores in the early phase of disease with more and larger CNS lesions and increased T cell infiltration compared to their wild type litter mates. However, in the late phase of disease the clinical scores of wild type and knock out mice were comparable, but leaving the wild type mice with larger lesions, stronger tissue destruction and slightly increased T cell infiltration. Interestingly, injecting mice with the specific A2aR agonist CGS-21680 in already developed EAE also resulted in more and larger lesions, an increased inflammatory cell infiltration and a higher mean clinical score. I was able to reproduce the visualisation of myelin damage with the Oil-Red-O lipid staining. Inactivating the A2aR with its specific antagonist SCH-58261 leads to less and smaller lesions in comparison with the control group. A later onset of disease and a milder clinical outcome were also observed. With the LPS injection model it was shown that triggering the A2aR causes *in vivo* microglial activation.

Conclusions: Taken together, the results indicate that activation of the A2aR in the early phase of EAE has a positive impact on the outcome of the disease. Nevertheless, a continuous activation of the receptor results in unfavourable clinical and histological findings. Apparently, activation of the A2aR has a detrimental effect in established neuroinflammation.

## Zusammenfassung

Hintergrund & Hypothese: Adenosin entfaltet im Immunsystem eine anti-entzündliche Wirkung. Frühere Studien implizieren, dass der immun-modulatorische Effekt des Purin-Nukleosids durch die Bindung an den Adenosin 2a Rezeptor (A2aR) getriggert wird. Im zentralen Nervensystem (ZNS) ist der genaue Effekt weiterhin ungewiss, insbesondere bei autoimmuner Neuro-inflammation.

Methoden: Mit meinen Versuchen untersuchte ich klinisch und histologisch den Effekt der A2aR-Signalwirkung in *experimental autoimmune encephalomyelitis* (EAE), einem Mausmodell der Multiplen Sklerose (MS). Mittels genetischer Ablation und sowohl pharmakologischer Aktivierung, als auch Inaktivierung des A2aR untersuchte ich die funktionelle Bewegungseinschränkung und verglich diese mit Gewebeveränderungen in Gehirn und Rückenmark. Um diese morphologischen Veränderungen auf zellulärer Ebene sichtbar zu machen, ist Immunhistochemie ein Schlüsselwerkzeug. Diese Technik ermöglicht es, Immunzellinfiltration, Demyelinisierung und das Ausmaß an Gewebezerstörung zu visualisieren. Um den Defekt zu bestätigen, fertigte ich außerdem Oil-Red-O Fettfärbungen an. Zusätzlich wurde auch das Lipopolysaccharid (LPS) Mausmodell angewandt, wodurch die Untersuchung von Mikroglia ohne Kontamination von peripheren Makrophagen ermöglicht wurde.

Ergebnisse: Im Einklang mit der Vorstellung, dass A2aR einen anti-entzündlichen Effekt hat, zeigten A2aR-defiziente Mäuse ($A2aR^{-/-}$) stärkere klinische Symptome in der frühen Phase der Erkrankung mit mehr und größeren Läsionen und mit gesteigerter T Zell Infiltration im Vergleich zu ihren Wurfgeschwistern. In der späten Krankheitsphase waren keine klinischen Unterschiede zwischen den Gruppen erkennbar. Allerdings zeigten die Wildtyp-Mäuse größere Läsionen, mehr Gewebezerstörung und mehr T Zell Infiltration. Interessanterweise resultierte die Verabreichung des spezifischen A2aR-Agonisten CGS-21680 in Mäusen mit bereits entwickelter EAE ebenfalls in einer ausgeprägteren Läsionslast, mehr entzündlicher Zellinfiltration und einer schwereren klinischen Beeinträchtigung. Mittels Oil-Red-O Fettfärbung konnte die Visualisierung des Myelindefektes reproduziert werden. Die Inaktivierung des A2aR mit dem spezifischen Antagonisten SCH-58261 führte zu weniger und kleineren Läsionen im Vergleich zu der Kontrollgruppe. Darüber hinaus konnten ein späterer Krankheitsbeginn und ein milderer Krankheitsverlauf festgestellt werden. Mit dem LPS Mausmodell konnte gezeigt werden, dass das Triggern des A2aR Mikroglia *in vivo* aktivieren kann.

Zusammenfassung: Die Ergebnisse zeigen, dass die Aktivierung von A2aR in der frühen Phase der EAE einen positiven Einfluss auf die Krankheit hat. Nichtsdestotrotz führt eine dauerhafte Aktivierung letztlich zu schlechteren klinischen und histologischen Befunden. Die Ergebnisse lassen darauf schließen, dass eine Aktivierung vom A2aR einen schädlichen Effekt in etablierter Neuroinflammation auslöst.

**Abbreviations**

| | |
|---|---|
| AMP | adenosine monophosphate |
| ATP | adenosine triphosphate |
| A1R | adenosine 1 receptor |
| A2aR | adenosine 2a receptor |
| A2bR | adenosine 2b receptor |
| A3R | adenosine 3 receptor |
| c / chet-AMP | cyclic / 2-cyclohexylethylthio-AMP |
| CD | cluster of differentiation |
| CFA | Complete Freund's Adjuvant |
| CNS | central nervous system |
| CREB | cAMP response element-binding protein |
| CX3CL1 | chemokine (C-X3-C motif) ligand 1 |
| DMSO | dimethyl sulfoxide |
| EAE | experimental autoimmune encephalomyelitis |
| Fig. | figure |
| GFAP | glial fibrillary acidic protein |
| Iba-1 | ionized calcium-binding adapter molecule 1 |
| IL | interleukin |
| LAG-3 | lymphocyte-activation gene 3 |
| LPS | lipopolysaccharide |
| MBP | myelin basic protein |
| MOG | myelin oligodendrocyte glycoprotein |
| mRNA | messenger ribonucleic acid |
| MS | multiple sclerosis |
| MTB | Mycobacterium tuberculosis |
| PBS | phosphate buffered saline |
| PFA | paraformaldehyde |
| PKA / C | protein kinase A / C |
| PTX | pertussis toxin |
| SEM | standard error of the mean |
| Th cells | T helper cells |
| TNF-α | tumor necrosis factor-α |

**Contents**

# 1. Introduction

## *1.1 Adenosine signaling*

Adenosine, a purine nucleoside composed of an adenine molecule and a ribose sugar molecule, plays a vital role in many physiological processes. For instance, it allows the organism to perform an effective energy transfer and cellular signal transduction. In the central nervous system (CNS), adenosine is a physiological modulatory messenger which is essential for the function of the CNS immune system and the organ itself [1].

There are two ways to increase the extracellular amount of adenosine. First, it can be transported out of the cell via specialized nucleoside transporters. Second, any kind of stress which leads to tissue damage and/or cell death releases adenosine triphosphate (ATP). ATP will then be degraded to adenosine by membrane-bound ecto-enzymes such as CD39 and CD73 [2]. High level extracellular adenosine concentration has been described as a “damage associated molecular pattern (DAMP)” [3].

The four adenosine receptor subtypes (A1, A2a, A2b and A3) belong to the group of G-protein coupled receptors with seven transmembrane domains. They are ubiquitarily expressed, including on the cells of the immune system and the CNS [1]. This work focuses on the immune modulatory role of the A2a receptor (A2aR). After adenosine activates the A2aR, a dual signaling is set in motion, as described for striatal cholinergic nerves [4]. The receptor is associated with two $G_s$-proteins [5], which trigger the adenylyl cyclase to produce intracellular cyclic adenosine monophosphate (cAMP) [6, 7]. Next, cAMP mediates the protein kinases A and C [4]. The protein kinase A (PKA) activates P-type calcium channels, whereas the protein kinase C (PKC) activates N-type calcium channels [4]. Additionally, PKA can affect gene expression directly by enhancing transcriptional activity via the cAMP response element-binding protein (CREB) [8]. Recently, the PKC pathway was implicated as a mechanism which is necessary for the induction of hypoxia inducible factor 1 in macrophages [9]. A cAMP-independent pathway has also been described for human neutrophils: The A2aR modulates stimulated

superoxide anion generation via activation of a plasma membrane-associated serine/threonine protein phosphatase [10].

*1.2 Adenosine in the immune system*

It is known that A2aR activation strongly down-regulates the immune cell function and is therefore capable of preventing detrimental tissue destruction [11-13]. The immune system achieves this effect with various mechanisms. For instance, T regulatory cells and primed precursor T helper (Th) cells express CD39 and CD73, which makes them capable of producing adenosine and inducing a local immune suppression by inhibiting the cytokine production and proliferation of Th1 and Th2 cells [14]. Elevated extracellular adenosine increases cAMP in peripheral T cells, blocking the T cell receptor-triggered CD25 upregulation [15]. Although A2aR is not expressed on B220-positive cells *i.e.* B cells [16], a stimulation of the A2aR elevates the intracellular cAMP level *in vitro* [17]. Correspondingly, natural killer cell activity is reduced by A2aR activation via increased cAMP [18]. In macrophages, A2aR ligation suppresses the production of the pro-inflammatory cytokines IL-12 and TNF-α [19]. Additionally, Th1 and Th17 cell infiltration can be reduced by activating the A2aR [20]. These anti-inflammatory effects were demonstrated in multiple tissue damage animal models, for instance in the lung, liver and heart [21-24]. In addition, a selective CD73-dependent activation of the A2aR via the prodrug chet-AMP leads to a suppression of joint inflammation in collagen-induced experimental rheumatoid arthritis [25]. Concerning regulatory T cells, adenosine signaling also plays an important role. On the one hand, stimulated T cells under the influence of the A2aR-specific agonist CGS-21680 *in vitro* show more transcripts of lymphocyte-activation gene 3 (LAG-3) than T cells in the presence of vehicle. LAG-3 is a protein of the immunoglobulin superfamily which binds to HLA class-II antigens and is involved in lymphocyte activation. On the other hand, an upregulation of LAG-3 leads to more regulatory activity *in vivo* and can therefore prevent tissue destruction and promote tolerance [20].

*1.3 Adenosine in the CNS*

Interestingly, concerning the CNS a quite different picture evolves. In models of stroke [26-28], cortical brain injury [29, 30] and spinal cord compression [31, 32], inactivation of the receptor by selective antagonism or genetic deletion caused a

more beneficial outcome. Chen *et al.* showed that concerning the transient focal ischemia model of stroke, a transgenic inactivation of A2aR in mice leads to a better clinical score and less overt tissue damage compared to wild type [26]. Accordingly, a reconstitution of the A2aR knock out mice with A2aR-positive immune cells after inducing stroke leads to larger brain injury [27]. By inactivating the A2aR in a stroke model pharmacologically, brain tissue is protected from myelin disorganization [28]. In an animal model of cortical brain injury, A2aR deficiency results in reduced neuron apoptosis, less brain edema and reduced neurological functional deficit compared to wild type [29]. This A2aR effect on tissue destruction in the brain is dependent on local glutamate levels [30]. When focusing on the animal model of spinal cord compression, a pharmacologic inactivation of A2aR reduces demyelination and functional impairment [31] just like a deletion of the A2aR gene [32]. Thus, a rather detrimental (than protective) effect of A2aR has been shown in the CNS. In order to better understand the cellular immune-related mechanisms in the models described above, the focus needs to be set on the CNS-resident immune cells, microglia. Microglia, the macrophages of the central nervous tissue, can reveal diverse properties: Activating the PKA via A2aR stimulation leads to an anti-inflammatory effect, whereas activating the PKC via A2aR stimulation in combination with glutamate signaling causes a pro-inflammatory effect [30, 33]. These cells are CNS-resident from embryonal stages on, play a very important role in neuroinflammation and are in constant exchange with other cell types: “perivascular microglial cells of the CNS are bone marrow-derived and present antigen *in vivo*” [34]. The mechanisms underlying this remarkable Janus-like function of adenosine in the CNS compared to other organ systems are not known.

### *1.4 Adenosine in EAE*

Multiple sclerosis, modelled in the following experiments by inducing EAE in mice, is a human disease which can either impress as a relapsing and remitting or as a progressive clinical activity, causing demyelination, neuronal damage and glial scaring [35]. Having in mind the effect of adenosine on inflammatory processes, an inhibition of the A2aR may have a beneficial influence on this disease. Using the most common model of MS, the EAE of the mouse, recent studies have shown that adenosine is involved in the development and progress of the disease. The

A2aR seems to play a role in EAE as it is upregulated on mRNA level in the CNS [36]. In order to promote lymphocyte migration in the brain, elevated local adenosine levels are necessary: It is believed that the major influence of adenosine on EAE takes place in the choroid plexus, an initial entry point for these immune cells, because very high levels of A2aR were detected in this anatomical structure [37]. Additionally, the expression of CX3CL1, a chemo-tactic and adhesion molecule, is increased by A2aR activation [16]. Extracellular adenosine is known to increase the permeability of the blood-brain barrier temporarily [38], allowing pro-inflammatory molecules and cells to enter the central nervous tissue. When focusing on bone marrow derived cells, mice lacking the A2aR on hematopoietic cells develop a severer and mice lacking the receptor on non-hematopoietic cells develop a milder EAE compared to wild type animals [39]. The role of A1R in EAE has been deeply investigated by Tsutsui *et al.* [36], but there is still a gap of knowledge regarding the other receptors.

