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TITOLO:

Behavioral phenotyping of RPTPz KO mice: a conclusion; and study of an early curative treatment with immunosuppressant Mitoxantrone in a mouse model of multiple sclerosis

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INTRODUCTION

Protein phosphorylation status is a key factor for survival and internal biological processes of many cells. Activity of protein kinases and phosphatases is thus essential for maintaining cell homeostasis and protein tyrosine phosphatases (PTPs) play an important role in this equilibrium. PTPs are a large family of structurally diverse enzymes. One group of PTPs, the receptor like protein tyrosine phosphatase (RPTPs) exhibits structural features that are common to cell adhesion molecules (CAMs) as well as cell surface receptors, that are an extracellular domain, a single transmembrane domain, and a cytoplasmic portion that contains one or two tyrosine phosphatase domains, suggesting that these receptors may play a role in cell/cell communication [1,2].

RPTPzeta (also known as RPTPz, RPTPβ, or PTPξ) is a member of a subfamily of RPTPs that contains, in its extracellular domain, a region with sequence homology to the enzyme carbonic anhydrase (CAH). The CAH domain is followed by a fibronectin type III (FNIII) repeat, and by a unique sequence termed spacer domain, a serine-glycine rich region [3,4,5]. The extracellular domain of RPTPz binds various cell adhesion molecules (Contactin, N-CAM, Ng-CAM, etc…) and growth factors (Pleitrophin, etc…) [6,7,8,9]. Natural ligands, for the intracellular domain of RPTPz, such as β-catenin, Fyn or β-adducin have been recently discovered [10,11,12].

RPTPz is predominantly expressed by astrocytes, oligodendrocytes, Schwann cells, but also neurons throughout the developing and adult nervous system [13,14,15]. Previous studies have suggested that RPTPz might be involved in maturation of oligodendrocytes, stimulate neuron-glial cells interaction as well as neurite outgrowth, and neural migration, and might potentiate regeneration after injury [16,17,18]. Therefore, RPTPz appears to be a major phosphatase involved in various key cellular functions in the nervous system, although its deletion does not induce major Central Nervous System (CNS) abnormalities, as observed in Knock-Out (KO) mice, except for a minimal alteration in the ultra-structural conformation of myelin sheaths in optic nerves [19].

In the last years reports, the steps of the KO characterization corresponding to the behavioral phenotyping in baseline conditions and in inflammatory condition after intraplantar injection of a carrageenan or formalin solution were illustrated. RPTPz -/- and +/+ littermate male mice were tested in an extensive battery of test designed to explore different domains controlled by CNS, like exploratory behavior, coordination and strength (grip strength test, openfield, foot print, and accelerated rotarod), memory (Y maze), nociception (tailclip, hotplate, Hargreaves, VonFrey), sensorimotor gating, susceptibility to seizure induced by Penthylenetetrazole, and electrophysiological readouts for nerve conductivity at two different ages (10-18 weeks and 40-48 weeks).

RPTPz KO mice showed no significant differences compared to wild type littermates in strength (grip test), exploratory behavior and anxiety (open field, nb of entries in Y maze), sensorimotor gating, moreover -/- mice showed susceptibility to seizure in early phases but not in the late phases of the PTZ test.
No differences were detected in nerve conduction velocity measured by in vivo electrophysiology.

RPTPz -/- mice displayed a marked phenotype linked to sensorimotor responses to nociceptive stimuli, showing reduced responsiveness to thermal and tactile stimuli

compared to control +/+ mice. No differences were observed for mechanical pain (tail clip).

To further assess this hypothesis, local and central inflammation and pain readouts were measured after challenge of the animals with carrageenan or formalin respectively.

After formalin-induced inflammation, -/- mice showed an impaired sensitivity in the overall course of the formalin test. This reduction in pain sensitivity is significant both in phase 1, corresponding to C-fibers activation due to peripheral stimulus (tissue damage and injection of the solution) and phase 2, corresponding to a combination of the inflammatory reaction in the peripheral tissue and functional changes in the dorsal horn of the spinal cord initiated by the C-fibers activation in the early phase.

After carrageenan-induced inflammation, -/- mice showed a significant hyposensitivity (increased latency) to thermal stimulus compared with +/+ mice in the vehicle treated paw. This result confirms the previously observed phenotype. Carrageenan injection triggered hyperalgesia in both -/- and +/+ mice, but the withdrawal reaction is enhanced in -/- mice. The significant difference observed between -/- and +/+ mice has disappeared in the carrageenan treated paw. Different findings were observed in tactile sensitivity. -/- mice showed a significant hyposensitivity (increased applied force for inducing withdrawal) to tactile stimulus compared with +/+ mice in vehicle treated paw, confirming previous results obtained in the phenotyping. Moreover, carrageenan injection triggered hyperalgesia in +/+ mice (reduced force threshold), but not in -/- mice.

These results clearly demonstrate the role of RPTPz in nociception. However, question on stimulus intensity or modulation during the pain sensitivity pathway remained open have been investigated in the last year by hotplate and Hargreaves' plantar test associated with stimulus intensity variation.

To better characterize this KO and confirm data already published [20], RPTPz KO mice and wild type (WT) littermate were subjected to Experimental Autoimmune Encephalomyelitis (EAE), animal model for multiple sclerosis. The results showed no difference between the 2 genotypes. Both genotypes developed a severe chronic EAE without remission phase. To verufy if remission could have happened as Harroch demonstrated [21], a second study was effectuated with a milder immunization procedure. In addition, both genotypes were treated with Mitoxantrone (MTX), a potent immunosuppressant/immunomodulator. No difference between the 2 genotypes was observed on disease severity and no remission was recorded. MTX induced a significant reduction of clinical score on both WT and KO. Hypoalgesia was observed in EAE animals vs. healthy and treatment with MTX did not influence the responsivity to thermal stimulus.

Therefore in this last year, I further investigate on the MTX effects dichotomy between clinical parameters and pain. A mild EAE was induced on normal C57bl/6 mice. Deeper analysis such as pain read out, spinal cord inflammatory infiltrates, demyelination and axonal loss, analysis of the structure of the peripheral nerves, and measurement of the level of pro-inflammatory cytokines were included.

Material and methods

Thermal sensitivity associated to stimulus intensity variation:

In order to investigate PTPRz KO pain-related behavior associated to stimulus intensity variation, we subjected 27 weeks old of age mice (8 male/genotype) to the hotplate test (Ugo Basile, Italy) with the temperature of the plate set at 50±1°C, 52.5±1°C, and 55±1°C according to the previous described protocol.
One week after hotplate testing the response of the animals was assessed in the plantar Hargreaves' test according to the previous described protocol with radiant heat set at 3 different intensities (137mW/cm^2/s, 164mW/cm^2/s, and 190mW/cm^2/s).
For both test, mice were distributed in 3 groups and were subjected on consecutive days to different stimulus intensity according to a Latin square experimental design.

