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Role of the Receptor like Protein Tyrosine Phosphatase zeta (RPTPz): deeper *in vivo* characterization.

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INTRODUCTION

Phosphorylation and dephosphorylation of proteins are two important processes in a cell allowing signal transduction and amplification all along the so call phosphorylation cascade. Thus Protein Tyrosine Phosphatases (PTP), and Receptor like Protein Tyrosine Phosphatases as part of this family are important proteins playing a role in the maintenance and survival of the cell. Because of the important role of the phosphorylation cascade, big effort has been placed in studying protein kinases. Comparatively, the study of protein phosphatases is just started.

Receptor like Protein Tyrosine Phosphatase zeta (RPTPz) is a member of transmembrane receptor like protein tyrosine phosphatases family that is widely express in the nervous system. As a receptor and a phosphatase, RPTPz has different ligands and substrates. One of the main substrate is phosphorylated βcatenin and one of the main ligand is pleiotrophin 18 (PTN18) (1) indicating a possible role of this RPTP in migration and differentiation of cells. In line with the supposed role of RPTPz in oligodendrocyte differentiation, previous studies showed that RPTPz KO are more severely affected by Experimental Autoimmune Encephalomyelitis (animal model for multiple sclerosis) (EAE) showing a greater susceptibility to the disease, an impaired recovery, together with an intensive degeneration of the spinal cord due mainly to increased oligodendrocyte apoptosis compared to +/+ (2).

Based on what is known on RPTPz functions in CNS and PNS it is surprising that no clear functional alteration are observable due to KO of the gene in vivo.
To further characterize the potential role of RPTPz, RPTPz KO mice were generated by replacing the genome sequence from exon 3 to exon 9 by Lac Z gene (LacZ KI) followed by stop codons. The sequence from exon 3 to 9 encodes for the extracellular part of the protein.

In the last year report, I've illustrated the first steps of the KO characterization corresponding to the behavioral phenotyping in baseline conditions. RPTPz -/- and +/+ littermate male mice were tested in an extensive battery of test designed to explore different domains controlled by CNS, like exploratory behaviour, coordination and strength (grip strength test, openfield, foot print, and accelerated rotarod), memory (Y maze), nociception (tailclip, hotplate, Hargreaves, VonFrey), sensory motor 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 behaviour and anxiety (open field, nb of entries in Y maze), sensory motor gating,

and -/- mice are more susceptible to seizure in early phases but not in the late phases of the test.
No differences were detected in nerve conduction velocity measured by in vivo electrophysiology.
RPTPz -/- mice displayed a marked phenotype linked to sensory-motor 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). Hargreaves' plantar test and Von Frey are two of the most commonly used test for the assessment of nociception, used also in Clinical practice. Polymodal C and Aδ nerve fibers are the fibers used for the transmission of the painful stimuli in general and for thermal and tactile stimuli in particular (3,4). The fact that results show a hyposensitivity of the -/- mice compared to +/+, demonstrate a potential role of RPTPz in the pain nerve impulse transmission along the C and Aδ fibers.
RPTPz -/- presented locomotor coordination difficulties and impaired working memory confirming the phenotype reported by another group on PTPRz KO (8).

In the tentative to reproduce the results obtained by Harrochs on RPTPz KO mice showing no recover for KO mice from an Experimental Autoimmune Encephalomyelitis (EAE, animal model for multiple sclerosis), an EAE a long term (80 days) was induced on KO and wild type littermate using the same protocol previously used by Harroch (2). At the end of the experiment, thermal pain sensitivity and nerve conduction velocity were measured. EAE performed on our -/- and +/+ mice show no significant differences in the overall course of the disease (time onset, maximal clinical score, and mortality), and no recovery was observed neither in -/- nor in +/+ mice. These results are not in line with the published data. This difference can be explained by the fact that our KO design is different from the one used in the Harrochs study. Moreover the strain of mouse used is different, swiss Webster for Harrroch and C57bl/6 for us. And finally housing environnement is not the same. Thermal pain readouts measured at the end of the EAE show that EAE induced hypoalgesia in +/+ mice but not in -/- that show no difference in thermal sensitivity between healthy and EAE animals.