## 2. Aims

Adenosine and its receptors A1, A2a, A2b and A3 are endogenous mediators of anti- and pro-inflammatory response, which emphasizes the adenosine signaling for further scientific investigation especially in autoimmune neuroinflammation.

This work is focused on different facets of the adenosine 2a receptor. Its mechanisms and functions are still incompletely understood. My goal is to focus on cellular aspects with histological methods regarding this field of immunology. Knowledge of the adenosine system and particularly its effects on CNS tissue may eventually be transferred to the clinical setting and potentially help MS patients.

Immunohistochemistry is an elaborate but effective way of dissecting overt tissue injury. In order to visualize CNS injury and inflammation, brain and spinal cord will be stained for microglia and other macrophages with ionized calcium-binding adapter molecule 1 (Iba-1) primary antibodies [40-43] and for demyelination with myelin basic protein primary antibodies [44]. CD3 primary antibodies will be used to detect T lymphocyte migration through the blood-brain barrier. The quantity of infiltration interests because lymphocyte infiltration and tissue destruction are important early determinants of MS and EAE [45]. Another focus needs to be set on the quality of inflammation. It is known that microglia change their morphology when they are under the influence of any kind of cellular stress [46]. Therefore, these changes in structure will be detected by immunohistochemistry.

My first aim is to follow the course of EAE after genetic deletion and pharmacological activation and inactivation of A2aR and to compare these results with the morphological evidence in the central nervous tissue.

The second aim of this study is to investigate the behaviour of microglia in the CNS under the influence of inflammatory stress. This will be accomplished by staining for the microglial marker Iba-1 at different time points of EAE and by using an LPS-triggered mouse model.

## 3. Materials & Methods

### *3.1 Materials*

#### *3.1.1 Laboratory equipment*

| | |
|---|---|
| Aspiration system | Vacusafe comfort, INTEGRA Biosciences GmbH, Fernwald, Germany |
| Bandelin Sonopuls | Bandelin electronic GmbH & Co. KG, Berlin, Germany |
| Cover slips | BB024060A1, Menzel-Gläser, Braunschweig, Germany |
| Dako Pen | S200230-2, Dako Deutschland GmbH, Hamburg, Germany |
| Fine surgical instruments | Fine Science Tools GmbH, Heidelberg, Germany |
| F-View fluorescence camera | Soft Imaging System, Munich, Germany |
| Leica CM1900 UV cryostat | Leica Microsystems, Wetzlar, Germany |
| Microscope slides | Histobond® glasses, Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany |
| Olympus BX51 microscope | Olympus, Hamburg, Germany |
| Omnifix®-F syringes | Braun Melsungen AG, Melsungen, Germany |
| Pipettes | Eppendorf, Hamburg, Germany |
| Shaker | 3D gyratory rocker SSM3, Bibby Scientific Limited, Stone, Staffordshire, UK |

#### *3.1.2 Chemicals, proteins*

| | |
|---|---|
| Bovine serum albumin | #A2153, Sigma-Aldrich, Munich, Germany |
| CFA | #263810, Difco, New Jersey, USA |
| CGS-21680 | C141-5MG, Sigma-Aldrich, Munich, Germany |
| DMSO | Sigma-Aldrich, Munich, Germany |
| Hoechst 33258 | #H3569, Molecular Probes, Thermo Fisher Scientific, Pittsburgh, USA |
| Immu-Mount | 9990402, Thermo Fisher Scientific, Pittsburgh, USA |
| Isoflurane | PZN-7253744, Actavis, Luxemburg |
| LPS from E.coli O26B6 | Sigma-Aldrich, Munich, Germany |

| | |
|---|---|
| Normal goat serum | #G9023, Sigma-Aldrich, Munich, Germany |
| $MOG_{35-55}$ peptide | Pepceuticals, Enderby, Leicestershire, UK |
| MTB H37RA | #263810, Difco, New Jersey, USA |
| Oil-Red-O staining kit | ab150678, Abcam, Cambridge, UK |
| PFA | Roth, Karlsruhe, Germany |
| PTX | List Biological Laboratories, Inc, Campbell, USA |
| SCH-58261 | S4568-5MG, Sigma-Aldrich, Munich, Germany |
| Tissue Tek® | Sakura Fintek, Alphen aan den Rijn, Netherland |
| Triton-X-100 | Merck, Darmstadt, Germany |

Buffers and solutions were prepared with double distilled $H_2O$.

*3.1.3 Antibodies*

The following primary antibodies were used:

| | |
|---|---|
| anti-A2aR, mouse (1:200) | 7F6-G5-A2, Santa Cruz Biotechnology Inc., Dallas, USA |
| anti-CD3, rat (1:200) | MCA500GA, AbD Serotec, Kidlington, Oxfordshire, UK |
| anti-GFAP, guinea pig (1:500) | 173 004, SySy, Goettingen, Germany |
| anti-Iba-1, rabbit (1:400) | 019-19741, Wako Chemicals GmbH, Neuss, Germany |
| anti-iNOS, rabbit (1:500) | PA5-16855, Thermo Fisher Scientific, Pittsburgh, USA |
| anti-MBP, rat (1:500) | MAB386, Millipore, Billerica, USA |
| anti-MHC I, mouse (1:200) | ab33752, Abcam, Cambridge, UK |

The following secondary antibodies were used:

| | |
|---|---|
| anti-rabbit Cy 2-labelled (1:500) | Invitrogen, Karlsruhe, Germany |
| anti-rat Cy 3-labelled (1:500) | Invitrogen, Karlsruhe, Germany |
| anti-mouse Cy 3-labelled (1:500) | Invitrogen, Karlsruhe, Germany |
| anti-guinea pig Cy 5-labelled (1:500) | ab102372, Abcam, Cambridge, UK |

The quotient in brackets indicates the degree of dilution.

*3.1.4 Software*

| | |
|---|---|
| Adobe Illustrator CS5 | Adobe System Inc, San Jose, USA |
| Adobe Photoshop CS5 | Adobe System Inc, San Jose, USA |
| Cell A software | Soft Imaging System, Munich, Germany |
| FracLac add-in | Karperien, Charles Sturt University, Australia |
| GraphPad Prism 5 software | GraphPad Software, La Jolla, USA |
| ImageJ software | National Institutes of Health, USA |
| Office Professional 2010 | Microsoft Corporation, Redmond, USA |

## *3.2 Methods*

### *3.2.1 Induction, treatment and clinical evaluation of EAE*

6-8 weeks old female mice with C57BL/6 background purchased from The Janvier Laboratory were used for the experiments. Animals were 6 weeks old and weighed between 19 and 23 g. Each mouse was immunized subcutaneously (s.c.) at day 0 with 200 µg $MOG_{35-55}$ peptide and 800 µg MTB H37RA dissolved in 100 µl PBS and 100 µl CFA. Emulsion was sonificated with a Bandelin Sonopuls at 50 % interval and 50 % power for 3 minutes. Additionally, 400 ng of PTX was applied intraperitoneally (i.p.) at day 0 and day 2. Omnifix®-F syringes were used for all injections.

The clinical EAE score of a mouse has been assessed as described in Aktas *et al.* 2003 [47]: 0, no disease; 1, tail weakness; 2, paraparesis; 3, paraplegia; 4, paraplegia with forelimb weakness or paralysis; and 5, moribund or dead animals. Mice with a persisting score over 3 got euthanized. Animals which died due to EAE were included as score 5 until the end of experiment and animals which died due to other circumstances were excluded. Mean clinical scores at separate days and mean maximal scores were calculated by adding scores of individual mice and dividing by number of mice in each group.

### *3.2.2 Genetic deletion of A2aR*

6-8 weeks old A2aR-deficient mice generated by Chen *et al.* [26] and bred on a C57BL/6 background, or wild type littermates of the A2aR-deficient mouse strain were immunized as described above.

*3.2.3 Pharmacologic modulation of A2aR*

Treatment was started at the peak of disease at day 12 every day with 0.1 mg/kg bodyweight of the A2a-receptor agonist CGS-21680 dissolved in 5 % DMSO [48]. For preventive antagonist treatment, 1 mg/kg bodyweight A2a-receptor antagonist SCH-58261 dissolved in 5 % DMSO was applied starting one day before EAE immunization [49]. Both treatment groups were compared with a control group treated with 5 % DMSO in PBS. All treatments were applied intraperitoneally.

*3.2.4 Preparation of tissue*

After sacrificing the mice by a deep isoflurane narcosis, blood was removed by a transcardial saline perfusation. Brain and spinal cord were isolated with fine surgical instruments. Specimens were then postfixed in 10 ml 4 % PFA for at least 6 hours on a shaker at +4 °C. Next, tissues were washed 3 times for 30 minutes with 10 ml PBS on a shaker at +4 °C. Dehydration was achieved by leaving specimens in 10 ml of a 30 % sucrose solution over night on a shaker at +4 °C. After brain and spinal cord parts have been embedded in Tissue Tek®, they were frozen with dry ice and stored at -80 °C. A Leica CM1900 UV cryostat was used to cut the samples. Slices were collected on Histobond® glasses.

*3.2.5 Immunohistochemistry*

After drying the slices and removing the Tissue Tek®, the contour of the tissue was traced with a Dako Pen. Next, 50 µl of 4 % PFA was applied on each slice for 15 minutes. The glasses were put in a humidity chamber at room temperature and on a shaker after every step. Between the steps, the liquids were removed with an aspiration system. After that, the tissue was treated with 50 µl of a 1 % Triton-X-100 in PBS solution for 3 minutes. After washing with PBS for 3 minutes, the tissue was blocked with 50 µl of blocking buffer for 2 hours. 10 ml of blocking buffer consists of 8 ml PBS, 1 ml of 100 % normal goat serum, 0.5 ml of a 10 % Triton-PBS solution and of 0.5 ml bovine serum albumin. The primary antibodies were diluted in a 50 % blocking buffer and 50 % PBS solution (staining buffer) before application on the slices. Incubation took place over night at +4 °C. The next morning, the tissue was washed 3 times with a 0.1 % Triton-X100 in PBS solution (washing buffer). Next, the secondary antibodies were diluted in staining buffer and applied on the tissue. The incubation took place in the dark for 1 hour.

After washing twice with washing buffer for 5 minutes, Hoechst staining was applied for 5 minutes. Finally, the slices were sealed with Immu-Mount and a cover slip.

*3.2.6 Oil-Red-O lipid staining*

Specimens were frozen in Tissue Tek® as described above. After drying the slices and removing the Tissue Tek®, the contour of the tissue was traced with a Dako Pen. The sections were incubated in 70 µl of 100 % propylene glycol for 2 minutes and then stained with 70 µl Oil-Red-O solution over night at room temperature in a humidity chamber on a shaker. Next, sections were differentiated for 1 minute in a mixture of 85 % propylene glycol in distilled water, rinsed in 2 changes of distilled water, then stained with 70 µl hematoxylin for 2 minutes and rinsed thoroughly in distilled water. Between all the steps, the liquids were removed with an aspiration system. All chemicals necessary for this staining were included in the Oil-Red-O staining kit.

*3.2.7 Evaluation of histology*

An Olympus BX51 microscope, an F-View fluorescence camera and the associated Cell A software were used to assess the tissue slices. A perivascular cuff was determined to be Hoechst- and Iba-1-positive signals around a vessel. A parenchymal infiltration without demyelination was determined to be Hoechst- and Iba-1-positive signals in an intact MBP-positive signal. A parenchymal infiltration with demyelination was determined to be Hoechst- and Iba-1-positive signals in a negative MBP signal due to tissue destruction (Table 1). In order to get a better picture of the inflammation, a healthy mouse brain and spinal cord were also examined. For the CD3 quantification, all CD3-positive cells were counted.

**Table 1: Evaluation of histology, lesion types and characteristics.**

| **Lesion type/characteristic** | Hoechst signal | Iba-1 signal | MBP signal | location |
|---|---|---|---|---|
| Perivascular cuff | + | + | + | vessel |
| Parenchymal infiltration without demyelination | + | + | + | parenchyma |
| Parenchymal infiltration with demyelination | + | + | - | parenchyma |

*3.2.8 LPS-induced microglia activation*

6-8 weeks old female mice with C57BL/6 background were used for this model described in Qin *et al.* 2007 [50]. On day 0, 5 mg/kg vehicle (PBS), LPS + vehicle (PBS) or LPS + CGS-21680 were applied intraperitoneally. After 24 hours, brains and spinal cords were obtained as described above.

The advantage of this model is that microglial function can be assessed without contaminating peripheral macrophages or T cells, as those cells are difficult to distinguish in immunohistochemistry and may react differently to A2aR signaling. Inactive microglia are ramified, whereas activated microglia appear to be dense in their structure (Fig. 1).

