J Neuroimmune Pharmacol (2016) 11:214–226
DOI 10.1007/s11481-015-9649-2

GUEST COMMENTARY

The Second Insubria Autumn School on Neuroimmune Pharmacology: Repurposing Established Drugs for Novel Indications

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Received: 4 December 2015 / Accepted: 9 December 2015 / Published online: 16 December 2015

Abstract The Second Insubria Autumn School on Neuroimmune Pharmacology was held at the University of Insubria in Varese (Italy) in November 16th–20th 2015, under the auspices of the Society on NeuroImmune Pharmacology, the Italian Society of Pharmacology, and the Italian Association for Neuroimmunology. The School was dedicated to the repurposing of established drugs for novel indications, considering the enormous opportunities for drug repositioning provided by the prominent innovative and interdisciplinary approaches peculiar to neuroimmune pharmacology. More than 40 graduate students and postdoctoral fellows from 12 european universities, research institutes and hospitals attended the School, and lectures were given by 20 internationally acknowledged experts in the fields of basic and clinical neurosciences, immunology, and pharmacology. A poster session provided young researchers the opportunity to present their results, and the best poster was given the Journal of Neuroimmune Pharmacology Young Investigator Award.

Keywords Neuroimmune pharmacology · Insubria Autumn School · Drug repurposing

The Second Insubria Autumn School on Neuroimmune Pharmacology was held at the University of Insubria in Varese (Italy) in November 16th–20th 2015, under the auspices of the Society on NeuroImmune Pharmacology (https://s-nip.org/), the Italian Society of Pharmacology (http://www.sifweb.org/), and the Italian Association for Neuroimmunology (http://www.aini.it/). The School took place 4 year after the first edition, which was held in November 2011 (Cosentino and Gendelman 2013), and 2 years after the first Italian NeuroImmune Pharmacology (INIP) Conference sponsored by the Italian Society of Pharmacology and held in November 2013 (Cosentino et al. 2013), both at the University of Insubria, a young italian university which is nonetheless continuously and actively stengthening its tradition of research and teaching in the novel and rapidly growing discipline of neuroimmune pharmacology.

The second edition of the School was dedicated to the repurposing of established drugs for novel indications, a critical issue which is increasingly emerging as a suitable strategy to tackle the productivity gap in the pharmaceutical industry. Indeed, despite the enormous increase in speding over the last several years, productivity in the biopharmaceutical industry, assessed e.g., by the number of new drugs approved per dollar spent, has actually decreased since the mid-1990s (Ashburn and Thor 2004; Munos 2009). In comparison to the *de novo* drug discovery and development process, which usually takes 10–17 years and is burdened with a high attrition, with <10 % overall probability of success, drug repurposing usually takes 3–12 years and benefits from reduced safety and pharmacokinetic uncertainties, thanks to the availability of detailed information on drug tolerability in the clinical setting, as well as on pharmacokinetics and dose range (Ashburn and Thor 2004).

Neuroimmune pharmacology provides enormous opportunities for the repurposing of drugs. Indeed, neuroimmune pharmacology has been defined as a field encompassing "interdisciplinary research in pharmacology, immunology and neuroscience, providing original therapeutic approaches to investigations of the neuroimmune network. The overarching goal of the discipline is to identify novel pharmacological

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targets or to exploit more established medicines for unique indications" (Cosentino and Gendelman 2013). Neuroimmune pharmacology, thanks to its prominent inter/transdisciplinary approaches, provides unprecedented opportunities for drug repurposing in several key therapeutic areas. The program of the Second Insubria Autumn School on Neuroimmune Pharmacology focussed on both classical as well on emerging topics in neuroimmunology, including cancer, multiple sclerosis and other autoimmune disease, neuroinflammation and neurodegeneration, and stress. Dopamine, noradrenaline and adrenaline had the lion's share, in view both of their key role as transmitters linking the nervous and immune systems, as well as of the enourmous amount of directly and indirectly acting pharmacological agents targeting adrenergic and/or dopaminergic pathways, already in clinical use and with a usually good therapeutic index (Sarkar et al. 2013; Cosentino and Marino 2013; Cosentino et al. 2015). Moreover, compelling evidence on the role of peripheral immunity in neuroinflammation and neurodegeneration associated with Parkinson's disease, and the resulting opportunities to exploit immunomodulating approaches to prevent/rescue the neurodegerative damage were also the subject of several valued lectures (Blandini 2013; Kustrimovic et al. 2014; Olson and Gendelman 2015). Indeed, repositioning established drugs for novel purposes benefits patients as well as the health systems, providing novel therapeutic strategies with a usually favourable therapeutic index and expectedly at low price. The potential of dopamine and dopaminergic agents as safe and effective antiangiogenic drugs in cancer is possibly one of the best examples presently available, and attracted a lot of attention during the School (Sarkar et al. 2015). The program of the School was completed by seminars dedicated to the foundations of pharmacodynamics and pharmacokinetics for the neuroimmune pharmacologist, as well as by technical workshops dealing with the nonconventional use of flow cytometry and real time PCR in the study of the neuroimmune network (Kustrimovic et al. 2014).

As a whole, the School was attended by more than 40 graduate students and postdoctoral fellows in medical and biological disciplines from 12 european universities, research institutes and hospitals, who had the opportunity to listen to lectures from 20 internationally acknowledged experts in the fields of basic and clinical neurosciences, immunology, and pharmacology (Fig. 1). The opening addresses of the conference were given by Prof. Howard E. Gendelman, MD, Margaret R. Larson Professor of Infectious Diseases and Internal Medicine, Professor and Chair at the Department of Pharmacology & Experimental Neuroscience of the University of Nebraska Medical Center (USA), and Editor-in-Chief of the Journal of Neuroimmune Pharmacology (JNIP). The School also included a poster session, to provide an opportunity to young researchers to present their results in the context of an international environment. Submitted

Fig. 1 The Second Insubria Autumn School on Neuroimmune Pharmacology at the University of Insubria in Varese (Italy), November 16th–20th 2015. **a** The first announcement of the School. **b** and **c** the awarding of the JNIP Young Investigator Award by Marco Cosentino, on behalf of the Scientific Committee of the School, to Giulia Ambrosi. **d** Some of the speakers and participants in the School

abstracts underwent peer review by members of the Scientific Commitee of the School, and the finally selected abstracts are attached to this editorial (P#01–16). Most of the posters dealt with novel information on dopaminergic and adrenergic pathways in the modulation of innate and adaptive immunity in health and disease (P#01, 06, 09, 10, 12, 14), and with the increasing evidence that peripheral immunity likely provides a major contribution in Parkinson's disease (P#01, 02, 10, 15). Cancer and anticancer immunity was the subject of several studies, including the direct effects of adrenergic and cholinergic agents on cancer cell proliferation (P#04, 06, 07). Dysregulation of cholinergic systems was studied also in multiple sclerosis (P#08), and several investigations addressed various aspects of inflammation in the brain (P#11, 13, 16). The use of adipose sem cells in diabetic neuropathy (P#03) and the role of NPY in wound healing (P#05) also deserved considerable attention. During the School, the speakers were invited to visit the poster session and to assign a score to each poster (speakers with any conflicts of interest were asked to abstain). On this basis, the Scientific Committee of the School finally awarded the poster P#02 by Giulia Ambrosi and coworkers (C. Mondino National Neurological Institute, Pavia, I) with the JNIP Young Investigator Award, established thanks to a generous grant from JNIP, on the condition that the selected poster will be submitted to the JNIP as full paper within 12 months, and it will be transferred upon acceptance of the manuscripts (Fig. 1).