Based on the obtained results, RPTPz appears to play an important role in pain sensitivity. To further assess this hypothesis, during the last year I measured pain readouts after challenge of the animals with carrageenan or formalin. The Carrageenan is a natural product that induces inflammation, the formalin is a chemical product that damages the injected tissues and secondary provokes inflammation.
The first EAE (induced by injection of 200µg of MOG peptide) was to strong to see a recover from the Wild Type mice, I decide to induce a milder (50µg injection of MOG peptide) and shorter (40 days) EAE on RPTPz -/- female mice and littermate +/+. And in order to reduce the severity of the pathology by decreasing the early inflammation (first phase of the disease), I used a treatment with a potent immunosuppressant (Mitoxantrone, MTX).

MATERIAL AND METHODS

Behavioral phenotyping of RPTPz -/- mice in challenge conditions.

Carrageenan-induced inflammation:

-/- and +/+ littermate male mice were tested for nociception (Hargreaves, VonFrey) after injection of 30µL of 1% carrageenan λ solution in saline in one hind paw, vehicle was injected in the controlateral paw as control. Carrageenan induces inflammation, hypersensitivity to nociceptive stimuli, tactile allodynia, and swelling. In our protocol, 2.5 hours after injection the animals were tested using Plantar Hargreaves and Von Frey on both paw for inflammatory pain sensitivity. Reaction of the carrageenan-injected paw was compared with the controlateral paw. Paw thickness was measured as index of inflammation-induced edema.

Formalin-induced inflammation test:

-/- and +/+ littermate male mice were tested after subcutaneous injection 10µL of a 2% Formalin solution in saline.
After formalin administration, animals are immediately observed and scored for pain behaviour every 30 seconds during 50 minutes long test. Score goes from 0 to 3
0= no pain behavior.
1= favoring injected limb (touch the ground without resting on it).
2= lifting injected limb.
3= licking injected limb.
Data are analyzed as cumulative score in 5 min bin, and as cumulative score for the 2 phases that are typically observed in this test:
Phase 1: pain due to tissue damage and injection of the solution.
Phase 2: pain due to formalin-induced inflammation.

Induction of a mild EAE in RPTPz -/- mice and early curative treatment with Mitoxantrone.

Induction of the disease:

On day 0 mice are immunized by injecting 0.1 mL in each flank of an emulsion composed of 50 µg MOG_{35-55} peptide in Complete Freund's Adjuvant containing 0.5 mg of *Mycobacterium tuberculosis*. Immediately after, they received an i.p. injection of 250 ng pertussis toxin.

Treatment:

At first sign of the disease, mice are recruited and treated for 10 days with Mitoxantrone (MTX) (0.5mg/kg daily) or vehicle.

Behaviour:

Plantar Hargreaves' test was performed on the forelimbs of the animals at the beginning of the treatment period and at the end of the experiment.

RESULTS

Behavioral phenotyping of RPTPz -/- mice in challenge conditions.

Carrageenan-induced inflammation:

Hargreaves' plantar test: -/- mice have shown 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. (Fig.1).

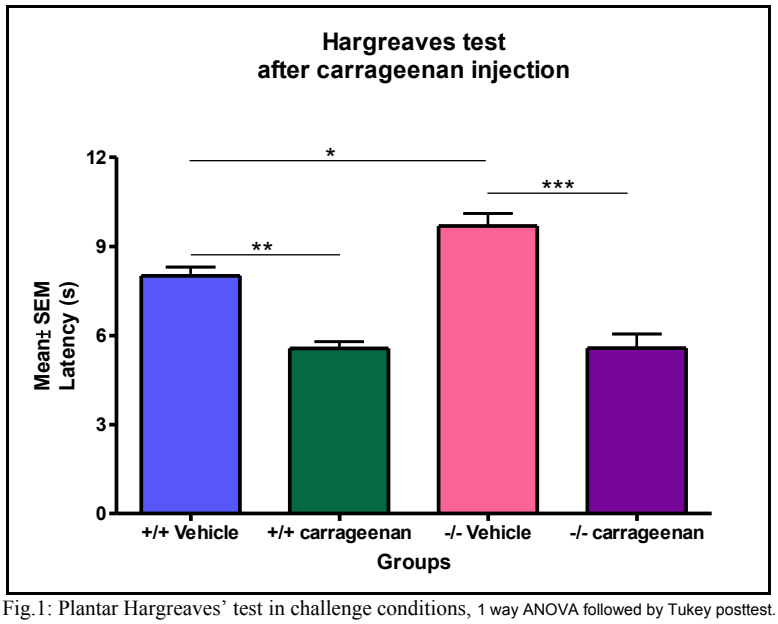


Fig.1: Plantar Hargreaves' test in challenge conditions, 1 way ANOVA followed by Tukey posttest.