Effect of an early curative treatment with immunosuppressant/immunomodulator MTX in a mild on C57bl/6 mice

1-Mice

30 naive C57BL/6 female mice (10 to 12 weeks old ; 19-22gr) were tested for a 40 days long EAE induced with MOG_{35-55} and other 10 naïves C57BL/6 female mice (10 to 12 weeks old ; 19-22gr) were used as controls.
Animals were housed singularly in top filter Plexiglas® cages (29X11X13cm) on a layer of wood shaving in an air –conditioned room (T°=22°C±2, relative humidity=55%±10) with a light/dark period of 12 hours (7am-7pm) at least 10 days before the study initiate. In this period, animals were observed daily to ascertain their fitness for the study.
GLP 4RF25 top certified pellet diet (Charles River Italia's feed licensee Muceloda S.r.l, Settimo Milenese) was used. To facilitate nourishment of sick animals, from day 7 wet pellets are placed every day on the cage bottom. The diet is available *Ad Libidum* to the animals. Water from the municipal main watering system is filtered and distributed *Ad Libidum* to the animals.

2-Induction of mild chronic EAE

Mice were immunized as follows:
Immunization was conduced by injecting s.c. 0.2mL in both flank (0.1mL in each flank) of an emulsion composed of 50µg MOG35-55 peptide (Neosystem, Strasbourg, France) in Complete Freund Adjuvant (CFA, Difco, Detroit, USA) containing 0.5mg of *Mycobacterium tuberculosis*. Immediately after, they received an i.p. injection of 250ng of *pertussis toxin* (List Biological Lab., Campbell, CA, USA) dissolved in 400mL of buffer (0.5M NaCl, 0.017% Triton X-100, 0.015M Tris, pH=7.5).

3-Recruitment of the animals

Animals were observed daily (seven days a week) from day 7 after immunization till day 40 post recruitment. Body weight and clinical score were recorded daily using the following scale: 0=healthy, 0.5=partial tail paralysis, 1=tail paralysis, 1.5= tail paralysis + partial unilateral hindlimb paralysis, 2= tail paralysis + hindlimd weakness

or partial hindlimbs paralysis, 2.5=tail paralysis + partial hindlimbs paralysis (lowered pelvis), 3= tail paralysis + complete hindlimbs paralysis, 3.5= tail paralysis + complete hindlimbs paralysis + incontinence, 4= tail paralysis + hindlimbs paralysis + weakness or partial paralysis of forelimbs, 5=moribund or dead.
At first clinical sign, animals were recruited and assigned to a group for treatment.

4-Treatment

Animal from group 1 were not immunized and not treated (Healthy). Animals from group 2 were immunized and treated daily by i.p. injection of Mitoxantrone 0.5mg/kg in saline. Animals from group 3 were treated by daily injection of saline (vehicle used to dilute Mitoxantrone). The treatment, performed in the early afternoon, started from recruitment and lasted for 10 days. Dose, duration and period of treatment were chosen based on literature to diminish toxicity and mortality [22].

5-Behavioral tests

To test the response to thermal on unrestrained animals and allow detection of hyperalgesia, we used a method adapted from the protocol described by Hargreaves and al. Plantar Hargreaves' test was performed, before immunization, on the forelimbs of the animals due to the coming paralysis of the hindlimbs. The data obtained were used as basal values for the animals. The test was repeated 1 week after immunization, at recruitment, and at sacrifice of the animals. All behavioral tests were performed during the light phase. Animals were allowed 1-hour habituation to the test room before testing. Testing sequence was randomized, and all apparatus were completely cleaned between two consecutives sessions. Animals were accustomed to the apparatus for 1 hour, for 2 days preceding the test day for the first test period. For the other periods of test no acclimatizing was necessary.
For the test, animals were placed in a clean acrylic box on a glass platform (Plantar test, Ugo Basile, Italy) and a removable infrared generator (radiant heat 137mW/cm^2/s) was placed underneath the animal's paw. The apparatus' controller automatically detects the withdrawal of the paw and the latency is recorded. A cut-off of 25 seconds is used to avoid tissue damage in case of absence of noxious response.

6-Sacrifices and samples

Mice were anesthetized with an overdose of thiopental solution (5%, 10mL/kg body weight). Blood was taken from left ventricle and put in EDTA coated tubes (K2EDTA BD microtainer, BD, Franklin lakes, USA). Tubes were then centrifuged (10 minutes at 2800rpm), and supernatant (plasma) stored at –80°C. Mice are then perfused with a 4% paraformaldehyde solution (PFA) in PBS pH8. Spinal cords were then taken and post-fixed in a 4% PFA solution in PBS pH8 for 2 days at 4°C. Samples were then transferred in a 30% sucrose solution in PBS at 4°C for 2 days. After this period of time, they were embedded in OCT and maintained at –20°C for sectioning. Anterior and Posterior peripheral nerves were also taken for analysis. 1 Anterior and 1 Posterior peripheral nerve per animal were put in a 2% glutaraldehyde solution in PBS. 1 Anterior and 1 posterior peripheral nerve per animal were snap frozen in liquid nitrogen and stored at –80°C.

7-Histological analysis

In order to analyze if there is an area of the spinal cord more susceptible to the disease, 20µm thick sections of spinal cords embedded in OCT were made at the cryostat (Leica) and stained for demyelination, axonal loss, and inflammatory cells quantification using respectively Luxol fast blue stain, Bielschowsky silver stain, Haematoxylin and Eosin (H&E) stain. Sections were then quantified at microscope, number of infiltrated inflammatory cells per mm^2 was counted, and areas of demyelination and axonal loss in % of total spinal area calculated.

8-Protein extraction and quantification from peripheral nerves

Snap frozen nerves samples were digested in 200µL of a triple detergent buffer (TDB) + protease inhibitor solution (protease inhibitor cocktail, Sigma-Aldricht Germany) and sonicated (Vibro Cell, Sonics and Materials Inc, USA). Samples were then put in ice and left as for 1 hour. After 1-hour digestion, samples were then centrifuged (15 minutes, 1400rpm, 4°C) and supernatant taken and frozen at –80°C. The protein content was measured with BCA protein assay: 1µL of each sample was put in 1mL of a 20% Bradford's reagent in water and left for 5 minutes. After 5 minutes optical densitometry was read using a spectrometer (model Shimadzu UV-1601) and protein concentration was calculated

9-Cytometric bead assay for inflammatory cytokines

After quantification of the protein content in the nerves extracts, Cytometric Bead Array (CBA) analysis was performed using the Mouse Inflammation Kit (BD Biosciences, San Diego, CA) as per the manufacturer's instructions with a sample volume equivalent to 150µg of protein per assay. CBA data were analyzed on a FACScalibur with Cellquest software and the CBA analysis software package (BD Biosciences).