As it happened with the First Insubria Autumn School on Neuroimmune Pharmacology and with the first Italian NeuroImmune Pharmacology Conference, also this second edition of the School probably succeeded in breaking traditional boundaries among established disciplines and academic

departments (Cosentino and Gendelman 2013), providing exciting opportunities for inter- and transdisciplinary confrontation and exchange of knowledge. We are moving now to the 22nd annual scientific conference of the Society on Neuroimmune Pharmacology, which for the first time will be held in Europe, in Krakow (PL) from 6th to 9th of April 2016, with the aim to further establish and develop fruitful international scientific collaborations and exchanges involving both sides of the ocean. As a whole, anyway we might state without too much baldness that neuroimmune pharmacology is increasingly becoming a vibrant and profitable area of basic and clinical research worldwide.

Abstracts accepted for the Poster Session of the Second Insubria Autumn School on Neuroimmune Pharmacology

P#01 - In vitro models for the study of dopaminergic modulation of human CD4+ T lymphocytes ad its relevance in Parkinson's disease

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Peripheral adaptive immunity is involved in the pathogenesis and progression of Parkinson's disease (PD), and dopaminergic agents are the mainstay in the treatment of PD. Dopamine (DA) is a key transmitter in the neuroimmune network, however the immune effects of dopaminergic agents are presently unknown. The aim of the present work was to use in vitro models to investigate the role of DA pathways in CD4+ T lymphocytes, likely contributing to microglial activation resulting in neuroinflammation, finally leading to the neurodegeneration occurring in PD, namely: (i) CD4+ T naïve, T central memory (TCM) and T effector memory (TEM) cells, and their responses to recall antigens; (ii) CD4+ T naïve cells, and their ability to differentiate towards different T helper (Th) lineages (Th1/Th2/Th17), and (iii) CD4+ T regulatory cells (Treg), and their suppressive effects on T effector cells (Teff).

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood by centrifugation, and CD4+ T naïve cells, Treg and Teff were purified by immunomagnetic sorting. Cells were cultured for 48 h or 7 days, as appropriate. Flow cytometric analysis was used to identify CD4+ T naïve/TCM/TEM cells (by staining CD3/CD4/CD45RA/CCR7), or Th1/Th2/Th17 (CD4/IFN-γ/IL-4/IL-17A), as well as to measure Treg-induced inhibition of Teff proliferation (by means of CPD staining).

The recall antigen tetanus toxoid (TTd, 3 μg/ml) decreased T naïve (mean ± SD: −16.3 ± 13.3 %, $n = 8$; $P < 0.001$), and increased TEM cells (33.6 ± 38.2 %, $n = 8$; $P < 0.002$). Monomeric or fibrillar α-synucein (α-syn) (an endogenous protein which might act as a neo-antigen on peripheral immunity in PD), reduced T naïve cells (−12.8 ± 9.6 % and −10.6 ± 9.7 %; in both cases, $n = 8$ and $P < 0.001$) and increased TEM cells (43.8 ± 42.0 % and 46.0 ± 48.1 %; in both cases, $n = 8$ and $P < 0.003$). Co-incubation with either the D1-like DA receptor (DR) agonist SKF-38393, or the D2-like DR agonists pramipexole did not affect T naïve/memory cell frequency, and did not modify the effects of TTd, and monomeric or fibrillar α-syn.

In preliminary experiments ($n = 2$), treatment of CD4+ T naïve cells with IL-12 + neutralizing anti-IL-4 ab increased IFN-γ + cells (Th1) by 15.1 ± 1.4 %, treatment with IL-4 + anti-IFN-γ ab increased IL-4+ cells (Th2) by 19.4 ± 4.4 %, and treatment with IL-1β + IL-6 + TGF-β + anti- IFN-γ ab + anti-IL-4 ab increased IL-17A+ cells (Th17) by 33.4 ± 38.4 %, suggesting that experimental conditions are likely appropriate to study lineage-specific differentiation of CD4+ T naïve cells and the effects of drugs. Experiments with dopaminergic agents on differentiation of CD4+ T naïve cells, as well as experiments on Treg, are still on going.

Studying the effects of dopaminergic agents on CD4+ T cells from PD patients will provide useful data to establish whether current treatments for PD may affect the peripheral immune response and in particular CD4+ T cells, and to what extent such influence might be relevant for treatment response as well as for disease progression.

This study was supported by a grant from Fondazione CARIPLO to Marco Cosentino (Project 2011–0504: Dopaminergic modulation of CD4+ T lymphocytes: relevance for neurodegeneration and neuroprotection in Parkinson's disease - The dopaminergic neuro-immune connection).

P#02 - Evaluation of the progressive changes in the innate and adaptive immunity in a rodent model of Parkinson's disease

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Introduction: inflammation is one of the main etiopathogenic factors in Parkinson's disease (PD), a common neurodegenerative disease caused by degeneration of the nigrostriatal pathway in the brain. Such neurodegenerative process is associated with activation of microglia and astrocytes, the brain resident immune cells, and infiltration of peripheral lymphocytes, especially T cells, in the lesioned areas. Neurodegeneration in PD is progressive and can be recapitulated in animal models. For instance, intrastriatal injection of 6-hydroxydopamine (6-OHDA) in the rat provides gradual neurodegeneration resembling the lesion occurring in humans, and is supposedly paralleled by similar inflammatory processes.