Automated Von Frey: -/- mice have shown a significant hyposensitivity (increased applied force for inducing withdrawal) to tactile stimulus compared with +/+ mice in vehicle treated paw. This result confirms the one obtained during the phenotyping. Carrageenan injection triggers hyperalgesia in +/+ mice (reduced force threshold), but not in -/- mice. (Fig.2)

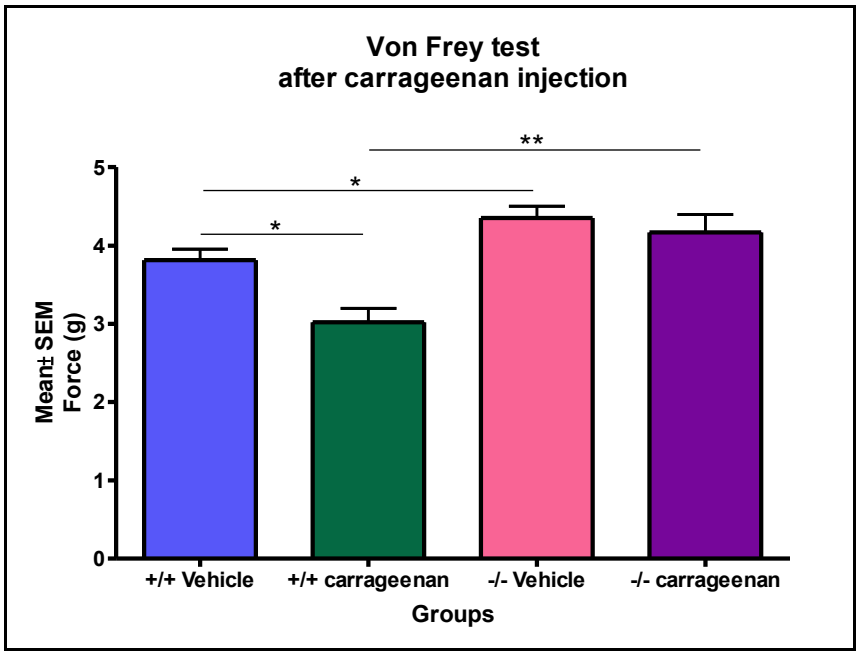


Fig.2: Von Frey filament test in challenge conditions, 1 way ANOVA followed by Tukey posttest

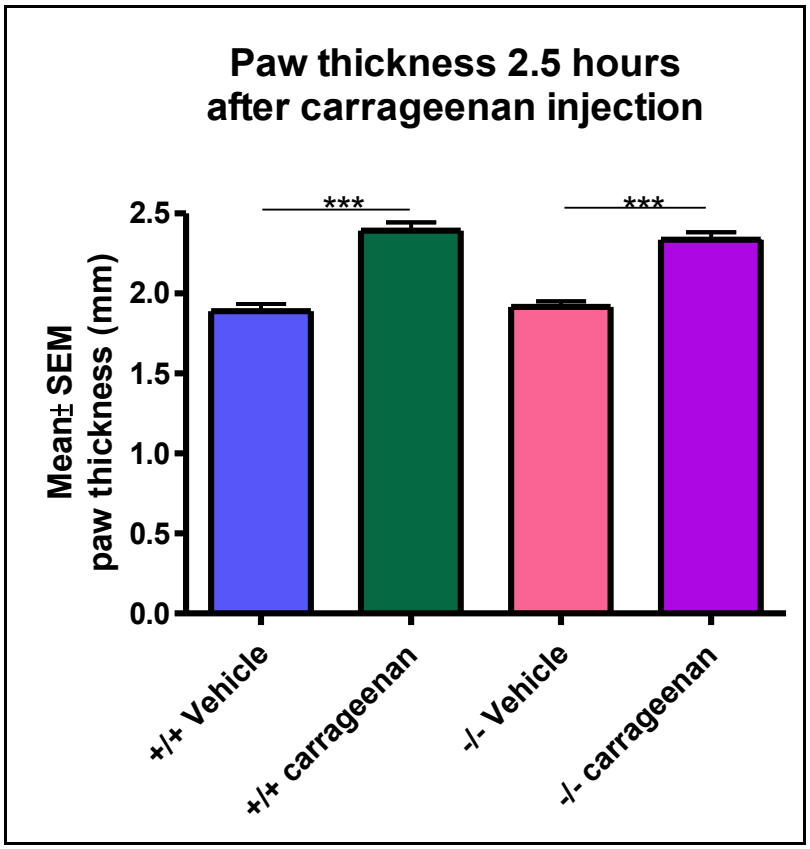


Fig 3: Paw thickness after carrageenan injection. 1 way ANOVA followed by Tukey post test