10-Peripheral nerves semithin sections and G-ratio measurement

Portions of peripheral nerves obtained from the mice and fixed in glutaraldehyde were postfixed in 1% osmium tetroxide for 2 hours at room temperature. After alcohol dehydration, these sample were embedded in Epon. Transverse sections (1µm thick) were stained with toluidine blue and examined by light microscopy (microscope Leica). To assess the severity and the extent of demyelination, remyelination, 3 different sections of each nerves, coming from all the animals were analyzed at 100X magnification and 3 different fields were used to semi-automatically measure the G-ratio (Leica's QUIPS software + G-ratio calculation plug-in developed by Leica)

Statistical analysis

All results are expressed in mean ± SEM. A two-tailed Student's t-test was used to analyze differences between two groups mean (i.e. daily clinical score). Where more than two groups (i.e. spinal cord histological analysis) were compared, the significance of the difference among group means was evaluated by one-way Analysis of Variance, and further statistical analysis for post hoc comparison performed with a two-tailed Tukey's multiple comparison test. When two factors (i.e. group and time,

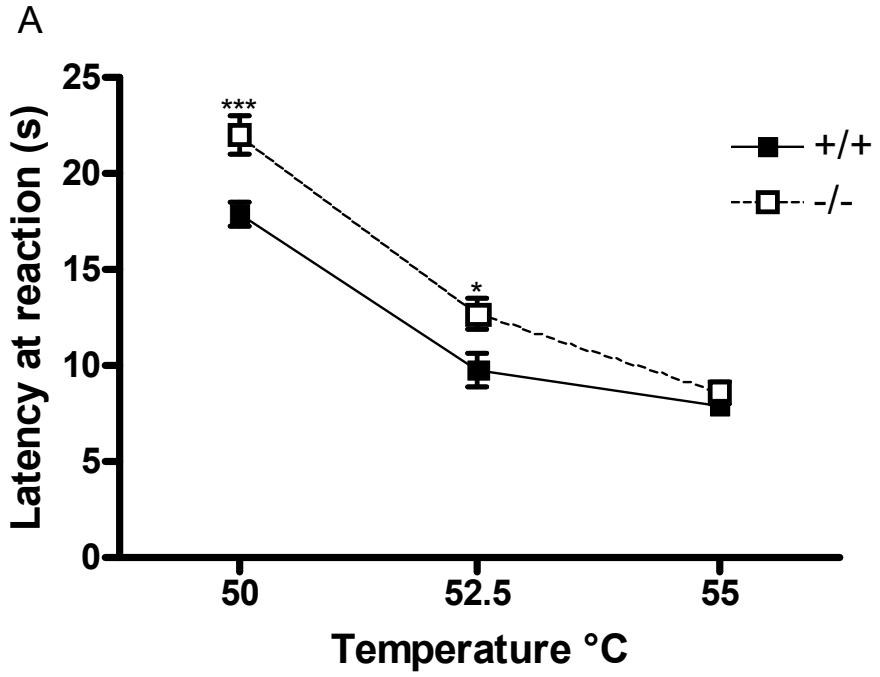
or group and intensity) were compared, the significance of the difference among group or time means was evaluated by two-way Analysis of Variance, followed by post hoc comparison with Bonferroni post test (two-tailed). Statistical significance was considered for $p<0.05$. All analyses were performed using GraphPad Prism 4 for Windows (GraphPad software Inc, San Diego, California).

Results

Thermal sensitivity associated to stimulus intensity variation:

A significant delayed thermal pain reaction was observed in the hotplate test in -/- mice for plate temperatures of 50±1°C and 52.5±1°C (respectively $p<0.001$ and $p<0.05$ -/- vs. +/+), while no significant difference was observed for plate temperature of 55±1°C (2 way ANOVA temperature $p< 0.0001$, genotype $p<0.001$, interaction NS, followed by Bonferroni posttest. Fig 1A).

A significant, delayed response of the -/- mice was observed for radiant heat in the plantar Hargreaves' test with intensities of 136mW/cm^2/s and 164mW/cm^2/s ($p<0.001$ -/- vs. +/+ for both beam intensities), but no differences were observed for radiant heat of 190mW/cm^2/s (2 ways ANOVA intensity $p< 0.0001$ genotype $p<0.0001$, interaction 0.001, followed by Bonferroni posttest, Fig 1B).



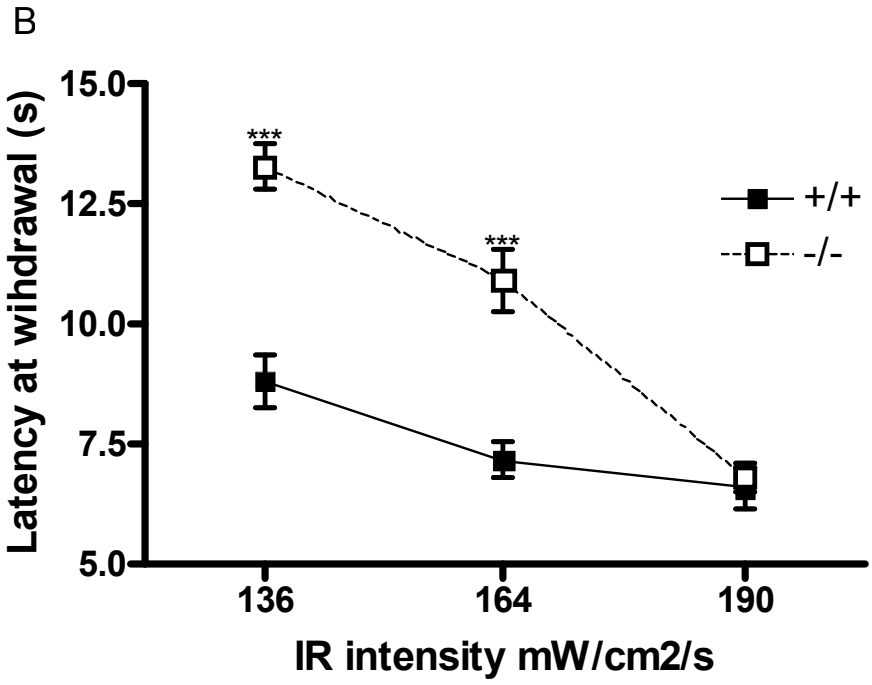


Fig 1: Thermal sensitivity associated to stimulus intensity variation in hotplate test (A) and plantar Hargreaves' test (B). (A) Significant delayed thermal pain reaction observed in -/- mice for plate temperatures of 50±1°C and 52.5±1°C (2 ways ANOVA temperature $p< 0.0001$, genotype $p<0.001$, interaction NS, followed by Bonferroni posttest). Reversion of the phenotype was observed for plate temperature of 55±1°C. (B) Hyporesponsivity to radiant heat of 136mW/cm^2/s and 164mW/cm^2/s was observed in -/- animals (2 ways ANOVA intensity $p< 0.0001$ genotype $p<0.0001$, interaction 0.001,followed by Bonferroni posttest). Reversion of the phenotype was observed for radiant heat of 190mW/cm^2/s. * $p<0.05$, *** $p< 0.001$ vs. wild type littermates.

Effect of an early curative treatment with immunosuppressant/immunomodulator MTX in a mild on C57bl/6 mice

1-Clinical score

Chronic EAE was induced and monitored as described in material and methods. Disease incidence was about 92% and disease onset took place from day 13 to day 19 post-immunization with an average of 15.74 days post-immunization. Maximum clinical score (2.11±0.17 for Mitoxantrone treated animals and 2.86±0.14 for vehicle treated animals) was reached 5 days after animals' recruitment (Fig 2). Significant decrease of clinical score of MTX treated animals compared to Vehicle treated animals was observed starting from day 5 post-recruitment till the end of the experiment (Fig 2). A slight rebound of the severity of the clinical score was observed in MTX treated animals from day 26 post-recruitment (10 days after the end of the treatment period). Howerver this severity remained statistically lower than vehicle treated animals (Fig 2). In addition, mortality rate in MTX treated animals was lower than in Vehicle treated animals (respectively 0% and 46% of the animals), and no animal died in the non immunized group.