Aims: the aim of this study is to investigate changes in the innate and adaptive immunity in response to the progressive nigrostriatal damage occurring in the 6-OHDA model of PD.
Methods: male Sprague–Dawley rats received a unilateral stereotaxic infusion of 6-OHDA (or vehicle) in the striatum. Rats were sacrificed at different time-points post-surgery. Upon sacrifice blood was drawn from each rat. Brains were collected after perfusion and sliced. The nigrostriatal lesion was assessed by immunohistochemistry for the dopaminergic marker tyrosine hydroxylase (TH) on coronal sections of both striatum and substantia nigra pars compacta (SNc). Immunofluorescent analysis of CD11b, Glial Fibrillary Acidic Protein (GFAP) and CD3 was also performed on SNc-containing slices to determine the activation of microglia, astrocytes, by using the Colburn's scale, and the number of infiltrated lymphocytes, respectively. Peripheral blood samples were used for flow cytometric determination of the most relevant CD4 T cell subsets. Blood samples were lysed, washed and incubated with monoclonal antibodies cocktails before acquisition. The following panels were employed: CD3 + CD4 + CD8-CD45RA- to define CD3 + CD4+ T cells; CD4/CD45RA/CD45RC/CD90 to define naïve and memory T cells as well as recent thymic emigrants. In order to define T regulatory cells (Treg), additional steps of fixation and permeabilization were introduced before incubating with Foxp3 antibody.

Results: In the lesioned SNc of 6-OHDA-operated animals, the activation of astrocytes increased over time reaching maximal levels at 7 days and remaining stable until 14 days post-operation, while activation of microglia reached maximal levels at 14 days. No significant lymphocytes infiltration was observed in the lesioned SNc. In peripheral blood of rats we observed decreased percentage of Treg (CD4 + CD25highFoxp3+) in animals with nigrostriatal lesion in comparison to sham operated rats. Interestingly, 24 h after lesion the percentage of Treg is higher in 6-OHDA-operated animals and then decreases at the following time-points. Other examined subsets of CD4+ T cells were not affected by nigrostriatal lesion as compared to sham-operated animals.
Conclusions: This study represents the preliminary step towards investigating the cross-talk between the peripheral immune system and the development of nigrostriatal neurodegeneration in a preclinical model of PD, with the aim of eventually identifying new therapeutic targets and possibly disease-modifying strategies capable of modulating such bidirectional loop.
This study was supported by a grant from Fondazione CARIPLO to Marco Cosentino (Project 2011–0504: Dopaminergic modulation of CD4+ T lymphocytes: relevance for neurodegeneration and neuroprotection in Parkinson's disease - The dopaminergic neuro-immune connection).

P#03 - Immunomodulatory effects of the intravenous administration of human adipose stem cells and their conditioned medium in an experimental model of painful diabetic neuropathy.

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Painful neuropathy (PN) is a neurological disorder that is a common complication of diabetes mellitus. Stem cell-based therapies are promising strategy for the treatment of PN. In this study we evaluated the effect of mesenchymal stem cells isolated from human adipose tissue (hASC) and their conditioned medium (CM-hASC) on the neuropathic symptomatology and neuroinflammation, in an experimental mouse model of diabetic neuropathy induced by Streptozotocin (STZ, 80 mg/Kg for 3 days, ip). The development of mechanical allodynia after STZ was monitored by using a Dynamic Plantar Aesthesiometer. When allodynia was well established (14 days after STZ), mice were therapeutically treated by intravenous administration with either 1*106 hASC or CM-hASC obtained from 2 × 106 serum-free cultured cells. As control we evaluated the effect on neuropathic pain of the CM obtained from a human fibroblasts cell line (CM-hF). Both hASC and CM-hASC were able to significantly reduce allodynia, on the contrary CM-hF was unable to reverse painful neuropathy. In STZ-mice treated with hASC or CM-hASC the anti-allodynic effect is very rapid in fact after only three hours from therapeutic treatment we observed a slight but significant pain relief, moreover the effect on pain is long lasting, in fact it is still evident 12 weeks from therapeutic treatment. When the pain relief started to decrease (6 weeks after STZ), some mice were treated again with hASC or CM-hASC (2nd treatment) and we observed that the anti-allodynic effect is fully restored. Moreover, treatments were effective also when performed at a very advanced stage of the disease (6 week after STZ). Both hASC and their CM were able to contrast the loss of body weight registered in STZ treated mice. In order to understand the mechanisms at the basis of the observed effects on pain, we started to study the involvement of neuroinflammation evaluating the levels of the cytokines IL-1 and IL-10 in the main stations of pain transmission, such as: sciatic nerve, dorsal root ganglia and spinal cord, 7 days after hASC or CM treatment. In all the nervous tissues obtained from neuropathic mice we observed a proinflammatory profile, characterized by high IL-1 and low IL-10 levels. Both hASC and CM treatments were able to restore a correct pro/antinflammatory cytokine balance. Furthermore it is well known that in diabetes also peripheral immunity is altered and in particular it is present an alteration of lymphocytes toward a T-helper 1 (Th) profile. We confirm the presence of Th1 profile in splenocytes, characterized by high IFN and low IL-10

levels, in STZ-animals. Also in this case treatments were able to restore the Th1/Th2 balance by decreasing IFN and increasing IL-10.

The data obtained in this study suggest that hASC treatment may be a favorable approach for neuropathic pain treatment and indicate that cells may eventually be substituted with their CM moving toward a cell-free therapy.

P#04 - Study on the ability of natural killer cells to recognize carbohydrate antigens

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Oncogenic transformation is often associated with the aberrant glycosylation of cellular proteins. This alteration in the glycan structures can lead to the expression of new carbohydrate antigens, which accumulate in high density, and often in a novel conformation, at the tumor cell surfaces.

A prominent example of these Tumour-Associated Carbohydrate Antigens (TACA) is the mucin-associated Tn antigen (GalNAc -O-Ser/thr), expressed at high levels in many tumours.

TACA can be recognized as non-self by immune cells, through highly specialized receptors, such as the C-type lectin receptors (CLRs), stimulating specific immune responses. CLRs include, among others, proteins whose specificity for N-Acetylgalactosamine (GalNAc) and Galactose (Gal) has been well studied and characterized, such as the hepatic Asialoglycoprotein receptor (ASGPR) and the Macrophage galactose-binding lectin (MGL).

Natural Killer (NK) cells, originally identified for their ability to lyse certain tumours in the absence of previous stimulation, are strongly regulated by oligosaccharides. To date, however, it is not clear if: i) carbohydrate antigens, such as the GalNAc monomer of Tn antigen, can modulate NK cell activity; ii) NK cells express specific Tn antigen receptors, such as the MGL and the ASGPR receptor.

The aim of our study was to investigate the expression of specific receptors, able to bind the GalNAc monomer on the surface of NK cells. For this purpose, residues of GalNAc or galactose (Gal) were first coupled with a fluorescent probe (FITC), then, their ability to bind to lectin receptors were measured by both direct and competitive binding assays.

We also assessed on NK cells the transcription of known genes coding for proteins which bind residues of GalNAc and Gal, such as ASGPR and the MGL, by molecular biology methods (RNA extraction, retrotranscription, and analysis of mRNAs through PCR).