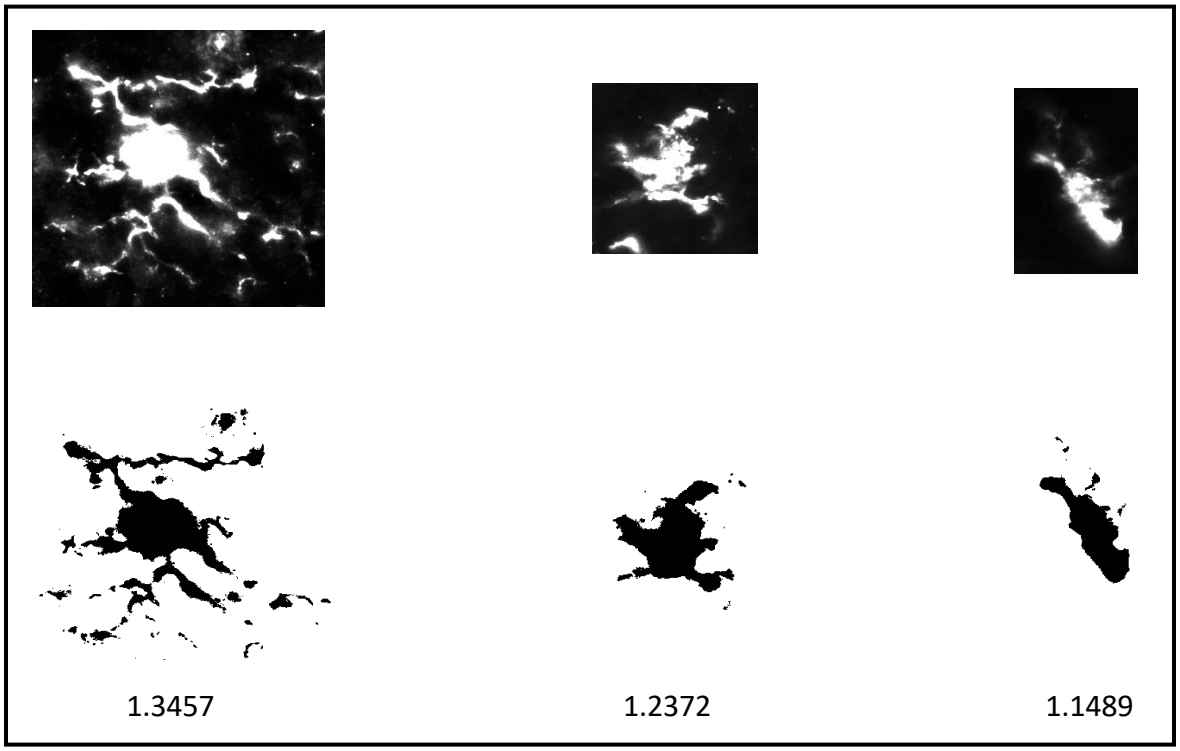



**Fig. 1: Calculation of fractal dimensions to quantifiy microglial ramification.** After immuno-histochemical staining for Iba-1 (upper row) single cells were converted to binary pictures (middle row). These were analysed by ImageJ and FracLac software in order to calculate D-value of fractal dimension (lower row). High D-values represent a higher state of ramification. Microglial activation comes along with a process retraction and a lower D-value. Magnification 400x.

*3.2.9 Calculation of microglial fractal dimension*

The degree of microglial activation was analysed morphometrically by calculating the D-value of fractal dimensions in the hippocampus, a brain region known to react strongly to systemic LPS. Three groups were compared in this experiment: vehicle, LPS + vehicle and LPS + CGS-21680.

In order to quantify the activation state and the morphological complexity of microglia, the method described by Soltys *et al.* [51] was applied. The frozen brain sections were stained for Iba-1. An Olympus BX51 microscope was used to take pictures of randomly acquired hippocampal microglial cells of each animal (Fig. 1, upper row). With Adobe Photoshop CS5 software the colours of the pictures were transformed into levels of grey. Next, the pictures were inverted with the same program. The pictures were saved as a GIF-image and further processed in ImageJ software. After binarizing the images (Fig. 1, middle row), FracLac add-in was used to calculate the Mean D of the fractal dimension (Fig. 1, lower row).

*3.2.10 Statistics*

GraphPad Prism 5 software was used to process all the data and perform statistical analysis. $P<0.05$ was determined to be statistically significant by Student's t test and the non-parametric Mann-Whitney *U* test. The Student's t test was used for the histological analysis, the CD3-positve cell quantification and the morphometrical analysis of microglial activation.

*3.2.11 Ethics*

All procedures were conducted according to protocols approved by the local animal welfare committees. The author participated in a one week animal research education course. File number of the animal research approval: 8.87-50.10.37.09.192. File number of the animal research institution at the Heinrich-Heine-University: G/192/2009.

*3.2.12 Shared data and mice*

Daily evaluation of EAE animals was performed by several group members. Graphs of EAE scores are used as well for projects that focus on other aspects (*i.e.* in particular Dissertation by Britta Wingerath 2015, Master Thesis by Klaudia Lepka 2012, Master Thesis by Maxi Hofrichter 2012). Furthermore, different tissues were used for different projects from the very same mice.

## 4. Results

### *4.1 Histological evaluation of healthy murine central nervous tissue*

A healthy mouse brain and spinal cord have been analysed histologically before the EAE experiments in order to get a better understanding of the neuroanatomy. The results were used to set a baseline for the further assessment of morphological changes caused by neuroinflammation. Figure 2 shows physiological Iba-1-positive signals in murine central nervous tissue.

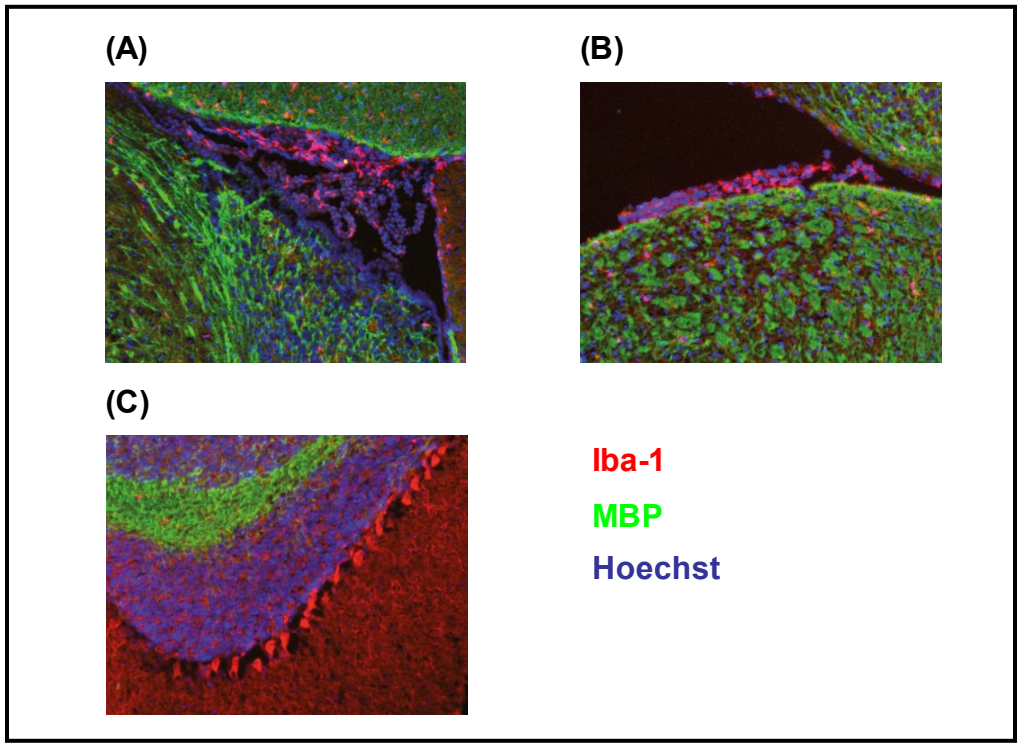



**Fig. 2: Physiological Iba-1+ signals in murine CNS tissue.** Magnification 100x. (A) choroid plexus (B) meninx (C) Purkinje cell layer in the cerebellum.

*4.2 A2aR in EAE*

*4.2.1 EAE course in mice lacking A2aR*

In order to show whether the adenosine 2a receptor plays a functional role in autoimmune neuroinflammation, the clinical and histological neuropathology was assessed from A2aR knock out mice (hereafter $A2aR^{-/-}$) and their wild type litter mates (hereafter $A2aR^{+/+}$) in an EAE model. $A2aR^{-/-}$ mice developed normally and no morphological abnormalities have been observed [26]. Staining for the A2aR in an EAE wild type mouse revealed that the receptor is expressed on Iba-1-positive cells (Fig. 3) and on CD3-positive cells (Fig. 4).

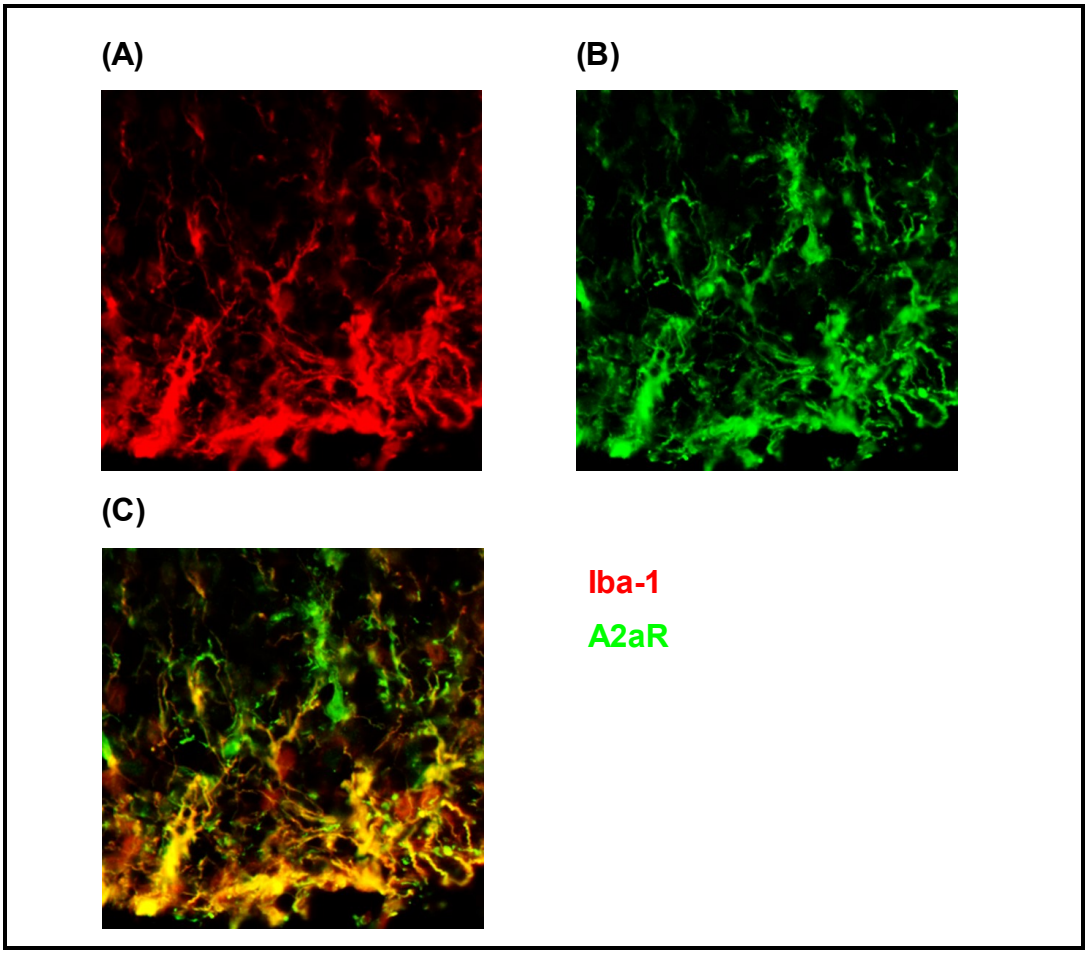



**Fig. 3: Staining for Iba-1 (A) and A2aR (B) in $A2aR^{+/+}$ EAE murine CNS tissue.** Overlapping Iba-1 and A2aR signals are yellow in (C). Magnification 200x.