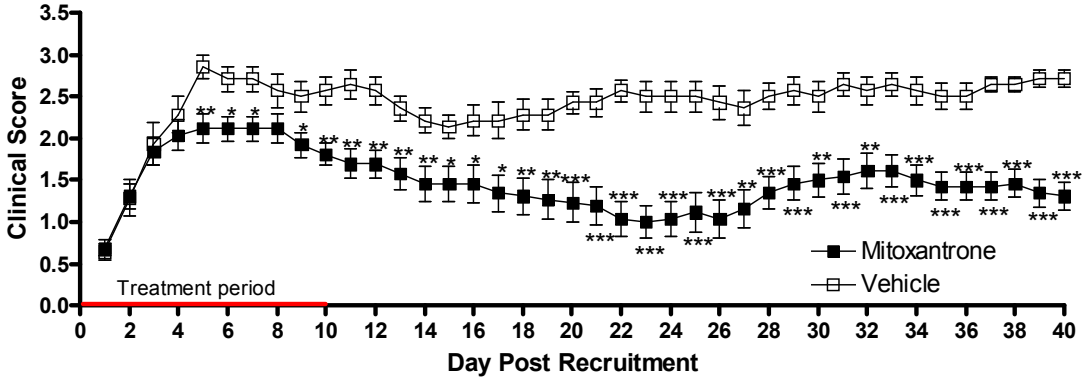


Fig. 2: Mean EAE scores from EAE female mice are shown during the course of the disease starting from the first day of treatment till the end of the experiment 40 days later. A significantly lower clinical score was observed in MTX treated animals starting at day 5 post recruitment. No significant difference in the onset of the disease and in the maximal clinical score was observed between groups.

2-Inflammation

Infiltrating cells were found exclusively within the spinal cord of EAE animals (Fig 3). Number of infiltrates in all the areas of the spinal cord was very low in EAE animals compared to historical data. MTX treatment induced a significant decrease in the number of infiltrate in cervical area ($p<0.001$ MTX Vs. Vehicle; 1 way ANOVA followed by Tukey multiple comparison post test) as well as in the thoracic area ($p<0.01$ MTX Vs. Vehicle; 1 way ANOVA followed by Tukey multiple comparison post test) but not for the lumbar area where only a trend was observed ($p>0.05$ MTX Vs. Vehicle; 1 way ANOVA followed by Tukey multiple comparison post test).

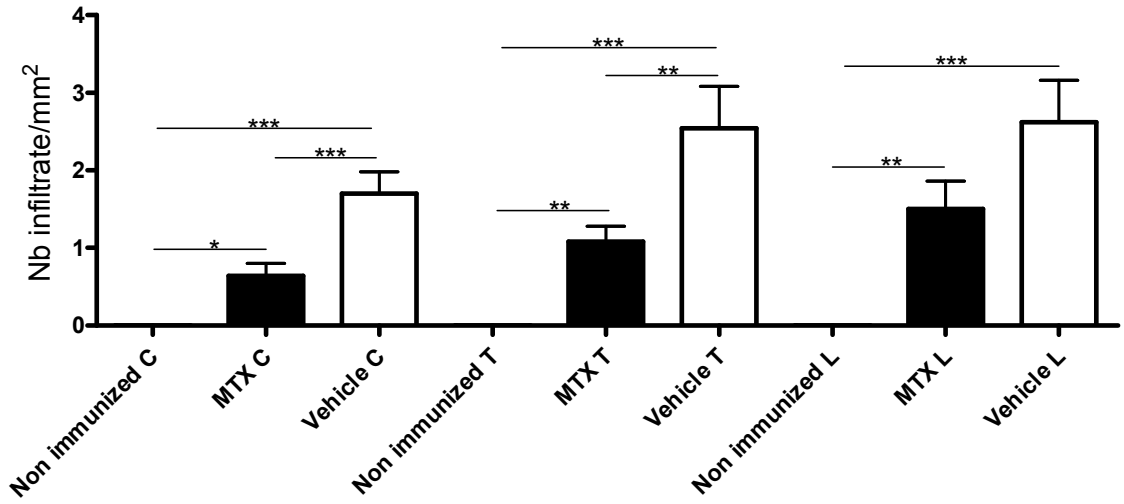


Fig 3: Quantification of the inflammatory cells in the spinal cord demonstrates that MTX treated animals always show less infiltrates than Vehicle treated animals. This difference is significant for the cervical and lumbar part of the spinal cord (***: $p<0.001$, **: $p<0.01$, *: $p<0.05$; 1 way ANOVA followed by Tukey multiple comparison post test). C stands for cervical, T for thoracic and L for lumbar.

3-Demyelination

Similarly to inflammation, areas of demyelination were found only in EAE animals but not in healthy (Fig 4). Greatest areas of demyelination were observed in animals

treated with Vehicle whereas in MTX treated group percentage of demyelination is significantly lower ($p<0.01$ for cervical and lumbar area, $p<0.001$ for thoracic area; MTX Vs. Vehicle; 1 way ANOVA followed by Tukey multiple comparison post test). No statistical differences were found between non immunized animals and MTX treated animals for the cervical and thoracic areas ($p>0.05$ MTX Vs. non immunized; 1 way ANOVA followed by Tukey multiple comparison post test). Unlikely a significant difference in percentage of demyelination can be observed between non immunized and MTX treated animals in the lumbar part of the spinal cord ($p<0.01$ non immunized Vs. MTX; 1 way ANOVA followed by Tukey multiple comparison post test).

4-Axonal loss

In the same manner, areas of axonal loss were observed only in EAE animals (Fig 5). Highest percentages of axonal loss were measured in vehicle treated animals, significantly different from MTX group in cervical and thoracic but not in lumbar areas ($p<0.01$ for cervical area, $p<0.001$ for thoracic area; MTX Vs. Vehicle; 1 way ANOVA followed by Tukey multiple comparison post test). No significant difference in percentage of axonal loss was observed between non immunized animals and MTX treated animals for the 3 areas of interest ($p>0.05$ MTX Vs. non immunized; 1 way ANOVA followed by Tukey multiple comparison post test).