Preliminary binding data showed a small reduction in the fluorescence intensity in the presence of increasing concentrations of unlabeled GalNAc monomer, suggesting the ability of NK cells to bind small carbohydrates. On the other hand, the PCR analysis clearly demonstrated that NK cells do not express neither ASGPR nor MGL receptors.

Taken together these results support the hypothesis that NK cells might express carbohydrate receptors, different from ASGPR and MGL, able to bind residues of GalNAc.

P#05 - NPY plays a central role in a new translational approach favoring limb wound healing

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NPY, a sympathetic neurotransmitter costored with norepinephrine, has emerged as a potent vascular growth factor, upstream of VEGF signaling. It is potently angiogenic by activating its Y1 and Y5 Gi-coupled receptors and previous results showed that platelet derived NPY is critical for sustained capillary angiogenesis in rodent hindlimb ischemia (Tilan et al., Faseb J 2013, 27: 2244–2255). We recently demonstrated that NPY may exert antiinflammatory effects by promoting a Th2 polarization (Buttari et al., Faseb J 2014, 28: 3038–49), thus favoring wound healing, and that NPY reduces the overall transcription of phospholipase C genes, in endothelial cells (Lo Vasco et al., Mol Cell Biochem 2014, 394: 43–52). Our present study was aimed at evaluating a new protocol for the treatment of human lower-extremity ulcers with autologous leukocytes and platelets containing NPY.

Lower-extremity ulcers show a low tendency to spontaneous healing as well as a high rate of complications and recurrence, often leading to a high risk for limb amputation. We assessed:1) the ability of autologous white blood cells and platelets injected at the border of wounds to promote neoangiogenesis, thereby overcoming hypoxemia and allowing limb salvage; 2) the presence of proangiogenic factors, such as VEGF and NPY, in biopsy samples collected periodically from patients.
Methods: Two groups of patients, including 50 patients with acute (18 cases) or chronic (32 cases) limb ischemia each, were treated with conventional therapies or with hyaluronic acid, supplemented with autologous cells collected by a multicell separator Haemonetics MCS + discontinuous flow ®. Leuco-platelet concentrate (LPC) was applied periodically and usually every 2 weeks at the border of the lesions. The response to therapy has been estimated taking into account the reduction in ulcer area and depth, and the presence of regenerative tissue.

The formation of new vessels as well as the presence of pro-angiogenic factors such as NPY and VEGF were

evaluated by immunohistochemistry on biopsy sample and quantitated by morphometric analysis.

Statistical analysis to evaluate the improvement of clinical results between groups was conducted with chi-square or Fisher exact test.

Results: Patients treated with advanced medications, including bioactive products but not with autologous LPC showed not only a longer healing time but also a greater percentage of failures. Samples obtained from LPC-treated subjects show an abundant presence of newly formed capillaries, characterized by a cubic, "reactive endothelium", that was detected near the site of infiltration of the leuco-platelet preparation, with an average number of 16 neocapillaries/mm2. Our data show that the presence of neoangiogenesis correlate with clinical outcome preceding wound healing. The newly formed tissue was abundantly stained by anti-NPY antibody, as well as by anti-VEGF antibody, in particular at the level of endothelial cells and adipocytes.

Conclusion: This is the first report, at the best of our knowledge, showing that NPY, thanks to its stimulatory effect on the migration of endothelial cells and several immune cells, such as monocytes and polymorphonuclear cells, may promote human wound healing, influencing both neoangiogenesis and anti-bacterial activity at the site of the lesion.

P#06 - β-adrenergic system in cancer cells: new targets for old drugs

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The association between stress and cancer has been described and well-studied over time and evidences seem to support that chronic stress increases cancer progression.

In the last years, many clinical and epidemiological studies were performed in order to clarify this association. The catecholamines (CA), adrenaline (A) and noradrenaline (NA), are crucial mediators of stress response, exerting their effects through interaction with α- and β-adrenergic receptors (AR). Persistent signaling of CA directly affects the expression of diverse genes in tumor, mesenchymal and immune cells and potently modulates the activities of various components involved in carcinogenesis. CA level is extraordinarily high in tumor microenvironment and β-AR are largely expressed in multiple malignant cell types. β-blockers, one of the most currently widely prescribed classes of drugs, represent a heterogeneous group of agents with distinct pharmacological properties. Given the high expression of β-AR in tumor cells, and the tight relationship between stress response and cancer progression, a significant amount of epidemiological studies have emerged to clarify the association between β-blockers use and mortality. Growing epidemiological evidences have revealed strong correlations between both progression-free and long-term survival and β-blockers usage in cancer patients.

Given the lack of studies, outlining the influence of β-adrenergic system on cancer cells proliferation, a review was elaborated focused on the following topics: 1) Expression of β1 and β2 receptors in human cancer cell lines; 2) Effect of adrenergic agonists and β-blockers on cancer cells proliferation and 3) Effect of β-blockers upon cancer cells proliferation induced by adrenergic agonists.

In summary: 1) β1 and β2-AR are expressed in all the cancer types included in this review, except in neuroblastoma; 2) The adrenergic agonists are able to increase the proliferation of several types of cancers; 3) The proliferative effect induced by the adrenergic agonists seems to be mediated by both β1 and β2-AR and 4) Binding to β-AR results in a cAMP transient flux which activates two major downstream effector systems: protein kinase A (PKA) and exchange protein activated by cAMP (EPAC). More recently, signalling mediated by β-arrestin was described as having distinct functional and physiological consequences from that mediated by G-proteins. However, "in vitro" studies exploring the underlying mechanisms involved in cellular response to CA through β-AR are lacking.

P#07 - Selective agonists for M2 muscarinic receptors inhibit cell proliferation and survival in human glioblastoma stem cells: possible implications in drug resistance

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Introduction. The involvement of muscarinic receptors in cancer has been largely documented. Recently, we have demonstrated that the activation of M2 muscarinic receptors, by the agonist Arecaidine Propargyl Ester (APE), arrests cell proliferation and induces apoptosis in glioblastoma (GB) cell lines. In the present work, we investigated the effects mediated by M2 receptors in glioblastoma cancer stem cells (GSC), an undifferentiated GB subpopulation characterized by high chemioresistance.