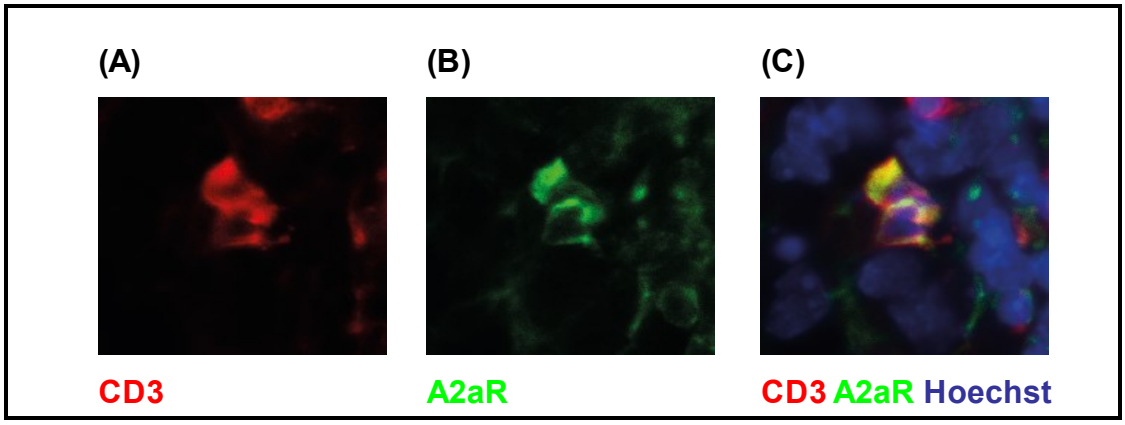



**Fig. 4: Staining for CD3 (A) and A2aR (B) in A2aR$^{+/+}$ EAE murine CNS tissue.**
Overlapping CD3 and A2aR signals are yellow in (C). Magnification 400x.

Additionally, immunohistochemical analysis showed that A2aR is not co-expressed on GFAP-positive cells, *i.e.* astrocytes (Fig. 5).

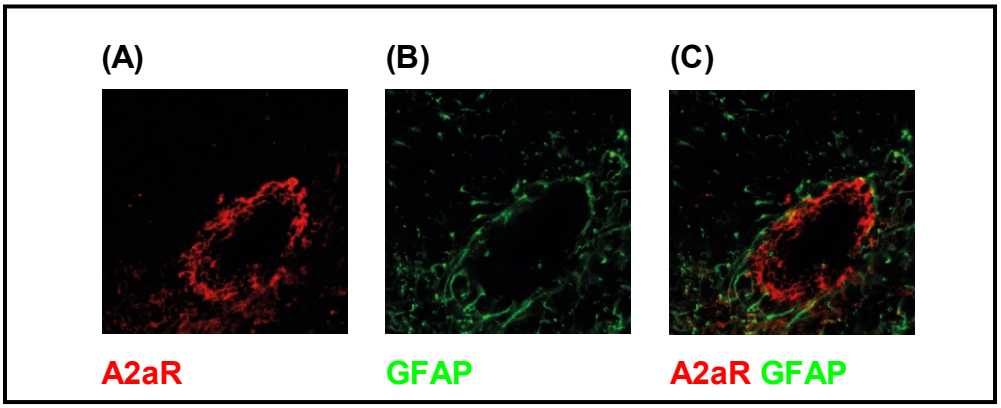



**Fig. 5: Staining for A2aR (A) and GFAP (B) in A2aR$^{+/+}$ EAE murine CNS tissue.**
No overlapping signals in (C). Magnification 200x.

Clinically, A2aR$^{-/-}$ mice presented an earlier and stronger increase of symptoms than the A2aR$^{+/+}$ mice, however, this difference diminished with progression of the disease (Fig. 6).

Therefore, animals were sacrificed at two time points for the histological analysis: The time point of a significant difference between the groups (day 14) and at end of the experiment, when no difference could be shown (day 42).

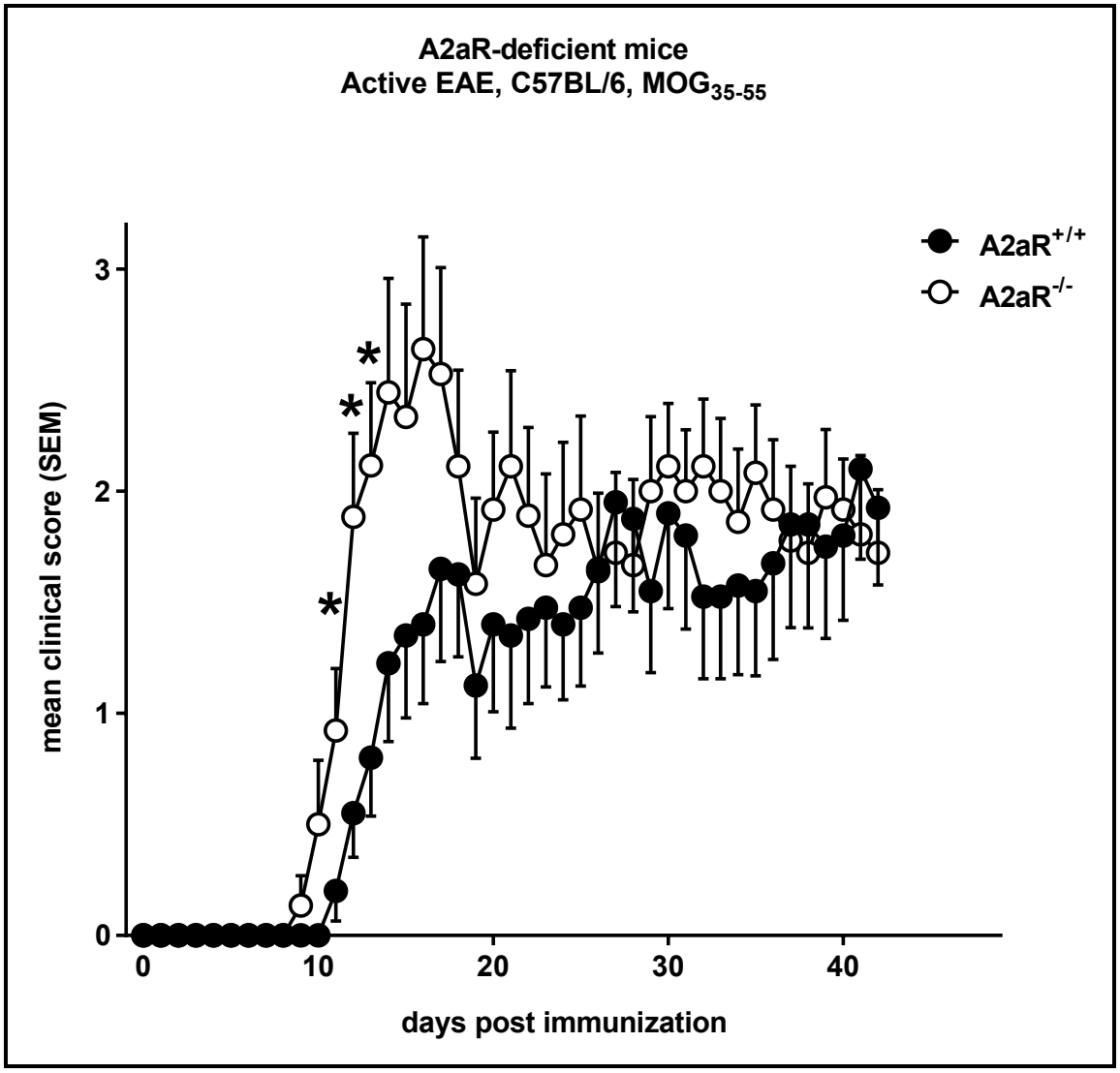



**Fig. 6: Statistical analysis of clinical EAE scores, genetic deletion of A2aR vs wild type.**
Mann-Whitney *U* test showed significant differences from day 11 to 13 ($p<0.05$).
Number of animals included: for d0 to d42; $A2aR^{+/+}$ n=8, $A2aR^{-/-}$ n=9.

At day 14, $A2aR^{-/-}$ mice developed significantly more Iba-1-positive lesions (Fig. 7A), more perivascular cuffs (Fig. 7C) and more parenchymal infiltrations (Fig. 7D). A tendency to larger lesions in $A2aR^{-/-}$ mice compared to $A2aR^{+/+}$ mice could also be shown (Fig. 7B).

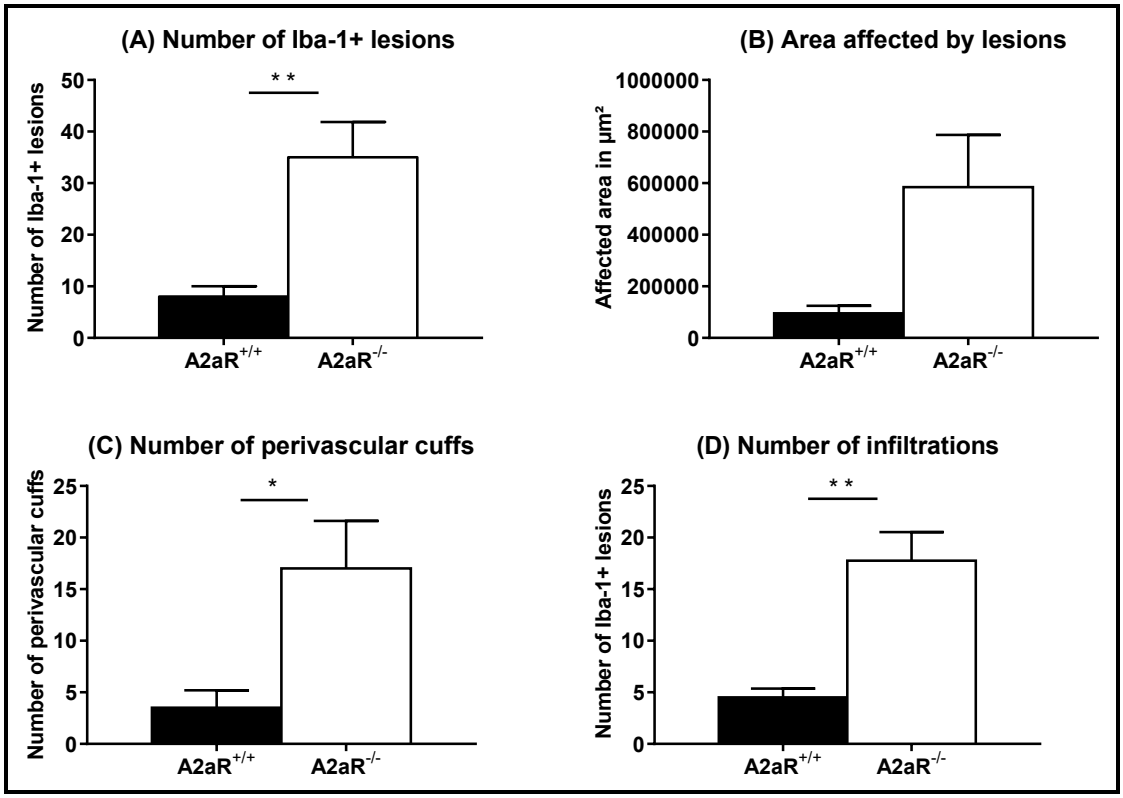



**Fig. 7: Histological analyses of A2aR knock out EAE day 14** (n=4 in each arm, SEM).
(A) number of lesions, p=0.0093 (B) area, p=0.0539 (C) cuffs, p=0.0337 (D) infiltrations, p=0.0039.

At day 42, both mouse groups had about the same clinical score (Fig. 6), underlining the quick recovery of the knock out mice. Histologically, A2aR$^{+/+}$ mice ended up with significantly larger lesions (Fig. 8A) and more neural tissue destruction (Fig. 8B) compared to wild type after a follow up of 42 days.

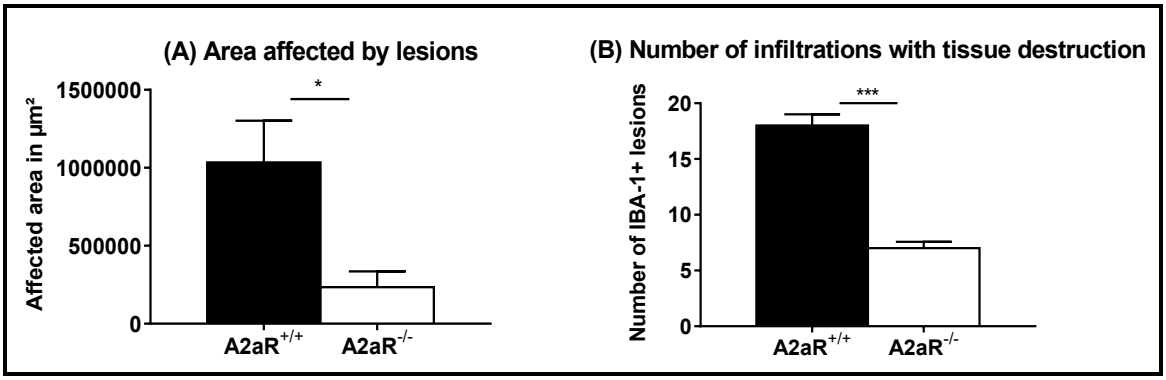



**Fig. 8: Histological analyses of A2aR knock out EAE day 42** (n=3 in each arm, SEM).
(A) area, p=0.0484 (B) infiltrations with tissue destruction, p=0.0007.

Representative images of the spinal cord show the difference in tissue destruction between the two arms of the EAE (Fig. 9, 10).