Paw thickness was significantly different between carrageenan-injected and vehicle-injected paw 2.5 hours after carrageenan injection, indicating the occurrence of inflammation-induced edema. No significant difference can be observed between the two genotypes in this parameter suggesting no differences in edema formation due to RPTPz KO. (Fig 3)

Formalin-induced inflammation test:

-/- mice have shown an impaired sensitivity (a lower score) in the overall course of the test (Fig. 4). This reduced pain sensitivity is significant both in phase 1 (early phase) and phase 2 (late phase) (Fig.5).

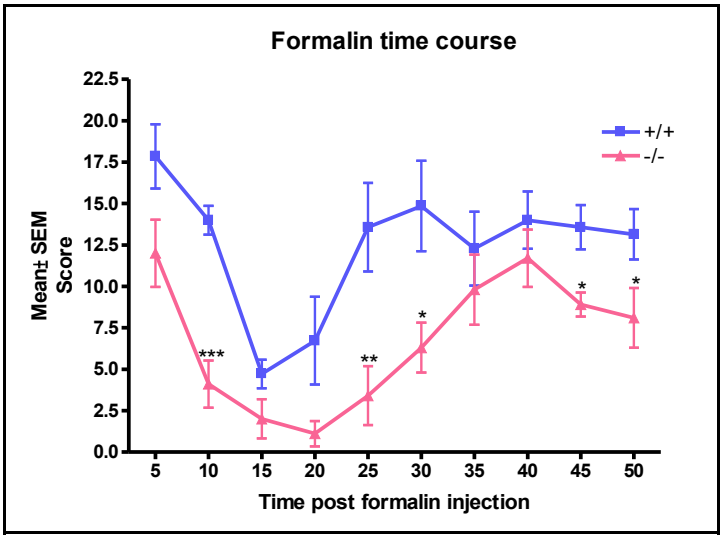


Fig 4: time course of the formalin-induced inflammation test. Student T-test for each time point

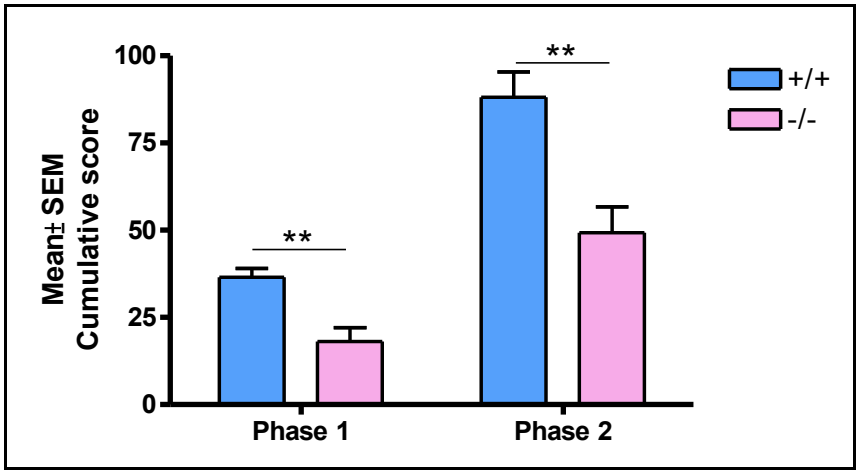


Fig.5: Formalin-induced inflammation test, student t-test.

Mild EAE in RPTPz -/- mice and early curative treatment with Mitoxantrone.

MTX significantly decrease the severity of the pathology from day 10 for +/+ mice and day 13 for-/- mice No significant differences in the course of the disease were observed between -/- and +/+ mice in each sub-group (treated with MTX or Vehicle) (Fig.6).
Interestingly, the pain readouts did not reflect the positive action of the MTX on the disease severity. No difference between MTX or with vehicle treatment was observable on forelimb thermal pain at the end of the experiment. EAE induces an increase in the time latency between recruitment and sacrifice of the animals (Fig 7).