Material and Methods. GB7 cell lines obtained from human biopsies were cultured in Euromed-N supplemented with N2, B27, EGF and FGF. MTT assay and trypan blue staining were used to evaluate cell viability and cell death, respectively. By

means of M2 silencing (by siRNA) and pharmacological competition we confirmed the ability of M2 agonists to selectively bind this receptor subtype. Transcript levels for muscarinic receptors and multidrug efflux pumps (e.g., ATP binding cassette, ABC) were analyzed by RT-PCR analysis.
Results. Our experiments were performed with the M2 agonist APE, the muscarinic orthosteric superagonist Iperoxo and its related dualsteric agonists P-6-Iper and N-8-Iper. In GB7, treatment with the M2 agonist APE (100 μM) decreased cell proliferation in a time and dose dependent manner. Also in GB7 cells, N-8-Iper inhibited cell growth and survival also at lower concentration (12.5 μM). The co-treatment of GB cells with M2 agonists (APE or N-8-iper) and different muscarinic antagonists confirmed that the decreased agonist-induced cell proliferation and survival were dependent on selective activation of M2 receptor. Similarly, the silencing of M2 receptor abolished the M2-mediated agonists effects. Moreover, APE and N-8-Iper decreased the mRNA levels for the ABC drug efflux pumps (C1 and G2).
Conclusions. Our data suggest that M2 receptor agonists represent a new relevant tools to investigate glioblastoma-related mechanisms. Furthermore, the ability of M2 agonists to decrease the drug efflux pumps expression suggests that they may have a role in reducing the GSC chemoresistance, and make them more responsive to conventional drugs (e.g., temozolomide).

P#08 - Cholinergic system alterations in multiple sclerosis: studies in RR-MS patients and in EAE model

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Introduction: Acetylcholine (ACh) modulates the immune system and inflammation by a mechanism identified as "non-neuronal cholinergic anti-inflammatory pathway". Muscarinic and nicotinic receptors expressed by immune cells differently modulate the inflammatory mediators.
Aim: To evaluate whether inflammatory state in MS may be related to cholinergic system dys-function we measured ACh levels, AChE and BuChE activity and expression and pro-inflammatory cytokines production in PBMCs and serum of RR-MS patients and healthy donors (HD). Moreover we evaluated the expression of cholinergic markers in brain and spinal cord of EAE mice.
Methods: ACh and cytokine levels were measured in serum using commercial kits, while serum AChE and BuChE activities were determined by Ellman test. qRT-PCR was performed to determine AChE, BuChE and cytokines mRNA expression. In situ hybridization was used to analyze ChAT, AChE and nicotinic alpha-7 receptor expression in EAE brain and spinal cord. Enzymatic histochemistry techniques and immunolocalization were used to correlate the expression of AChE and BuChE with glial markers.
Results: Lower levels of ACh and higher levels of AChE and BuChE were observed in MS patients than in HD. PHA-stimulated expression of IL-1beta and IL-17 was significantly higher in PBMCs of MS patients compared to HD. In PBMCs of MS patients, PHA plus nicotine co-treatment decrease the expression and production of these cytokines. Interestingly alpha-7 nAChR expression in PBMCs of MS patients was high compared to HD with increasing levels after PHA stimulation. In CNS of EAE mice, ChAT mRNA levels increased in CNS cholinergic areas when compared to control CFA injected animals, whereas AChE and BuChE expression and enzymatic activity decreased. The expression of BuChE increased in glial cells. Conclusions: Our results suggest that the decreased levels of ACh in MS may contribute to exacerbate the inflammatory state in MS. Reestablishment of cholinergic function may contribute to reduce the inflammatory state both in immune system and brain.

This work was supported by FISM (FISM 2013/R/25). MDB fellowship was financed by FISM and Ateneo Sapienza Funds.

P#09 - Dopaminergic receptors on human monocytes and peripheral blood dendritic cells

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Dendritic cells (DC) and monocytes (MO) are members of the mononuclear phagocyte system of antigen presenting cells, which includes different subtypes that vary in their origin, location, migration patterns and specialized immunological roles, and exhibit multiple functions during immune responses. Historically, DC and MO have been grouped together but recent studies showed that they may derive from different progenitors.

Dopamine (DA) acts on immune cells through the interaction with five dopaminergic receptors (DR) playing a physiological role in regulation of immune response. The presence of DR has been reported in human immature monocyte-derived DC as well as on human CD14+CD16+ MO, and recently bone-marrow derived DC were shown to express all DR as well as the machinery necessary to synthesize, store, and degrade DA. DR on DC have been proposed to play a role in a murine experimental allergic encephalomyelitis, the animal model of multiple sclerosis, by regulating the activity of various target T cell populations.

The aim of our study was to examine the expression of DR on human peripheral blood DC (bDC) as well as on circulating MO by means of flow cytometric assay.

Peripheral venous blood samples were collected from healthy volunteers ($n=4$; 3 F 1 M; age [mean ± SD]: 35.3 ± 7.4 years). Phenotyping of DR on bDC and MO was performed by two 5-color flow cytometric analysis, by use a two-step protocol which allowed the identification of all the five DR on classical (CD14 + CD16-), intermediate (CD14 + CD16+), and non-classical (CD14dim CD16++) MO, and on bDC (Lin1-HLADR+), plasmacytoid DC (pDC, LIN1-HLA-DR + CD11c-CD123+) and myeloid DC (mDC, LIN1-HLA-DR + CD11c + CD123low).

Preliminary results show that bDC frequency is (mean ± SEM) 0.7 ± 0.1 % of total mononuclear cells, with pDC and mDC being respectively 34.0 ± 7.6 % and 38.2 ± 7.4 % of total bDC. MO frequency is (mean ± SEM) 6.1 ± 0.3 % of total leukocytes with classical, intermediate and non classical phenotype being respectively 76.0 ± 2.9 %, 9.5 ± 1.5 % and 6.8 ± 1.2 % of total MO.

Staining for DR on bDC as well as pDC and mDC provided so far no evidence for their expression. On the contrary, all five DR seems to be expressed in circulating MO.

DC, MO and their relative subtypes represent a heterogeneous population with different phenotypic properties. No evidence was obtained so far for the expression of DR on bDC as a whole and/or in specific subtypes. Human MO, in particular classical and intermediate MO, express mRNA and surface protein of all five DR while less is known about non classical MO: this may explain why monocyte-derived DC express DR. Functional assays are now needed to assess the relevance of DR on MO as potential targets for therapeutic interventions.

P#10 - Expression of dopaminergic receptors on CD4+ T cells in Parkinson's disease: correlation with disease progression

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Parkinson's disease (PD) is a neurodegenerative disorder characterized by the progressive loss of dopaminergic neurons in the midbrain. Several lines of evidence however increasingly point to CD4+ T lymphocytes infiltrating the brain as contributors to glial activation and neuroinflammation, finally leading to neurodegeneration. Since dopamine is also an established transmitter in the neuroimmune network and CD4+ T cells express dopaminergic receptors (DR), the present study was devised to investigate DR expression on CD4+ T cells from PD patients and its possible correlation of disease severity.