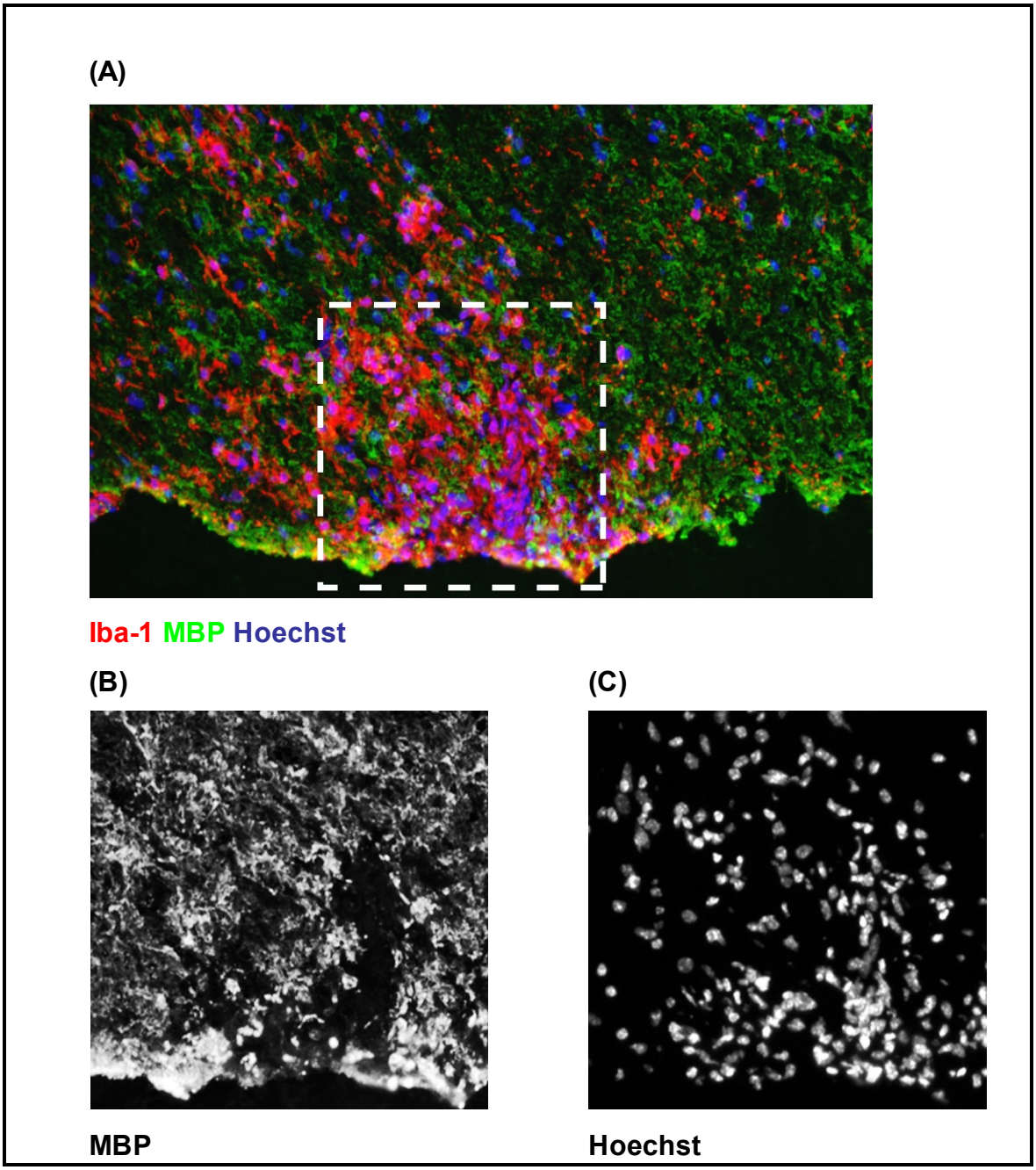



**Fig. 9: Representative images A2aR$^{+/+}$ mouse spinal cord day 42.** Magnification 200x. (A) Overview of a tissue infiltration with demyelination, framed area is shown in (B) and (C). (B) and (C) Picture detail of (A); loss of MBP signal in (B) indicates demyelination.

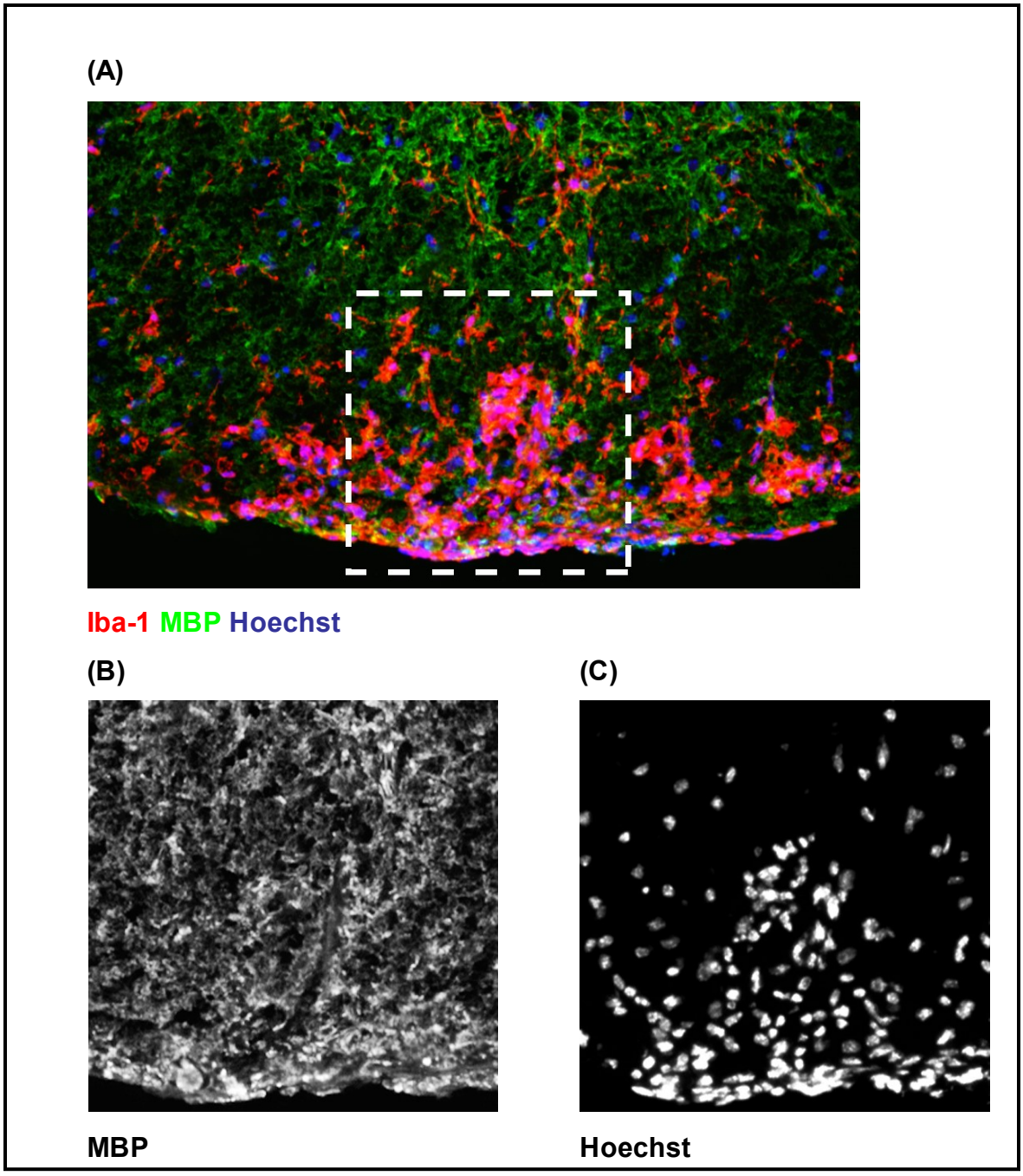



**Fig. 10: Representative images A2aR$^{-/-}$ mouse spinal cord day 42.** Magnification 200x. (A) Overview of a tissue infiltration without demyelination, framed area is shown in (B) and (C). (B) and (C) Picture detail of (A); intact MBP signal in (B) indicates no demyelination.

Accordingly, the extent of lipid-rich myelin debris visualised by Oil-Red-O lipid staining appears to be higher in A2aR$^{+/+}$ animals (Fig. 11).

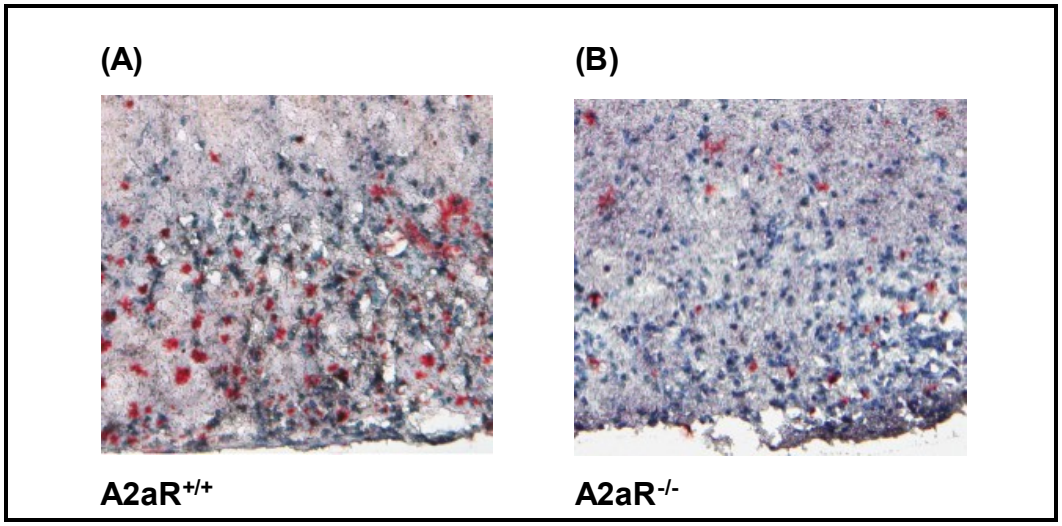



**Fig. 11: Oil-Red-O staining A2aR$^{+/+}$ (A) and A2aR$^{-/-}$ (B) mouse spinal cord day 42.** Amount of myelin debris appears to be increased in A2aR$^{+/+}$ mice (A). Magnification 200x.

T cell infiltration in the CNS was assessed by staining CD3-positive cells. At day 14, more T cell infiltration was encountered in the knock out animals than in the wild type group (Fig. 12A), while at day 42 the opposite trend was observed (Fig. 12B).

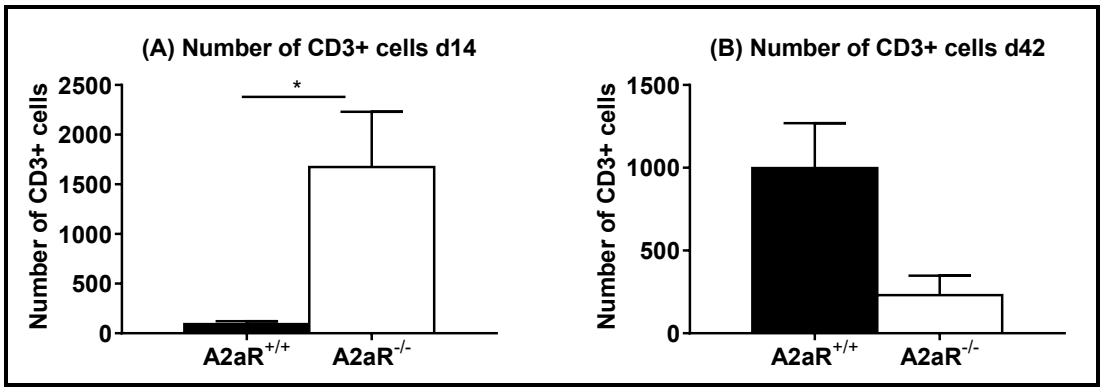



**Fig. 12: T cell CNS-infiltration in A2aR wild type and knock out mice** (n=3 in each arm, SEM). (A) CD3+ cells d14, p=0.0469 (B) CD3+ cells d42, p=0.06.

### *4.2.2 Pharmacologic modulation of A2aR in EAE*

#### *4.2.2.1 Pharmacologic activation of A2aR in EAE*

The remarkable recovery of A2aR$^{-/-}$ after the first peak of disease is of special interest. Additionally, after encountering a detrimental effect of A2aR$^{-/-}$ in the early phase of disease, the focus was set on the therapeutic effects of agonist treatment. Therefore, the pharmacological A2aR activation was started at the time of the first onset of symptoms (day 12) post immunization, when the crucial immunological processes in the periphery were already developed.

For this purpose, EAE animals were treated with the A2aR-specific agonist CGS-21680 (hereafter CGS) starting day 12 (daily i.p. injections of 0.1 mg/kg). The control group was treated with vehicle (DMSO 5 %).

Clinically, CGS-treated mice did not recover after the onset of disease. Therefore, the mice in the control group ended up with a better clinical score at day 25 (Fig. 13).