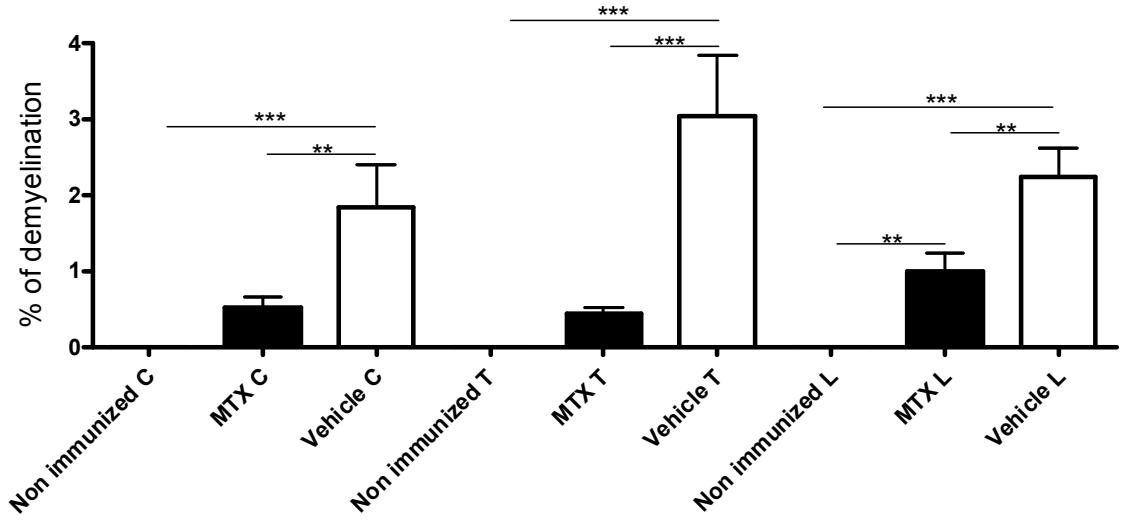


Fig 4: Quantification of the demyelination in the spinal cord demonstrates that MTX treated animals have a significantly lower percentage of demyelination compare to Vehicle treated animals. MTX treated animals have shown a significant difference in demyelination with non immunized animals only for the lumbar part of the spinal cord. (***: $p<0.001$, **: $p<0.01$; 1 way ANOVA followed by Tukey multiple comparison post test). C stands for cervical, T for thoracic and L for lumbar.

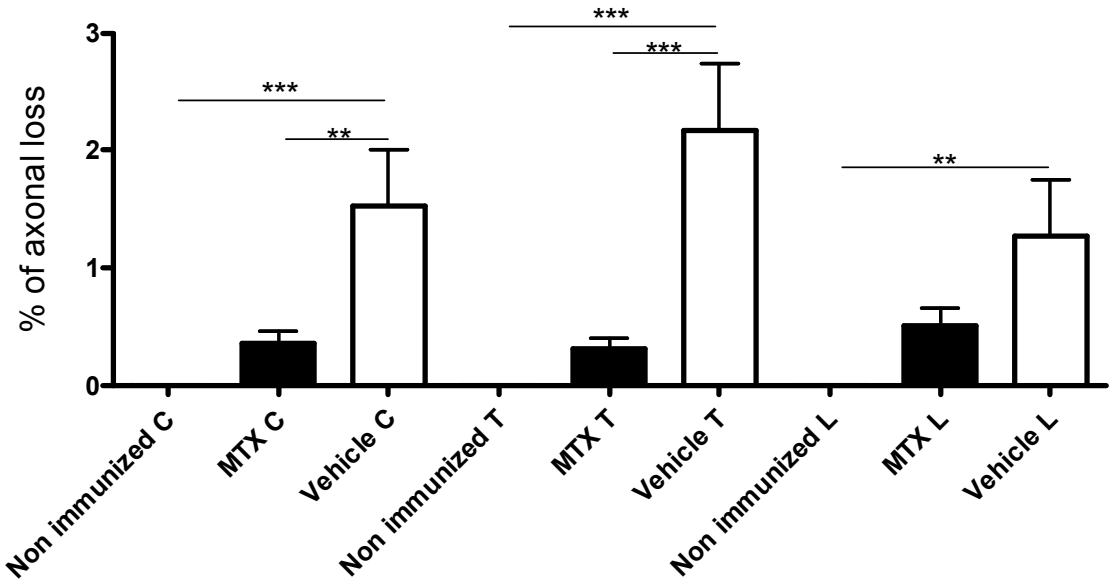


Fig 5: Quantification of the axonal loss in the spinal cord demonstrate that MTX treated animals always show less axonal loss compare to Vehicle treated animals, significant only in the cervical and thoracic part. No significant difference was observed between non immunized and MTX treated animals. (***: $p<0.001$, **: $p<0.01$; 1 way ANOVA followed by Tukey multiple comparison post test). C stands for cervical, T for thoracic and L for lumbar.

5-Areas involved in inflammation/demyelination/axonal loss

Analyses of areas of the spinal cord involved in demyelination or axonal loss were performed in order to better understand which parts of the spinal cord are more susceptible to the disease.

Inflammation:
Inflammatory infiltrates observed in spinal cords of diseased animals 40 days after recruitment were spread in the total white matter area in all the 3 zone considered (cervical, thoracic, and lumbar).

Demyelination (Fig 6):
In MTX treated group, 66% of the animals had demyelination in the dorsal column medial lemniscus system and more precisely the fasciculus gracilis, 50% in the anterior cortico-spinal tract, 33% and 25% respectively in the anterior and posterior spino-cerebellar tract for the cervical part of the spinal cord. For the thoracic part of the spinal cord 54% of the animals were demyelinated in the anterior cortico-spinal tract and 27% in the fasciculus gracilis. Concerning the lumbar part of the spinal cord, 36% of the mice were demyelinated in the anterior cortico-spinal tract, 18% in the anterior spino-cerebellar tract and in the fasciculus gracilis.
In the Vehicle treated group, 66% of the animals had demyelination in the fasciculus gracilis, 66% in the anterior cortico-spinal tract, 17% and 33% respectively in the anterior and posterior spino-cerebellar tract of the cervical area. In the thoracic part 83% of the animals had demyelination in the anterior cortico-spinal tract, and 33% in the fasciculus gracilis, anterior and posterior spino-cerebellar tracts. In the lumbar part of the spinal cord 66% of the animals had demyelination in the anterior cortico-spinal tract, 50% in the fasciculus gracilis and the anterior spino-cerebellar tract, and 33% in the posterior spino-cerebellar tract.