Blood samples were obtained from PD patients ($n=45$; age (mean ± SD): 69.9 ± 9.8 years) and age- and sex-matched healthy subjects (HS) ($n=28$; age 68.8 ± 8.1 years). Expression of DR on CD4+ T cells was assessed by flow cytometry (Kustrimovic N. et al., J Neuroimmune Pharmacol 2014, 9: 302–12). Peripheral blood mononuclear cells were separated from whole blood by density gradient centrifugation, and thereafter CD4+ T cells were isolated by immunomagnetic sorting. Real-time PCR of DR mRNA was performed according to Cosentino M. et al. (Neuroimmunomodulation 2012, 19: 283–92).

Complete blood counts of patients and HS were all within normal limits, however PD patients had less total lymphocytes, in terms of absolute number (−14 %) and percentage (−3.7 %). Reduction of lymphocytes in PD patients was accounted essentially by reduction of CD4+ T cells (on average −22 %; 1012.0 ± 439.1 vs. 793.4 ± 275.8, $P=0.037$). In comparison to CD4+ T cells from HS, cells from PD patients had higher mRNA levels for DR D1 and DR D3, and lower mRNA levels for DR D5 and DR D4. PD patients had lower CD4+ T cells expressing DR D5, both as absolute number and percentage. Correlation analysis between mRNA levels and membrane expression of DR on CD4+ lymphocytes was performed. mRNA levels of DR D2 correlated with absolute number ($P<0.001$) and percentage ($P<0.001$) of CD4+ cells in HS, while in PD patients mRNA levels of DRD3 ($P=0.007$) and DRD5 ($P=0.004$) correlated with percentage of CD4+ cells.

The relationship with disease severity was assessed by dividing PD patients into 3 groups according either to the UPDRS Part III score. DR D1, DR D5 and DR D2 mRNA levels decreased with increasing UPDRS. Similar correlations were observed for DR D1 and DR D5 expression on CD4+ T cell membranes. Decreased DR expression on CD4+ T lymphocytes and its relationship with disease progression deserves consideration, in view of the role of CD4+ T cells in PD as well as of dopaminergic agents in the treatment of the disease. In-depth investigation is strongly warranted to assess the influence of dopaminergic agents on peripheral immunity, and its possible consequences for the therapeutic response as well for progression of PD.

This study was supported by a grant from Fondazione CARIPLO to Marco Cosentino (Project 2011–0504: Dopaminergic modulation of CD4+ T lymphocytes: relevance for neurodegeneration and neuroprotection in Parkinson's disease - The dopaminergic neuro-immune connection).

P#11 - Anti-amyloid beta autoantibodies: treatment implications in cerebral amyloid angiopathy-related inflammation

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Cerebral amyloid angiopathy-related inflammation (CAA-ri) is a rare and aggressive meningoencephalitis characterized by the acute onset of neurological symptoms associated to the radiological evidence of cerebral vasogenic edema (VE) and sulcal superficial siderosis or cortical/subcortical microhemorrhages (MH).

CAA-ri represents a clinical challenge in neurological practice. A definitive diagnosis requires brain biopsy in order to confirm the presence of an inflammatory recruitment associated with amyloid deposits at the level of the affected vessels. However, brain biopsy must be considered a harmful procedure in CAA-ri, because of the high bleeding risk of these cases. This issue has severely affected the opportunity to make a correct diagnosis and treatment, with a negative impact on the patient's outcome. Despite the acute and aggressive course of the disease, sometimes leading to fatal outcomes, CAA-ri represents the most readily immunosuppressive-responsive form of CAA, if diagnosed and treated promptly, highlighting the urgent need of less invasive procedures and biomarkers.

In this perspective, we recently developed an ultrasensitive technique (patent pending) for the dosage of anti Amyloid-beta (anti-Aβ) autoantibodies in cerebrospinal fluid (CSF). The assay allowed to demonstrate an up to 3-fold increased of anti-Aβ antibodies during the acute phase, in association with augmented levels of soluble Aβ40, Aβ42, tau and P-tau, followed by a reduction of both autoantibodies and neurodegenerative markers after remission.

These results enlighten the key role played by anti-Aβ antibodies in the pathogenic mechanisms characterizing the disease, pointing out the dosage of CSF anti-Aβ antibodies as one of the most promising diagnostic biomarker of CAA-ri.

We are currently leading the largest cohort and biobank of CAA-ri, namely iCAβ International Network, a Consortium aimed to the discovery and validation of diagnostic biomarkers, with more than 100 cases recruited world-wide. Through the Network, we are shedding light on the clinical and treatment evolution of CAA-ri, e.g., that the rate of early- or late-recurrence is an extremely rare event, or that, in the case of relapse, VE and MHs are not necessarily affecting the same brain region involved during the first inflammatory episode.

Taking together, these evidences sustain that the abovementioned clinical effects can be positively modulated by decreasing autoantibody titer following immunosuppressive therapy, typically resolving after intravenous pulses of steroids, giving important treatment implications for the management of this challenging disease.

P#12 - Dopaminergic modulation of human neutrophil functions

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Background: Dopamine (DA) is an endogenous neurotransmitter belonging to the catecholamine family, which acts on five different dopaminergic receptors (DRs). Increasing evidence points to DA as critical in the connection between nervous and immune system, leading to the definition of DA as neuro-immunetransmitter.

Among immune cells, polymorphonuclear leukocytes (PMN) are considered to play a pivotal role in several immune-mediated diseases. Litle attention however has been so far dedicated to the possible functional modulation of PMN by DA. This study was aimed to investigate the presence of DR on human PMN and the ability of DA to modulate PMN functions.

Methods: PMN were obtained from venous blood of healthy donors. Apoptosis was measured by flow cytometry; DR

membrane expression was analyzed using flow cytometry; migration was measured by means of microscopic evaluation, and reactive oxygen species (ROS) production by spectrofluorometric assay.

Results: Incubation with DA did not affect the percentage of viable, early and late apoptotic PMN, with the only exception of DA 1 μM which slightly increased late apoptotic cells at 3 h.

All DR were expressed on PMN surface but to a different extent: D1-like DR were expressed on average by 82-89 %, whereas D2-like DR were expressed by 18–58 %. Stimulation with fMLP significantly reduced only the DR D1 expression.

DA 1 μM profoundly reduced fMLP-induced migration (14.9 ± 1.3 μm vs 22.0 ± 2.4 μm with fMLP alone, $n = 5$, $P < 0.01$) and this effect was reverted by the D1-like DR antagonist SCH-23390 (1 μM; 23.2 ± 2.3 μm vs 11.5 ± 1.5 μm, $n = 3$, $P < 0.05$) and not by the D2-like DR antagonist haloperidol (1 μM; 13.7 ± 1.4 μm vs 13.2 ± 0.8 μm, $n = 3$, $P > 0.05$). The D1-like DR agonist SKF-38393 (0.1 μM) reduced fMLP-induced migration (14.9 ± 0.6 μm vs 27.9 ± 2.1 μm, $n = 4$, $P < 0.05$), an effect similar to that exerted by DA. The D2-like DR agonist pramipexol (1 μM) did not affect fMLP-induced migration (21.2 ± 1.8 μm vs 23.0 ± 1.7 μm, $n = 8$, $P > 0.05$).