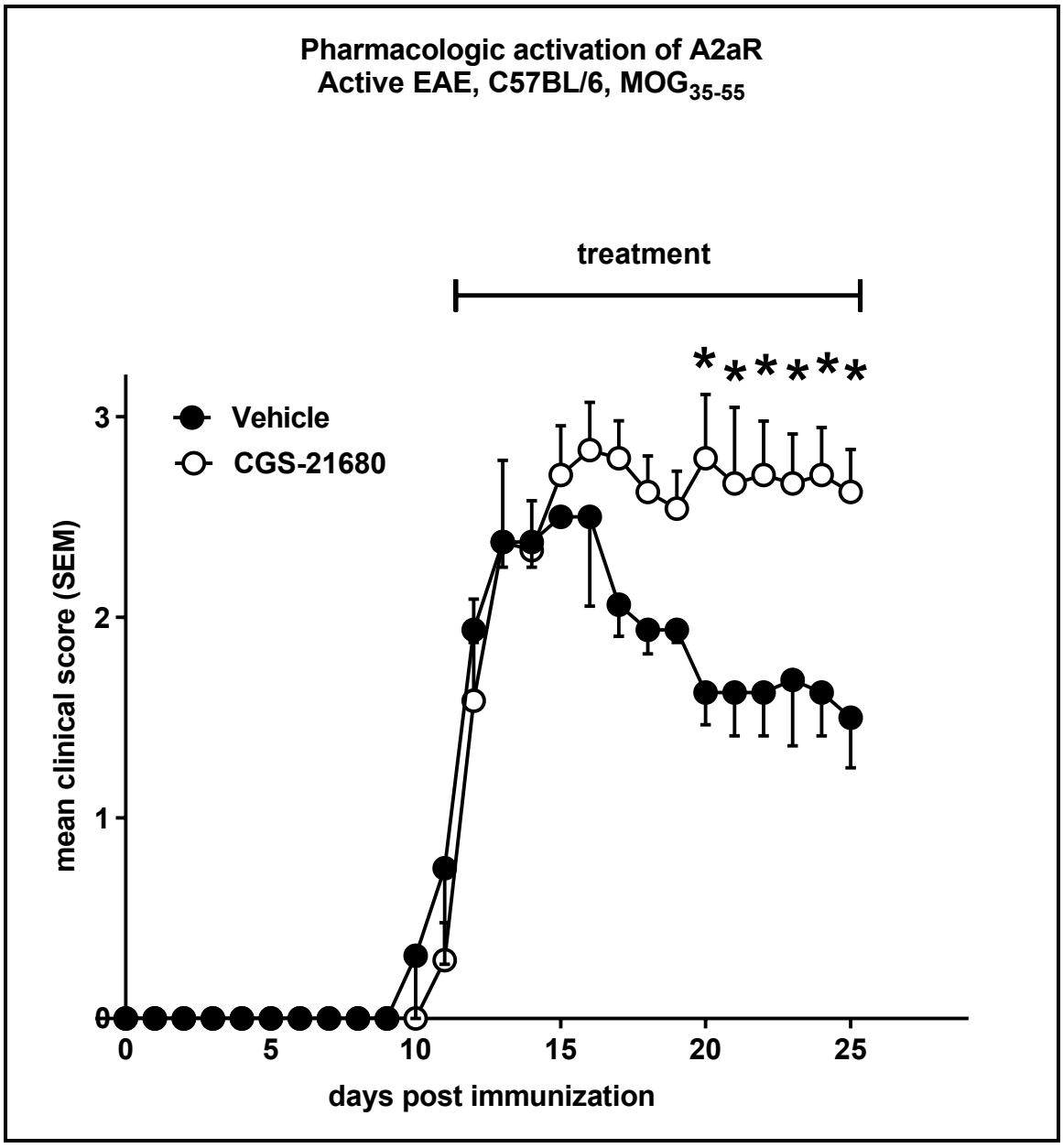



**Fig. 13: Statistical analysis of clinical EAE scores, pharmacologic activation of A2aR.**
Mann-Whitney *U* test showed significant differences from day 20 to 25 ($p<0.05$).
Number of animals included: for d0 to d25: Veh n=4, CGS n=6.

Histologically, the number of lesions was significantly higher (Fig. 14A) and the lesions were significantly larger (Fig. 14B) in CGS-treated mice compared to vehicle.

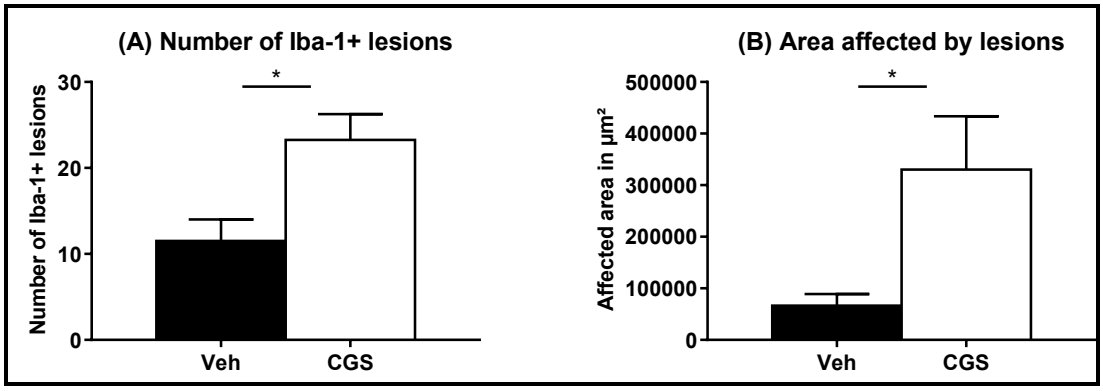



**Fig. 14: Histological analyses of CGS EAE, disease quantity** (n=4 in each arm, SEM). (A) number of lesions, p=0.0239 (B) area, p=0.0474.

Although there was no difference in the number of perivascular cuffs (Fig. 15A) at day 25, the number of infiltrations in the CGS group was significantly higher than in the vehicle group (Fig. 15B).

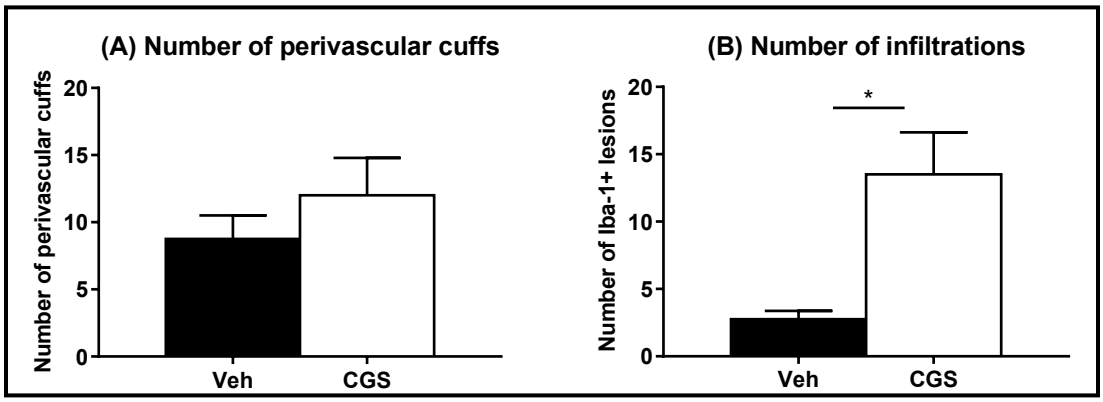



**Fig. 15: Histological analyses of CGS EAE, disease quality** (n=4 in each arm, SEM). (A) cuffs, p=0.3629 (B) infiltrations, p=0.015.

Interestingly, activation of the A2aR increased the number of lesions especially in the spinal cord (Fig. 16).

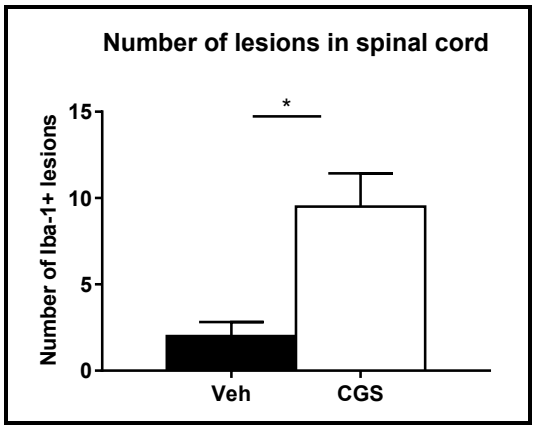
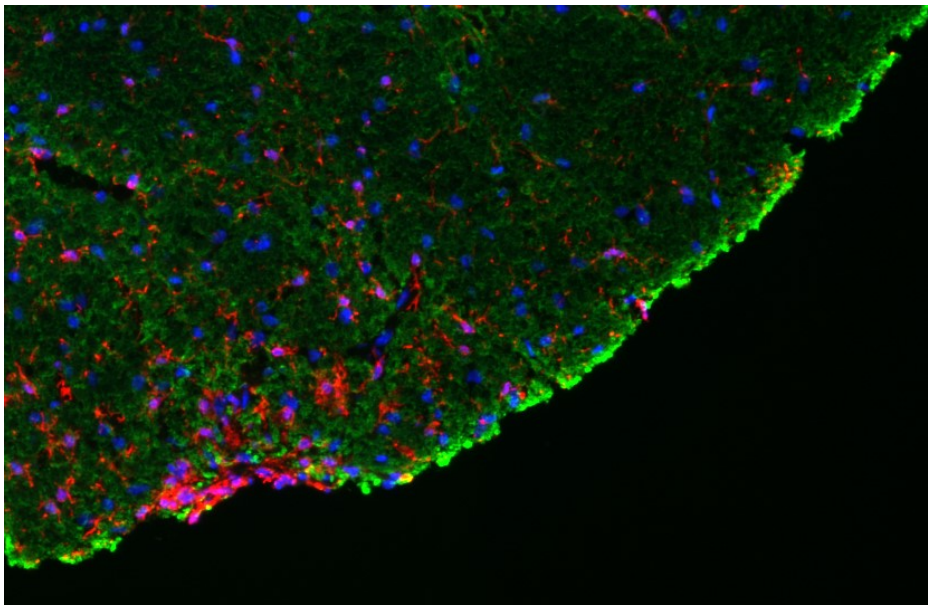



**Fig. 16: Histological analysis of CGS EAE, disease location** (n=4 in each arm, SEM). p=0.0118.

Taken together, CGS used at the late stage of disease lead to worse neurological symptoms (mean score 3), a quicker EAE progression, an inhibition of disease recovery and more myelin debris clumps, in contrast to vehicle.

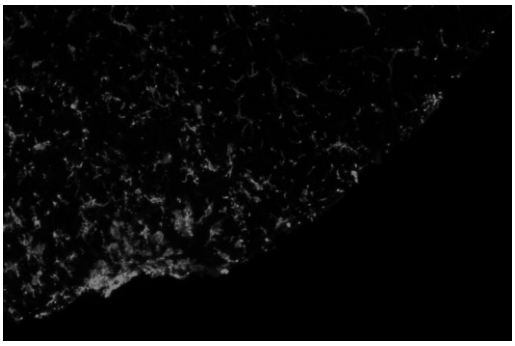
Representative images of the spinal cord stress the difference of the inflammatory progress (Fig. 17, 18).

(A)



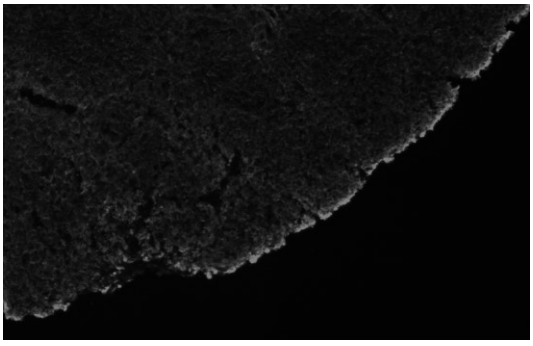
Iba-1 MBP Hoechst

(B)



Iba-1

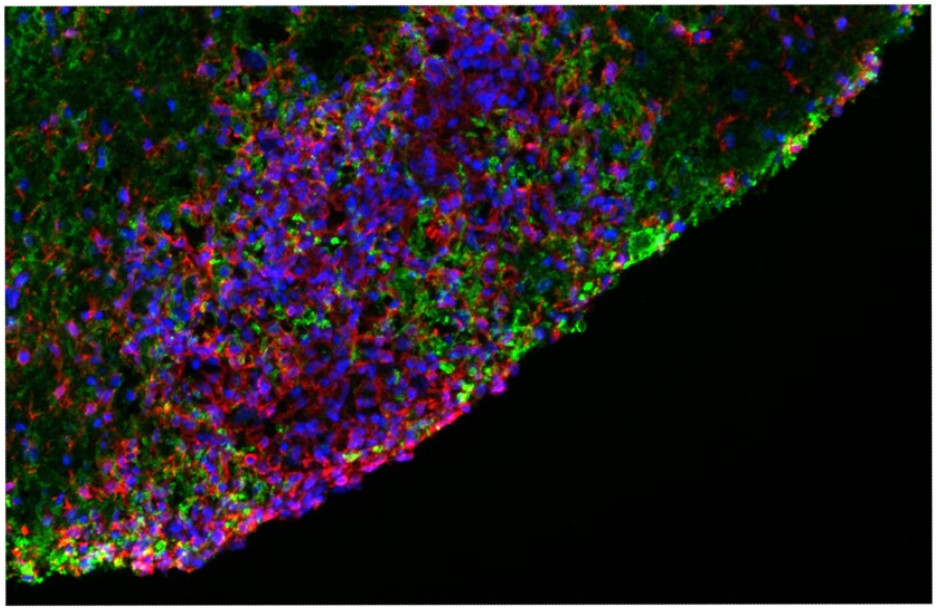
(C)



MBP

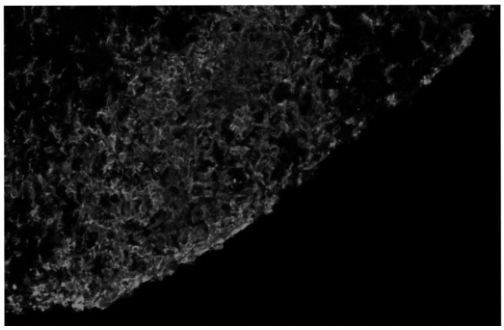
**Fig. 17: Representative spinal cord images for the vehicle-treated group.** Magnification 100x.
(A) Merged signals of Iba-1, MBP and Hoechst.
(B) Iba-1 and (C) MBP signals only.