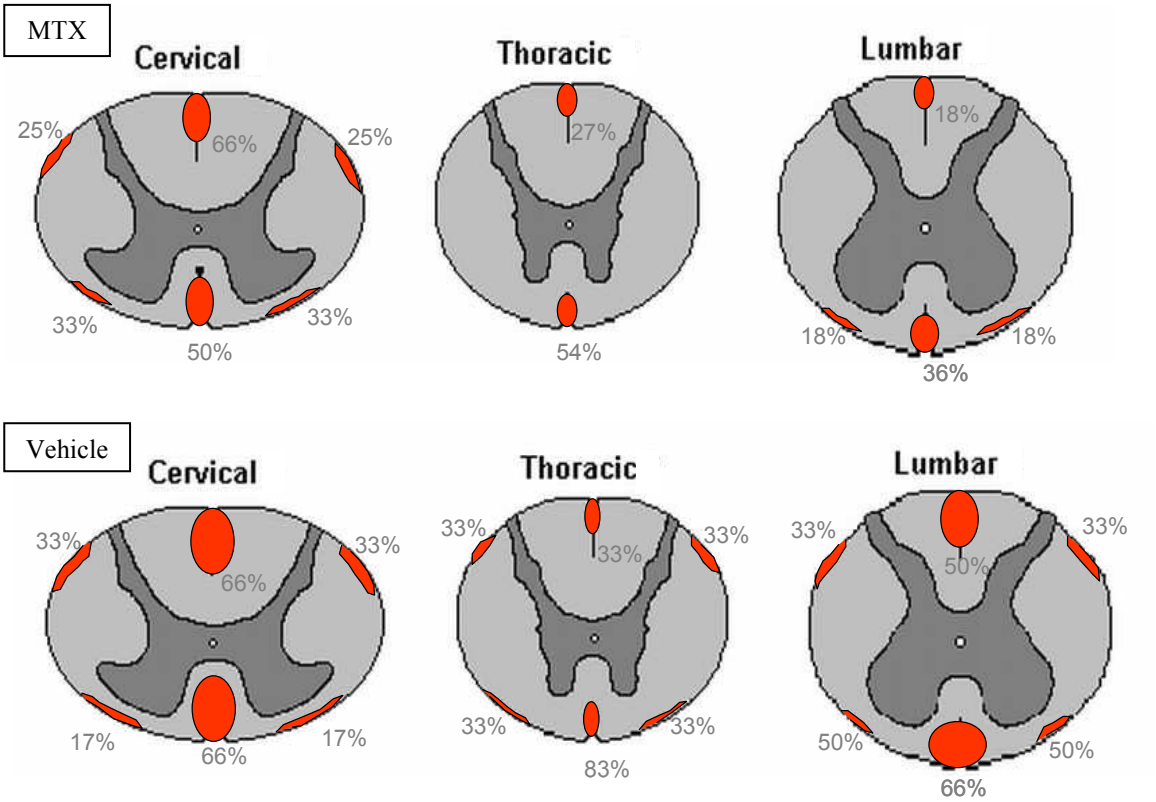


Fig 6: Schematic representation of the areas of the spinal cord involved in demyelination. Areas most involved for both MTX treated and Vehicle treated animals but in a different extend are the fasciculus gracilis, the anterior cortico-spinal tract, and the anterior and posterior spino-cerebellar tract. Spots on the schema indicate the extend of the demyelination for each group and part of the spinal cord, the numbers nearby each area represent the percentage of animals presenting demyelination in this area.

Axonal loss (Fig 7):

In the MTX treated group, 50% of the animals had axonal loss in the fasciculus gracilis and 17% in the anterior cortico-spinal tract for the cervical area. In the thoracic part, only the fasciculus gracilis presented axonal loss in 17% of the animals. In the lumbar part, the only area showing sign of axonal loss was the fasciculus gracilis in 33% of the animals.

In the vehicle treated group, 33% of the animals had axonal loss in the fasciculus gracilis and the anterior cortico-spinal tract for the cortical area. In the thoracic part, 17% of the animals had axonal loss in the fasciculus gracilis and the cortico-spinal tract. In the lumbar area, 66% of the animals had axonal loss in the anterior cortico-spinal tract, 50% in the fasciculus gracilis, and 17% in the posterior spino-cerebellar tract.

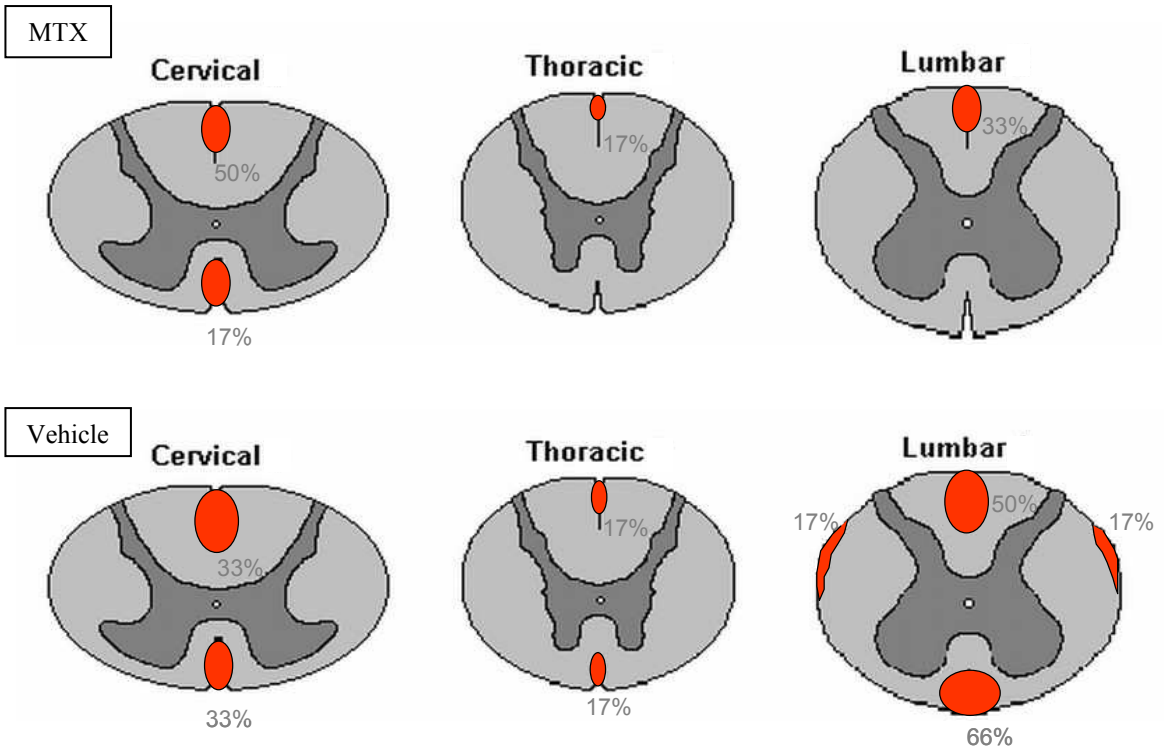


Fig 7: Schematic representation of the areas of the spinal cord involved in axonal loss. Areas most involved for both MTX treated and Vehicle treated animals but in a different extend are the fasciculus gracilis, the anterior cortico-spinal tract, and the posterior spino-cerebellar tract. Spots on the schema indicate the extend of the demyelination for each group and part of the spinal cord, the numbers nearby each area represent the percentage of animals presenting demyelination in this area.

6-Cytometric bead assay

At 40 days post recruitment, concentration of principal cytokines of inflammation was performed on plasma samples, anterior and peripheral nerves protein extracts.

In plasma, no difference was observed in concentration of IL12p70, Tumor Necrosis Factor alpha (TNFa), interferon gamma (INFg), and interleukins IL10, and IL6. Concentration of Monocyte chemotactic protein 1 (MCP1) in plasma of MTX treated animals (118±13.03 pg/mL) was significantly higher compared to other two groups ($p<0.01$ vs. non immunized, $p<0.05$ vs. Vehicle treated animals; 1way ANOVA followed by Tukey's multiple comparison posttest).

Concentration of inflammatory cytokines in both anterior and posterior peripheral nerves showed no differences between all the groups of animals ($p>0.05$ for all the cytokines in anterior and posterior peripheral nerves). (Table 1)

Cytokines concentration (pg/mL)	Blood			Anterior peripheral nerves			Posterior peripheral nerves		
	Non-immunized	MTX	Vehicle	Non-immunized	MTX	Vehicle	Non-immunized	MTX	Vehicle
IL12p70	30.70±5.66	37.42±6.77	33.55±7.05	27.46±5.12	24.13±3.33	21.15±2.48	33.06±5.61	23.09±3.45	29.42±2.61
TNFa	34.88±2.26	35.45±3.94	36.94±5.76	21.89±3.27	Nd	Nd	25.79±4.39	26.92±5.46	Nd
IFNg	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd
MCP1	59.11±9.57	118.0±13.03	63.90±11.30	58.73±6.83	64.67±5.49	66.62±9.10	79.59±13.07	59.38±6.44	62.73±14.12
IL10	53.40±16.81	43.45±9.56	28.70±0.00	123.6±19.97	92.63±10.56	94.17±5.29	158.14±23.94	104.60±22.49	97.23±15.48
IL6	Nd	28.73±2.27	41.13±19.99	Nd	Nd	Nd	Nd	Nd	Nd

Table 1: cytokine summary: concentrations of each cytokine tested are presented. Data given as Mean±SEM. Nd stand for non detectable.