DA 1 μM also reduced fMLP-induced ROS generation (278.7 ± 57.6 arbitrary fluorescence intensity units (FI) vs 415.3 ± 80.4 FI, $n = 8$, $P = 0.005$) and the effect was reverted by SCH-23390 (440.5 ± 194.8 FI vs 206.5 ± 106.0 FI, $n = 8$, $P < 0.001$) but not by haloperidol (244.6 ± 105.7 FI vs 234.3 ± 98.7 FI, $n = 7$, $P > 0.05$). The effect of DA on fMLP-induced ROS generation was mimicked by the D1-like DR agonist SKF-38393 (189.3 ± 90.1 FI vs 353.9 ± 90.9 FI, $n = 7$, $P < 0.05$). On the contrary, the D2-like DR agonist pramipexol did not affect fMLP-induced ROS generation (286.6 ± 69.4 FI vs 362.7 ± 192.7 FI, $n = 4$, $P > 0.05$).

Conclusion: The present results support the presence and the functional role of DRs on human PMN. To our knowledge, this is the first study showing that the effects of DA are mediated by DRs and in particular that D1-like DR are preferentially involved. D1-like DR agonists should be assessed in the treatment of inflammatory conditions involving PMN.

P#13 - Neuron and microglia cells: different LPS- induced cytokine profile offers a new target for the study of depression

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Many advances have been made to understand the biological and molecular mechanisms underlying depression. Most of the studies on depression were focused on studying the abnormalities in neuronal functioning. Recently, emerging evidence propose an involvement of non-neuronal brain cells in neurologic dysfunction, but this link remains to be thoroughly examined. In particular, microglia, the innate immune cells of the central nervous system, is the most interesting candidate mediator of abnormal brain-immune dialogue in depression. However, even if clinical evidence implicating microglial dysregulation in affective disorders is still limited, it has been demonstrated that enhanced microglial activation and amplified inflammatory cytokine production can impair normal neurologic function. This supports the correlation between inflammatory markers and psychiatric disorders that may be more than merely associative, because inflammation can actually contribute to mental disorders. Given this, the purpose of our study was to better understand the molecular mechanisms underlying neural-immune interaction by investigating the different response of neurons or microglia cells to an immunological challenge.

In doing so, we exposed hippocampal neuronal cells HT22 and N9 microglial cells to lipopolysaccharide (LPS, 100 ng/μl) for 2, 6 or 24 h and investigated the expression of the main pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) induced by LPS stimulation and of IL-18, a multifunctional cytokine that has been shown to regulate the expression of the aforementioned immune-mediators. Neurons and microglia cells showed a different transcriptional activity following LPS, moreover time of exposure to LPS differentially affected the expression of the considered targets. Surprisingly, neuronal cells already responded following a short term exposure to LPS, as microglial cells do. The strong induction of IL-1β, IL-6, TNF-α observed in HT22 cells after a 2 h treatment supported the hypothesis of a greater involvement of neurons in immune activity than expected. On the other hand, a different cytokine activation pattern appeared after 6 h of exposure to LPS in HT22 and N9: the expression of IL-6 and TNF-α was still enhanced only in N9 cells, while in neurons it returned to basal levels. After 24 h, the mRNA levels of the pro-inflammatory cytokines were not affected by LPS in both cell populations. Interestingly, the expression of IL-18 was strongly induced in HT22 cells as early as after a 2 h LPS exposure, while in N9 microglial cells the increase in IL-18 expression was delayed and was present only after 6 h of treatment. This time-dependent response suggests a potential role for IL-18 in mediating the communication between neuronal and microglial cells in the inflammatory response. The different LPS-induced cytokine profile and the specific time of activation in neurons and microglia indicate that both cell types are responsive to an inflammatory stimulus and they may modulate immune functioning by directly affecting cytokines activity. Consequently, these results can be the basis for further study of the interaction between neurons and microglia and for examining the role of microglia in an inflammatory condition, a known determining factor in the pathogenesis of neurodegenerative diseases and psychiatric illnesses, such as depression.

P#14 - Adrenergic modulation of interleukin-8-induced migration in human polymorphonuclear leukocytes

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Background: The catecholamines (CA) noradrenaline (NA) and adrenaline (A) are neurotransmitters and neurohormones in the central nervous system and in peripheral tissues, where they contribute to the integrated response to stressful stimuli. Increased production of CA during chronic stress may be however detrimental. Adrenergic modulation of adaptive immunity has been extensively characterized, while few information exist regarding innate immunity and in particular, about polymorphonuclear leukocytes (PMN), which are key players in acute and chronic inflammatory responses.

We recently reported that human PMN express mRNA for the various subtypes of α1- and α2-, as well as for all the β-adrenoceptors (AR), which are regulated upon cell activation, and that A inhibits PMN migration, CD11b/CD18 expression, and ROS production (Scanzano et al., Inflamm. Res. 2015;64:127–35).

In the present study we investigated the adrenergic modulation of human PMN migration induced by the chemotactic stimulus interleukin (IL)-8. Moreover, we examined the membrane expression of β-AR.

Materials and Methods: Venous blood was obtained from healthy subjects and PMN were isolated after sedimentation on dextran. Cell migration was assessed by optical microscopy and expression of β-AR by flow cytometry.

Results: Spontaneous migration of PMN was (mean ±SD)15±3.3 μm ($n=10$). IL-8 (10 ng/ml) increased migration by 6.6±5.8 μm ($n=10$; $P=0.006$)

A (0.01-1 μM), concentration-dependently reduced the effect of IL-8 reaching the maximum effect at 1 μM (Δ migration: −1.3±1.9 μm, $n=10$; $P<0.001$ vs IL-8 alone). In preliminary experiments, the effect of A was reduced by the α1-AR antagonist prazosin (1 μM) (6.4±7.0 μm, $n=4$; $P=0.075$ vs IL-8+A), but not by the α2-AR antagonist yohimbine (1 μM) (1.2±2.4 μm, $n=5$) or by the β-AR antagonist propranolol (1 μM) (1.3±2.3 μm, $n=5$).