(A)



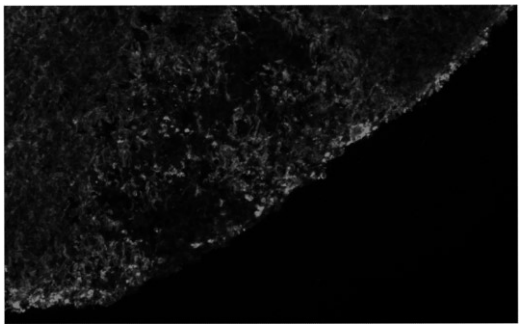
Iba-1 MBP Hoechst

(B)



Iba-1

(C)



MBP

**Fig. 18: Representative spinal cord images for the CGS-treated group.** Magnification 100x.
(A) Merged signals of Iba-1, MBP and Hoechst.
(B) Iba-1 and (C) MBP signals only.

Additionally, the visualisation of myelin pathology by Oil-Red-O lipid staining in EAE animals treated with CGS revealed a similar pattern as shown in the A2aR knock out EAE (Fig. 11), characterized by increased myelin debris accumulation in white matter. Representative images of the cerebellum show that myelin debris appears to form clumps (Fig. 19). Quantification was performed by counting the number of such myelin debris clumps per section in cerebellar white matter (Fig. 20).

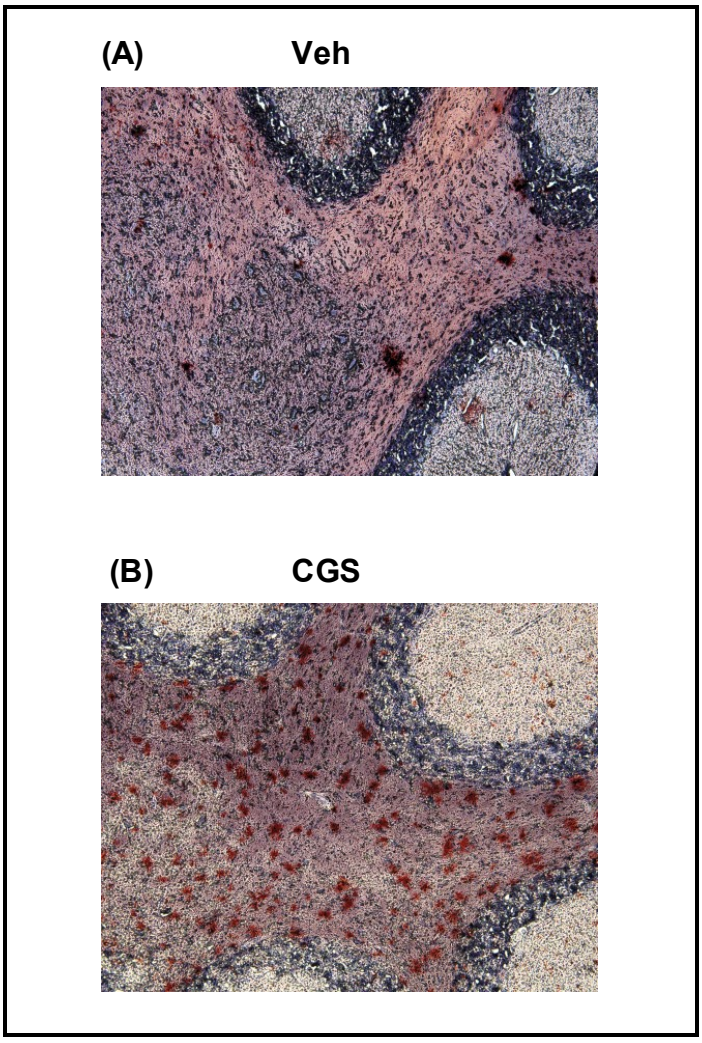



**Fig. 19: Representative cerebellum Oil-Red-O staining images.** Magnification 200x. (A) vehicle-treated animal (B) CGS-treated animal.

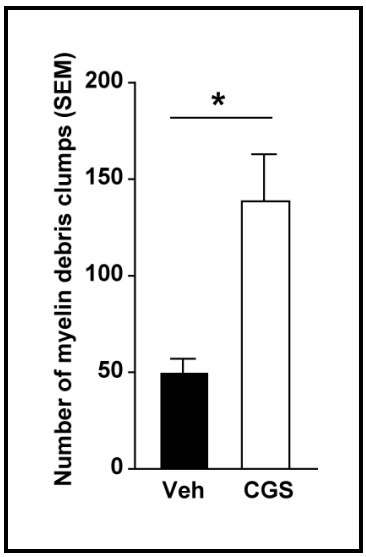



**Fig. 20: Myelin debris clumps per section in cerebellar white matter** (n=4 in each arm, SEM). p=0.0288.

No effects of CGS could be observed on molecules related to macrophage/microglia functions, *e.g.* expression of inducible nitric oxide synthase (iNOS) or major histocompatibility complex (MHC) class I, in immunohistochemical analysis (Fig. 21).

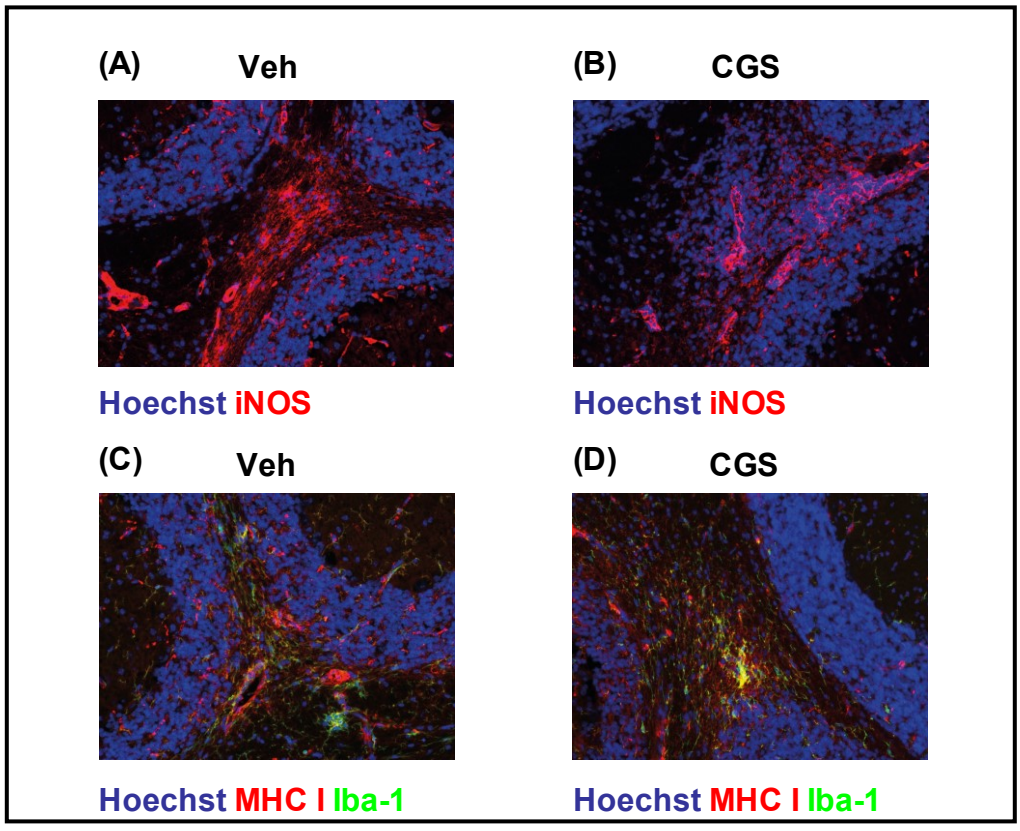



**Fig. 21: Histological analysis of molecules related to macrophage/microglia functions.** No overt differences for iNOS (A, B) and MHC class I (C, D). Magnification 200x.

*4.2.2.2 Pharmacologic inactivation of A2aR in EAE*

The impact of a pharmacologic inactivation of A2aR in EAE was also assessed. For this purpose, SCH-58261 (hereafter SCH), a specific A2aR antagonist, was administered (daily i.p. injections of 1 mg/kg, first application 24 hours before vaccination).

Clinically, the SCH-treated animals presented a delayed disease onset and a milder outcome than the vehicle mice (Fig. 22).

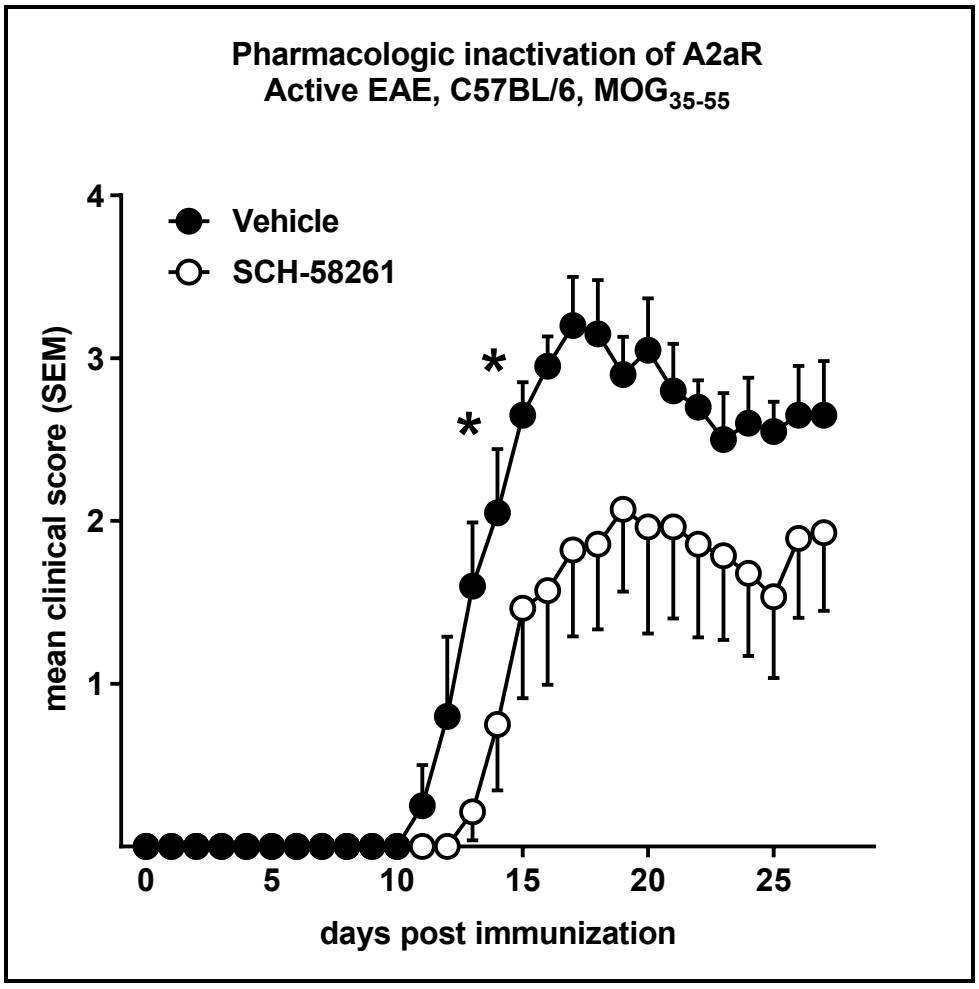



**Fig. 22: Statistical analysis of clinical EAE scores, pharmacologic inactivation of A2aR.**
Mann-Whitney *U* test showed significant differences on day 13 and 14 ($p<0.05$).
Number of animals included: for d0 to d27 Veh n=5, SCH n=7.

Histologically, the experiments show that the vehicle group had more (Fig. 23A) and larger (Fig. 23B) lesions compared to SCH-treated animals.