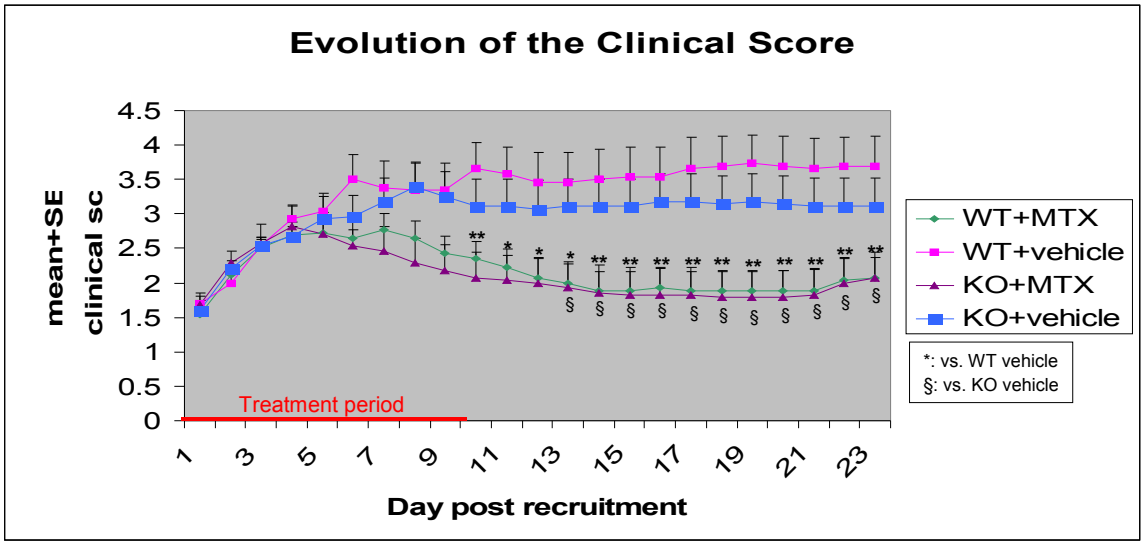


Fig.6: Evolution of the clinical score during the short mild EAE.

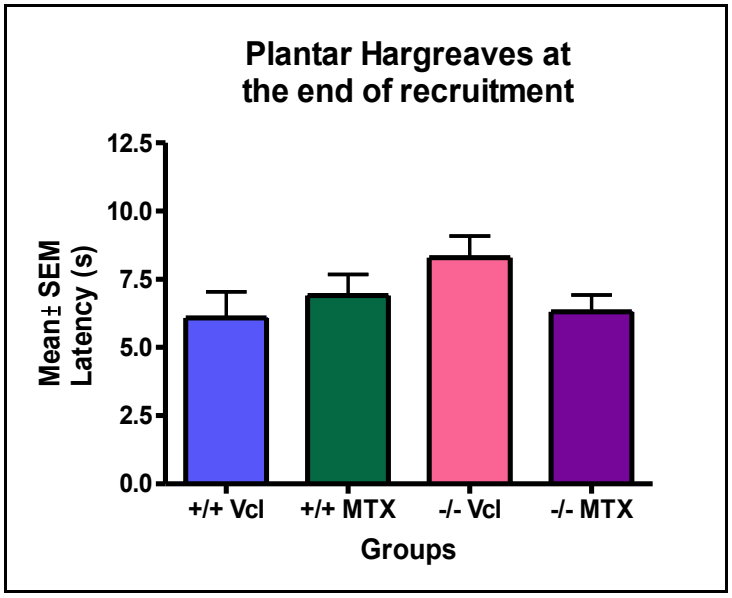
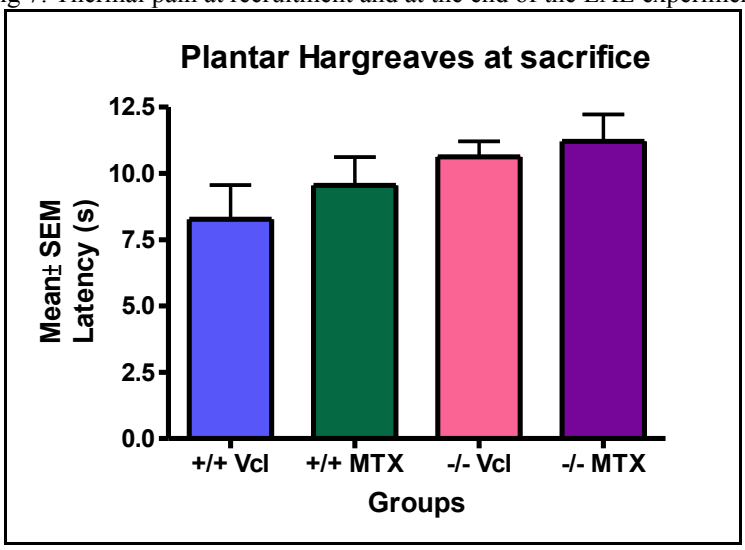