The effect of IL-8 was also reduced by NA (1 μM) (down to 33±33.8 % of IL-8 alone, $n=5$; $P=0.020$) and by the β-AR agonist isoprenaline (1 μM) (29.1±6.6 %, $n=3$; $P=0.004$). Flow cytometric analysis showed that 23.6±8.8 % of total PMN expressed β1-AR and 9.2±5.2 % expressed β2-AR. Analysis of β3-AR expression is presently ongoing.

Conclusions: PMN express both β1- and β2-AR on their membranes. Both A and NA reduced IL-8 induced migration, however pharmacological evidence does not so far allow to identify the relative contribution of α- and/or β-AR-mediated pathways. Further experiments with α- and β-AR selective agonists and antagonists, as well as investigation of the signal transduction mechanism involved, are now required. In addition, the membrane expression of α1-AR (and possibly of α2-AR) on PMN membranes, as well as their possible regulation during cell activation, must be assessed.

P#15 - Evaluation of gender's influence and possible molecular mechanisms in a murine model of Parkinson's disease

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Introduction: Parkinson's disease (PD) is due to progressive degeneration of dopaminergic neurons in the Substantia Nigra Pars Compacta (SNc) and dopamine depletion in the striatum. PD has a multifactorial ethiopathogenesis which includes neuroinflammation and microglia activation that contribute to exacerbating neuronal loss. Epidemiological data suggest a sexual dimorphism in PD, with women showing lower prevalence as compared to males. Such difference might be due to the sexual hormones estrogens.

Goals: The aim of this project is to understand if there are differences in the progression of nigrostriatal damage and in microglia activation among male, female, ovariectomized (OVX) and OVX+Estrogen-treated mice, after intrastriatal injection of dopaminergic neurotoxin 6-hydroxydopamine (6-OHDA), used to establish a mouse model of PD.

Materials and methods: Male, female, OVX and OVX+Estrogen (subcutaneous pellet of 17βestradiol 0.01 mg) mice (C57BL/6 N 9–11 weeks) under general anesthesia (Equithesin,3 mL/Kg, i.p.) received stereotaxic infusion of 6-OHDA (2 μg/μL saline 0.02 % ascorbic acid) into the right striatum. Animals were sacrificed at different time points after surgery (1, 2, 7, 14 days).

Coronal sections containing striatum and SNc were used for immunofluorescent (IF) staining with:

- tyrosine hydroxylase (TH, dopaminergic neurons/terminals marker, used to evaluate nigrostriatal damage) and IBA1 (microglia marker);
- IBA1 and TNF-α or CD206, respectively markers for M1 (cytotoxic) or M2 (cytoprotective) microglial phenotype.

Microglial activation was assessed by using the Colburn's scale, a qualitative score system which evaluates changes in microglia cell density and morphology.

Results: SNc damage was significantly higher in OVX mice as compared to the other experimental groups, both at 7 and

14 days after surgery. Microglial activation at 1 and 2 days after surgery was higher in the SNc as compared to later time-points, in all experimental groups except from OVX mice. As for the OVX group, higher microglial activation was shown at all time-points. The IF reactions indicate a slight microglial shift towards the M2 phenotype in all experimental groups 1 day after surgery In the OVX mice, microglia shows a M1 phenotype at 2, 7, 14 days after surgery. A slight polarization towards the M1 phenotype was detected also in male mice 2 days after surgery. In contrast, OVX+ Estrogen mice show milder activation of microglia, which is polarized towards a M2 phenotype at all time-points.

Conclusion: In our mouse model of PD, the endogenous availability of estrogens in males and females as well as the treatment with estrogen in the OVX group are associated with reduced nigrostriatal damage as compared to OVX. Furthermore, estrogens treatment in the OVX mice restore microglia activation state and M2, cytoprotective, phenotype in the SNc to the same level of males and females. Our data support a role for estrogen as a potential disease-modifying treatment in PD.

P#16 - Does blocking microglial activation prevent tinnitus onset?

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Tinnitus is a phantom auditory perception that can chronicize, affecting quality of life similarly to chronic pain. Although several risk factors (Kim et al. 2015) and brain areas involved (Chen et al. 2015; Lowe and Walton 2015) have been identified, tinnitus etiogenesis has not been clarified. Similarly, in animal models, reliability of tinnitus induction protocols is not optimal: a variable fraction of treated animals do not develop the symptom (Koehler and Shore 2013), and it appears unclear whether tinnitus induced with different experimental protocols is due to similar mechanisms.

Changes in neural plasticity at several stages of the auditory system have been observed in correlation with tinnitus (Henry et al. 2014). Neural plasticity is affected by both neuronal activity and microenvironmental conditions. We focused on glial responses in tinnitus, since these cells affect the microenvironment and their activation is a well-known nervous tissue response to insults. In particular, we studied tinnitus-associated microglia changes in DCN, a structure that is known to be necessary for tinnitus onset (Brozoski and Bauer 2005) and in higher auditory stations.

Tinnitus was induced (in 9/11 treated rats) with unilateral cochlear destruction, noise trauma (7/8) or salicylate (4/6), and microglia was observed with Iba-1 immunofluorescence in rat brain slices by confocal microscopy. Although all treatments were able to induce tinnitus (tested as in Turner 2006), noise trauma and salicylate treatment increased microglial density without inducing activation, whereas cochlear destruction increased both microglia density and activation. After noise trauma, microglia became less uniformly distributed, showing a cluster in the DCN region corresponding to noise trauma frequencies. We then used minocycline, a broad-spectrum tetracycline antibiotic, to inhibit microglia activation. Minocycline, in fact, is able to inhibit microglia polarization toward M1 pro-inflammatory state, by inhibit the expression of M1 marker, such as CD68, CD86, TNF-α, IL-1β, IFN-γ and by reducing the upregulation of the transcription factor NF-kB, (Guasti et al., 2009; Kobayashi et al., 2013)

Microglia activation was necessary for tinnitus onset after cochlear destruction and noise trauma, animals treated with minocycline, starting 2 h after surgery or acoustic trauma did not show microglial activation nor behavioural evidence of tinnitus. On the other hand, tinnitus induced by salicylate was observed regardless minocycline treatment. Microglial activation was also sufficient for tinnitus induction, in fact, after LPS intracranial stereotaxic injection in the DCN, behavioural evidence of tinnitus was observed in rats.

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Acknowledgments The School was supported by a grant from the Insubria International Summer School Program of the University of Insubria, and it was organized with the contribution of the PhD Courses in Clinical and Experimental Pharmacology and in Clinical and Experimental Medicine and Medical Humanities, thanks to the valuable support of the Center for Research in Medical Pharmacology (Director: Prof. Marco Cosentino). The valuable collaboration of Mrs. Paola Gervasini, Dr. Monica Pinoli and Dr. Angela Scanzano, who took the responsibility of the Organizing Secretariat, is gratefully acknowledged.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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