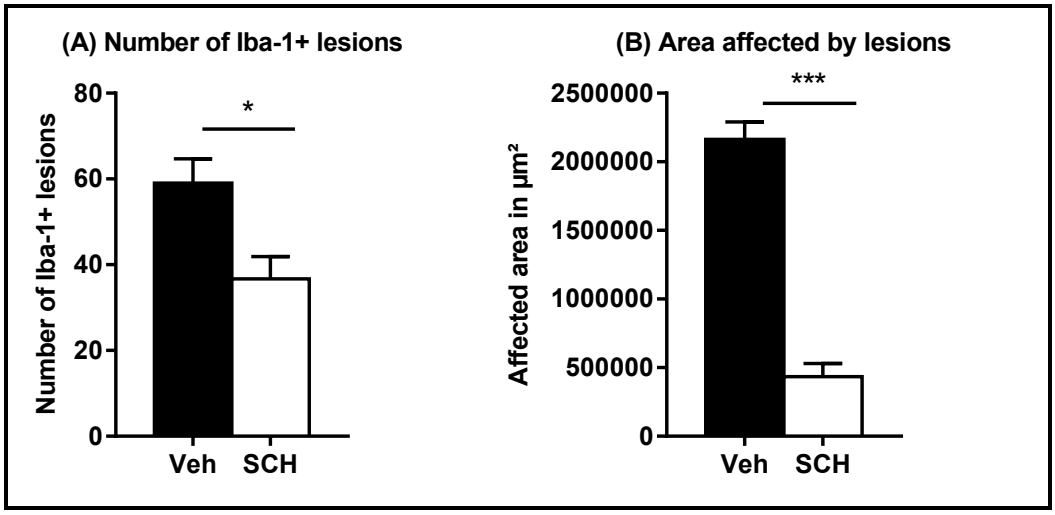



**Fig. 23: Histological analyses of SCH EAE, disease quantity** (n=3 in each arm, SEM). (A) number of lesions, p=0.0443 (B) area, p=0.0004.

*4.3 Role of A2aR in LPS-induced neuroinflammation*

In order to get a better picture of the role of A2aR in CNS tissue, microglia were activated *in vivo* by a systemic LPS injection (5 mg/kg i.p.) for 24 hours.

Hippocampal cells revealed a more activated phenotype after administering LPS, as compared to vehicle. An additional CGS challenge increased this effect further, as compared to the LPS arm and the vehicle-treated arm (Fig. 24).

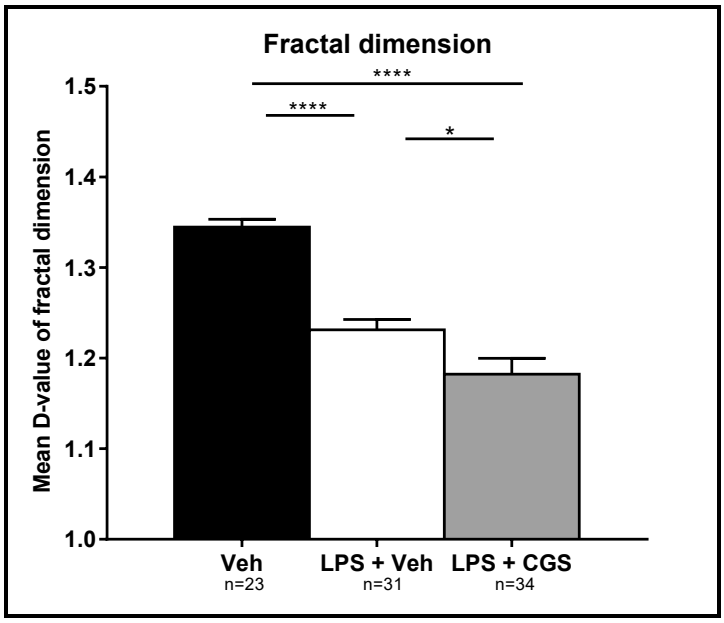



**Fig. 24: Morphometrical analysis of microglial activation, mean D-value** (SEM). Veh/LPS + Veh, p≤0.0001; LPS + Veh/LPS + CGS, p=0.0244; Veh/LPS + CGS, p≤0.0001.

## 5. Discussion

Maintaining an immunological homeostasis in inflammatory processes inside and outside the central nervous system is crucial. In order to better understand the underlying mechanisms of neuroinflammation, previous studies have shown that immune modulation via adenosine signaling is capable of regulating tissue damage in various pathologies and autoimmune diseases.

In this work, the A2aR has been genetically deleted and pharmacologically both activated and inactivated *in vivo*. The data gained from the knock out EAE implicate that A2aR may have both protective and detrimental properties. In the early phase the disease, $A2aR^{-/-}$ mice show more pronounced tissue damage than $A2aR^{+/+}$ mice. Having a look at the results in the late phase, the results were seemingly paradox: $A2aR^{-/-}$ recovered quickly and histological analysis points to even more pathologies in $A2aR^{+/+}$ mice. This led to hypothesize that A2aR activation may have detrimental effects in late stage EAE, *e.g*. an impairment of recovery and repair mechanisms.

In order to dissect these effects, a pharmacologic activation has been performed at a time point where disease has already developed (day 12). A protective effect of CGS-21680 on this autoimmune disease, as seen in many other autoimmune disease *e.g.* autoimmune hepatitis [21], was not observed. To the contrary, late stage application of CGS worsened disease significantly.

This result is in line with the late stage recovery in A2aR knock out animals. After an initial detrimental effect of the receptor absence, *i.e.* a protective effect of A2aR, it seems that in late stage EAE the absence of the receptor may have positive effects on the disease course. This is evidenced by the quick recovery seen after the disease onset in A2aR-deficient mice. In other words, it seems that A2aR has protective effects in early EAE and detrimental effects in late EAE. The notion of a detrimental effect in late stage EAE was impressively supported by the deleterious influence of late CGS injection, which is known to have an anti-inflammatory effect.

Consequently, the application of SCH-58261, a strong inhibitor of A2aR receptor signaling, proved to have opposite effects, *i.e.* a protective effect on EAE disease course, reproducing the results of another research group [37]. The proposed mechanism relies on a T cell entry promotion by adenosine via A2aR, which is in principle a plausible explanation. This notion is, however, completely based on their finding that A2aR is only detectable at the choroid plexus. This explanation seems to fall short for two reasons. First, I was able to detect A2aR with immunohistochemical analysis in EAE lesion tissue, not only in choroid plexus. Second, while immune cell entry via the choroid plexus has been described in EAE [52], EAE is predominantly a disease of the spinal cord. The vast majority of lesions are found there and in the cerebellum and by no means predominantly in the vicinity of the choroid plexus. To the contrary, these lesions are the absolute exception. The profound effects of A2aR signaling on EAE can hardly be entirely explained by choroid plexus-mediated immune cell entry controlled by A2aR. At a first glance, this seems rather unexpected as the blood-brain barrier at the choroid plexus is freely permeable for selective molecules [53]. Therefore, the central nervous tissue around the ventricles should be more vulnerable for any kind of systemic inflammatory process.

In search of a possible mechanism I concentrated on the above described paradox. There is a large body of robust data that A2aR plays an anti-inflammatory role in the immune system. The finding that A2aR knock out mice experience a worse disease onset points to the conclusion that the peripheral immune response in EAE, *i.e.* the immune reaction at the vicinity of the subcutaneous immunization, the draining lymph nodes, spleen *etc.*, is exaggerated in the one or two weeks before symptom onset, *i.e.* the immune system attack on the CNS. At a later time point the A2aR effect seems reverse as exemplified by the quick recovery and the comparable lesion load in the histological analysis in long term follow up. This idea is strongly supported by late application of CGS in EAE. I hypothesized that this effect is mediated by CNS cells.

Microglia, the macrophages of the CNS, share many features of peripheral immune cells, but also significantly differ from them [54]. Microglia express A2aR

and upregulate the receptor in stress conditions [55]. A systemic LPS injection is such a stress condition: This challenge results in an enhanced microglial activation compared to vehicle. The advantage of this LPS stimulation model is that the role of A2aR in microglia can be assessed *in vivo* excluding a contribution of peripheral macrophages. Interestingly, a co-stimulation with CGS, as demonstrated in my experiments with the LPS stimulation model, turns microglia in a more activated phenotype, indicating a clear tendency towards pro-inflammatory action in the central nervous tissue.

Consequently, the effect of the peripheral immune system on the immune system in the CNS needs to be discussed. My findings confirm in part that EAE is worsened at least at disease onset in A2aR$^{-/-}$ mice with more overt tissue damage and more infiltrating T cells. Thus, blocking the effect of A2aR in the brain might be good for the disease outcome, but antagonizing the receptor simultaneously in the peripheral immune system might lead to an increased migration of immune cells into the CNS. One reasonable and comprehensible link between these two compartments was given by Dai *et al.* in a model of traumatic brain injury and in *in vitro* experiments: When the local glutamate levels are low, the A2aR agonist CGS reveals an anti-inflammatory effect in microglia, while high local glutamate levels in the presence of CGS result in a pro-inflammatory effect [30]. This could explain the earlier and severer progression of EAE in knock out mice. In the beginning of the disease, glutamate levels might still be low, so the anti-inflammatory effect of A2aR stimulation is missing compared to the wild type animals. However, this does not give us the full picture of the adenosine impact on inflamed central nervous tissue. Earlier studies have shown that peripheral monocytoid cells are necessary for activating microglia in EAE [56] and, as stated above, that lymphocyte infiltration is important for the onset of EAE [45]. Additionally, “myelin-primed T cells entering the CNS in EAE have to be restimulated locally to evoke clinical disease” [57]. This corresponds with the findings in this work where the number of CD3-positive cells in the CNS tissue of A2aR knock out mice in the beginning of the disease is increased. They also show more clinical symptoms than the wild type control group.

These paradox findings give rise to the idea that adenosine and the A2aR have different effects on cells from different compartments. CD3-positive T cells do not cross the blood-brain barrier under the influence of A2aR stimulation in EAE as much as T cells without the A2aR. This effect, on the one hand, is clearly neuroprotective. Microglia are non-bone marrow derived cells and they show a more activated phenotype after LPS and CGS stimulation compared to LPS stimulation only. This effect, on the other hand, is neurodegenerative. Indeed, several reports suggest that A2aR activation on haematopoetic cells rather confers neuroprotection and A2aR activation on non-haematopoetic cells, *i.e.* brain tissue cells, rather confers neurodegeneration. This has been shown in a model for ischemic brain injury [27], spinal cord injury [31] and recently also for EAE [39].

Regarding the pharmacological inactivation of the A2aR, a different picture evolves in contrast to the A2aR-deficient mice. Animals treated with the specific antagonist SCH-58261 develop neuroinflammation which is alleviated compared to the control group treated with vehicle. These results indicate that blocking A2aR activation leads to less tissue destruction in EAE, but does not have the same effect as a lack of A2aR.

Concerning neuroinflammation with a leaky blood-brain barrier such as in EAE, it needs to be considered that serum itself is a danger signal for microglia and is therefore capable of activating these macrophages of the central nervous tissue [54]. Systemic LPS administration activates microglia, a simultaneous administration of the A2aR agonist CGS-21680 enhances this activation. This activation was measured by a change in morphology: Resting microglia are ramified, whereas stimulated microglia show process retraction. A recent study confirms that A2aR activation turns microglia into an activated phenotype [55], which is quite contrary to most findings implicating A2aR to down-regulate immune cells [58]. Taken together, the histological data implicates that CNS tissue damage is linked with A2aR stimulation and microglia/macrophage activation. These results underline that a therapeutic approach with CGS causes a detrimental outcome regarding inflammation of the central nervous tissue.

## 6. Conclusions

In summary, I demonstrate a crucial but complex involvement of A2aR in the pathogenesis of experimental autoimmune encephalomyelitis. The following conclusions can be drawn:

Genetic deletion of the A2aR in EAE results in a more severe disease in the acute phase. In later stages, the disease severity is lower with smaller lesions, less T cell infiltration and reduced tissue destruction compared to wild type.

Pharmacological activation of the A2aR in EAE leads to a worse disease outcome. Especially the number of lesions in the spinal cord is increased. Additionally, stimulating the A2aR turns microglia into a more activated phenotype.

Pharmacological inactivation of the A2aR in EAE alleviates the disease severity and leads to fewer and smaller lesions.

This study confirms and extends existing findings on the differential functions of the A2a receptor on T cells, macrophages and microglia in the course of autoimmune neuroinflammation, with beneficial, but also detrimental effects. A translation into a therapeutic setting of multiple sclerosis should be done with caution and must be preceded by further insight into the cellular mechanisms underlying the observations of this work.

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**Eidesstattliche Versicherung**

Ich versichere an Eides statt, dass die Dissertation selbständig und ohne unzulässige fremde Hilfe erstellt und die hier vorgelegte Dissertation nicht von einer anderen Medizinischen Fakultät abgelehnt worden ist.

29. August 2016, Jonas Maximilian Graf

Unterschrift

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