7-G-ratio

Semi-thin sections of peripheral anterior and posterior nerves were analyzed at the light microscope. When measuring the G-ratio, no differences were found between all the 3 groups for both anterior and posterior peripheral nerves. G-ratios measured were between 0.66 and 0.72, values considered as normal for the G-ratio of peripheral nerves (Fig 8). Moreover no obvious clues in difference in the number of remyelinated axons were observed.

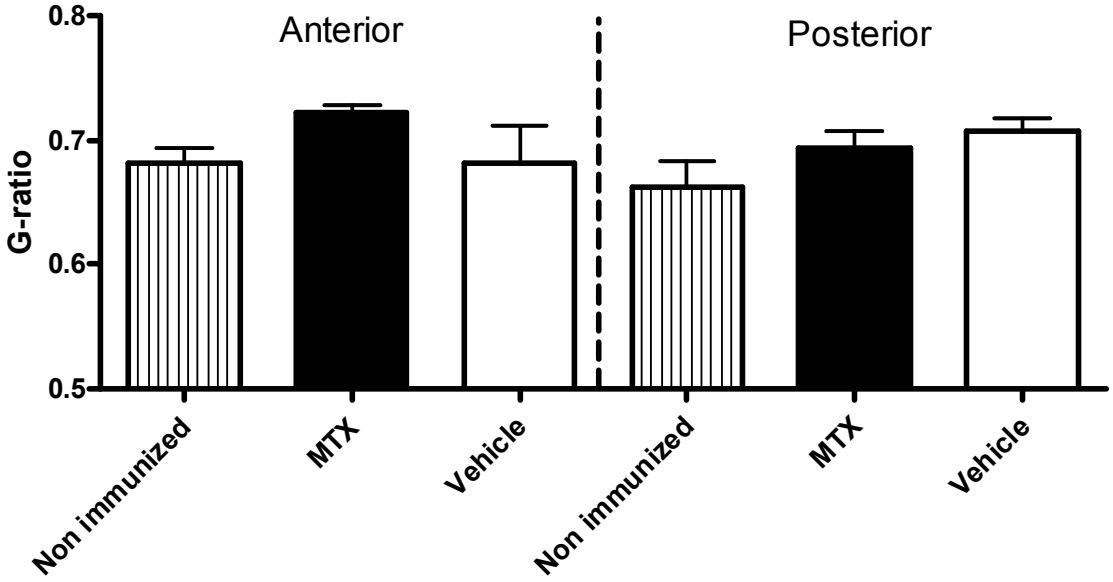


Fig 8: calculation of the G-ratio has shown that there was no difference between all the 3 groups for both anterior and posterior peripheral nerves.

8-Pain readouts (Hargreaves)

Thermal sensitivity assessed on forelimb of EAE vehicle and MTX treated mice as well as healthy animals by Hargreaves' plantar test at specific time points during the disease progression, was carried out to follow changes in response to a thermal stimulus.

No significant differences were observed between groups till period of recruitment at the onset of the disease (Fig 9). At this later time point, both MTX treated animals and vehicle treated animals have a significant delayed response to the thermal stimulus compared to non-immunized animals ($p<0.01$ MTX VS. Non-immunized and $p< 0.05$ Vehicle VS. Non-immunized; 2way ANOVA followed by bonferroni posttest). However, no differences were observed between MTX treated and Vehicle treated mice. At sacrifice, similar results were observed, that is to say that both groups of diseased animals showed an hyporesponsivity (longer time at paw withdrawal) compared to non immunized animal ($p<0.01$ for both MTX VS. Non-immunized and Vehicle VS. Non-immunized; 2 way ANOVA followed by Bonferroni posttest) and no differences between MTX treated and Vehicle treated animal were observed(Fig 9).

Healthy animals did not show any statistical differences all along the experiment period. Although a tendency to decrease latency at paw withdrawal was recorded (Fig 9).

MTX treated animals show no differences in thermal sensitivity between basal value and 1 week post immunization, but became hyposensitive (increase in time latency) at recruitment (onset of the disease) ($p<0.01$ recruitment VS. 1 week post immunization; 2 way ANOVA followed by bonferroni posttest) (Fig 9). Value observed at sacrifice is not statistically different neither from recruitment nor from basal and 1 week post immunization (Fig 9).
In vehicle treated animals, differences in response to a thermal stimulus were similar to that observed in MTX treated animals (Fig 9).

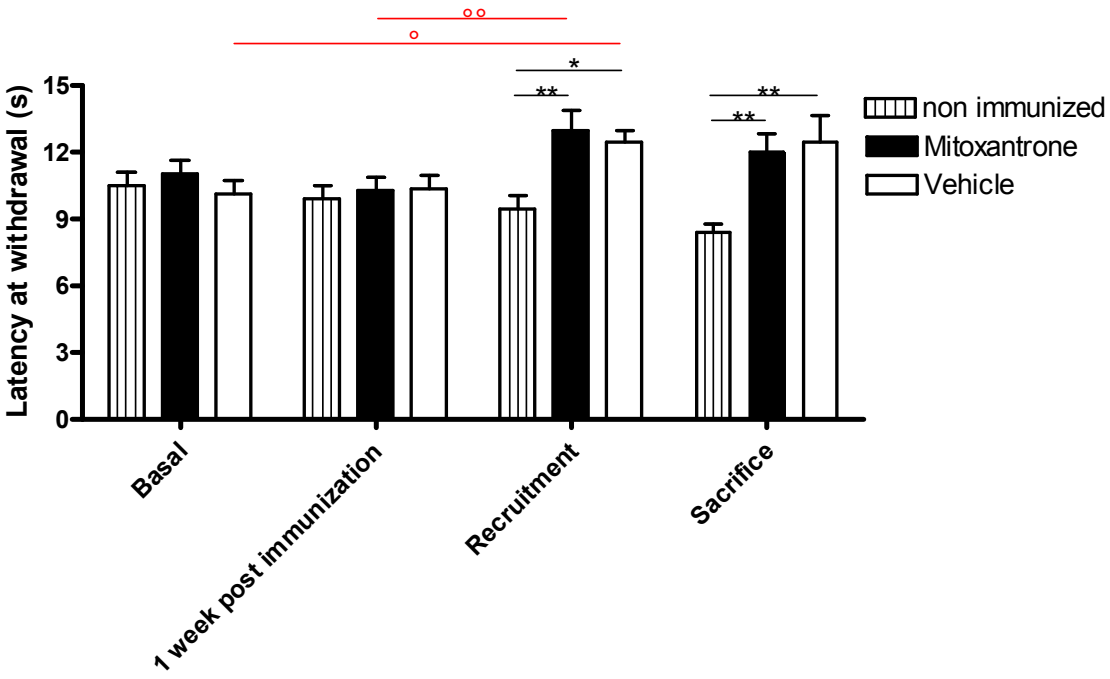


Fig 9: Evolution of the responsivity to a thermal stimulus during the EAE demonstrates that there is no significant difference between groups until the period of recruitment and the beginning of the treatment. At recruitment the two EAE groups have shown a significant delayed response to thermal stimulus compared to non immunized animals but no difference is observed due to the treatment. This hyporesponsivity of EAE animals compared to non immunized animals was still significant at the end of the experiment. At this time point no significant difference between groups was detectable. (*<0.05; **p<0.01; intergroup; °p<0.05; °°p<0.01; inter-timepoint; 2 way ANOVA followed by Bonferroni posttest).