Fig 7: Thermal pain at recruitment and at the end of the EAE experiment



DISCUSSION

The data obtained with the assessment of inflammatory pain reaction in RPTPz -/- mice confirm its role in nociception.

We observed that the impaired sensitivity in the Formalin-induced inflammation test is probably due to a problem of painful stimulus transmission all along the C-fibers. In fact, the formalin-induced inflammation test can be divided in two different phases (5). The phase 1 (also called early phase) correspond to C-fibers activation due to peripheral stimulus (tissue damage and injection of the solution). The phase 2 (also called late phase) correspond 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. RPTPz KO mice impaired pain sensitivity both in phase 1 and in phase 2 seems to indicate a possible role a RPTPz early in the activation of the nociceptors and C-fibers, but also later during the integration of the nerve signal at the level of the dorsal horn of the spinal cord.

After sensitization of the mice with the carrageenan, RPTPz KO mice show an enhanced hypersensitivity in the Hargreaves' plantar test. It is known that the fibers sensitive to this test are unmyelinated C-fibers. However, the same mice didn't show any hyperalgesia during the Von Frey test where the tactile fibers used are thought to be myelinated Aδ-fibers (6,7).

Alteration of the pain signal differs with the type of fiber used to conduct the nerve impulse and the type of sensitization of the nociceptive fibers.

In disease condition during an EAE, no difference was observed between RPTPz -/- and +/+ mice despite the decrease of the early inflammatory process. But the difference observed between MTX and Vehicle treated groups in the overall course of the disease indicates a possible dichotomy in the role of the MTX between the disease modifying role and the amelioration of the symptomatic consequences of the disease. To assess this hypothesis we will repeat this experiment including more readouts about pain, inflammation and nerve degeneration.

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elenco dei seminari frequentati:

- Apomorphine-induced prepulse inhibition-disruption, an animal model for sensory-motor gated deficiency (schizophrenic behaviour). Held by Dr Adage Tiziana. (Serono).
- TNBS - induced colitis model in mice, On-going validation. Held by Dr Ardissone Vittoria (Serono).
- MRI quantification of knee osteoarthritis. Held by Dr Ladel Christoph (Serono).
- Erythropoietin and its non-erythropoietic derivatives in EAE and peripheral neuropathies. Held by Dr Roberto Bianchi (" Mario Negri" Institute for Pharmacological Research).
- MMP inhibitors and Minocycline derivatives in EAE model. Held by Valeria Muzio (Serono).
- How the brain repairs itself: new therapeutic strategies in inflammatory and degenerative CNS disorders. Held by Gianvito Martino (DIBIT - San Raffaele Hospital).
- "Phenotypic and functional study of human neural cells". Held by Francesca Ruffini's (Istituto San Raffaele).
- Role of Pax2 in Kaposi's sarcoma cells. Held by Stefano Buttiglieri's (Universita' di Torino).
- "The role of dendritic cells in capturing, expanding and disseminating prions". Held by Etienne Levavasseur's (INSERM U712, Paris).
- "Revealing disease-relevant molecular mechanisms: The example of myosin VI". Held by Sarah Vreugde's.
- "Multiple sclerosis, parasites and the hygiene hypothesis". Stefano Sotgiu's Istituto di Clinica Neurologica Universita' di Sassari.

elenco dei corsi frequentati:

- Animal models for the study of neurological diseases: histological, molecular, and biochemical hallmarks. DiMI training Barcelona 26-30th June 2006.
- Immunohistochemistry on tissue and cell culture: photonic and electronic microscopy.

elenco dei congressi frequentati:

- Neurodegenerative Diseases: Molecular Mechanisms in a Functional Genomics Framework. Max Delbrück Center for Molecular Medicine (MDC), Berlin, September 6-9, 2006 (I didn't present my work during these congress)
- Myelin development and function: neuron-glial interactions in jealth and neurological disease. Kloster Irsee, Germany, August 17-21 2005. (I didn't present my work during these congress)