Discussion

Past years results obtained in the behavioral phenotyping, suggested a regulatory role of RPTPz in the integration of sensorimotor stimuli potentially through relevant neural network not only "physiologically" but also under inflammatory condition.

This year we observed that high intensity of thermal stimuli in KO mice triggered responses equal to the one observed in wild type (Fig 1) suggesting that this modulatory role might be specific to stimuli of low to moderate intensity. Indeed, the concept that nociceptive responses to stimuli of different intensity may be differently processed and mediated by various patterns of neurochemical mediators was already suggested by Cao and coworkers [23] that observed that tachykinin-1 knockout mice differed from their wild-type littermates only at intermediate intensities of thermal and chemical stimulation, and further confirmed by several studies performed on genetically modified mice [24,25].

The RPTPz modulatory role in the integrated sensorimotor responses appears to be specific for nociceptive stimuli, since no alteration in the motor response to sounds or sensorimotor gating as assessed by prepulse inhibition of the startle was observed in the KO mice during the behavioral phenotyping.

In the EAE study, we tested the efficacy of an early curative treatment with MTX in a chronic EAE model on C57BL/6 mice. Extends of the inflammatory process, demyelination, and axonal loss were measured at the end of the experiment and compared to non immunized and vehicle treated animals. Moreover, evolution of thermal pain responsiveness was monitored for all the duration of the experiment.
Several studies showed that immunosuppressive drugs can be effective in reducing the progression of disability in MS. However, such potent agents are usually used with care because of their side effects, which are potentially severe [26]. This is, for example, the case for MTX, which trigger severe impairment in cardiac function [27]. MTX has proved, since many years now, to be efficient to suppress EAE in animal models [28], and his mechanism of action as well as his therapeutic role are well documented [29,30,31].
In our experiment, MTX succeeded to reduce but not to stop, as already observed [25], the severity of the chronic EAE in mice (Fig 1), even if the process of inflammation was all ready triggered. A slight increase in mean clinical score observed day 26 post-recruitment (10 days after the end of the treatment period) may denote a loss of efficacy of the treatment and may signify that treatment of autoimmune diseases may require periodic cycles of therapy to block disease expression. The efficacy of MTX in reducing and /or modulating the inflammation not only reduced the severity of the disease but also led to a decrease in mortality.
In our experiment, no differences were observed when measuring the G-ratio of peripheral nerves of the mice (Fig 8) and no obvious presence of remyelinated fibers were noticed. This result was expected and is in line with the EAE model that is a central nervous system demyelination model where peripheral nervous system is not implicated. The difficulty to measure the concentration of pro-inflammatory cytokine and the similar concentration of these cytokines in all the groups (excepted for MCP1) (table 1) indicate that the process of inflammation is stopped and no other inflammatory cells will be activated.
Histological analyses are consistent with the observation of the clinical score. The low density of infiltrated cells in the spinal cords of EAE animals for both treatment (Fig 3) indicates that the inflammatory process of the EAE 40 days post onset of the disease is finished or reduced, as already observed in a previous study [32]. Nevertheless the effect of MTX is still visible confirming the efficacy of the treatment on the inflammatory process. Likely due to this decreased of the inflammation, demyelination of the spinal cord is drastically reduced in MTX treated mice (Fig 4) to the point that no statistical difference can be observed between MTX treated animals and non immunized animals for the cervical and thoracic area. Axonal damage in the spinal cord followed the same profile as demyelination (Fig 5), however it is not clear if it is an indication of a possible cause effect relationship or a secondary consequence of the decreased of the inflammation in parallel to decreased demyelination.
It is well admitted that EAE induced immune reaction against myelin proteins that cause disseminated inflammatory CNS lesions, which share important aspects of the pathology of MS lesions. Nevertheless, tracts of the spinal cord are known to be more susceptible to demyelination and axonal loss. This is for example the case of the cortico-spinal tract (CST) that conveys motor information. We have shown here that

not only the CST is damaged but also other afferent sensitive tracts (Fig 6 and 7). Major ascending pathways involved in demyelination are the fasciculus gracialis, that is the part of the dorsal column medial lemnicus system that convey signals from propriception and inocuous sensation of the lower part of the body, and the spino-cerebellar tracts (both posterior and anterior) that convey unconscious proprioceptive information to the cerebellum (Fig 6 and 7). However, these pathways are not involved in an equal manner in the 2 groups of EAE animals. MTX treated animals have shown a lower percentage of animals presenting demyelination or axonal loss in the previously mentioned tracts, and demyelination and axonal damage is not present in all the tracts (Fig 6 and 7), confirming the role of MTX as a disease modifying agent.

Pain is a major symptom of MS that has a dramatic impact on patients' quality of life. Really few studies have addressed this problem and examined nociceptive response in a mouse model of MS [33]. In our study we assessed sensorimotor response of EAE mice to a thermal stimulus and looked if the use of an immunomodulatory/immunosupressive agent known to be efficient to decrease the severity of the disease exerts also a role on pain sensitivity. Until onset of the disease (that correspond to the recruitment of the animals and the beginning of the treatment), EAE animals did not present any sign of hyper or hypo algesia compared to non immunized animals (Fig 9) indicating that the inflammatory process is not yet started. Similar results were already observed in literature [30]. Aicher showed, in a mouse model of MS, that animals became hypoalgesic at the onset of the disease, and that the same animals became hyperalgesic during the chronic phase of the disease [31]. In our results, we observed that both groups of EAE animals became hypoalgesic during the recruitment period (onset of the disease), but they remain hypoalgesic till the end of the experiment 40 days later (Fig 9). Our results demonstrate first that EAE induces hypoalgesia and then that treatment with MTX did not ameliorate the sensorimotor response indicating that pain in EAE and by extension in MS is probably not only due to a defect in myelination or to an axonal damage. As Bannerman demonstrate, in a chronic EAE on C57BL/6 mice [34], there is no significant loss of spinal cord motor neurons, however, early in the course of the illness, motor neuron dendrites disrupted. Motor neurons dendritic abnormalities persiste and motor neuron perikaryal atrophy appear over time. This could explain why despite the decrease of the inflammatory process due to the treatment with MTX, the pain sensitivity is not significantly different between MTX treated animals and vehicle treated animals. The early process of inflammation trigger the disruption of motor neurons dendrites and the perikaryal atrophy that together lead to a loss of pain responsivity observed in EAE mice.

In this study, we demonstrate that EAE induces hypoalgesia. Treatment with a potent immunomodulator/immunosupressant agent stops the process of inflammation, prevent demyelination and axonal damage, but fail to ameliorate the sensorimotor response to a nociceptive stimulus. These results indicate, for the MTX, a dichotomy between its disease modifying role and the amelioration of the consequences of the disease, that is to say dendrites abnormality and perikaryal atrophy that lead to motor hyporesponsivity.

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Sezione 2

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