



Shedding New Light on Spinal Cord Injury via a Spatio-Temporal Proteomic and Physiological Approaches

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Abstract

Spinal Cord Injury (SCI) represents currently incurable disorder of the CNS often accompanied by permanent health consequences-disability. In order to mimic a SCI, a balloon-compressive technique was performed at thoracic Th8-9 spinal level in adult rat. Shot-gun proteomic was used to identify proteins in each spinal cord segment-derived conditioned medium along the rostral-caudal axis after SCI with time course. In addition, 3D MALDI Imaging, tissue micro-proteomics were undertaken and combined with confocal imaging, *in-vitro* and *in-vivo* functional testing. Our data clearly described the spatial and temporal events which developed in acute phase after SCI. While we found some similarities in the segments above and below the lesion, the caudal segment has been identified as the therapeutic target. We then assessed in a rat SCI model the *in vivo* impact of a sustained RhoA inhibitor administered *in situ* via functionalized- alginate scaffold. In order to decipher the underlying molecular mechanisms involved in such a process, an *in vitro* neuroproteomic-systems biology platform was developed. Here, the pan-proteomic profile of the Dorsal Root Ganglia (DRG) cell line ND7/23 DRG was assessed in a large array of culture conditions using RhoA i and conditioned media obtained from SCI *ex-vivo* derived spinal cord slices. A fine mapping of the spatio-temporal molecular events of the RhoA i treatment in SCI was performed. The data obtained allow a better understanding of regeneration induced above and below the lesion site. Results notably showed a time- dependent alteration of the transcription factors profile along with the synthesis of growth cone-related factors (receptors, ligands, and signaling pathways) in RhoA i treated DRG cells. Furthermore, we demonstrate the inflammatory response process involvement via immunoglobulin's by binding to their specific receptors on the DRG cells upon neurite outgrowth initiation and thus modulating the neurite outgrowth process. We then validate our results by an *in vivo* proteomic studies along the spinal cord segments. Taken together, we expand knowledge on SCI proteins involved in inflammation, neurotransmission, immune response, re growth and repair, and other local responses to injury.

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Introduction

Spinal Cord Injury (SCI) is a devastating medical condition including irreversible damage to the Central Nervous System (CNS). Traumatic SCI can lead to paralysis with complete or partial loss of neurological function below the injury site (Figure 1). The overall impact of SCI on both the patient and the society depends on a range of factors, including: the age of patient, the extent of the injury, the availability and timing of appropriate health care, services and the environment in which the person lives (physical, social, economic and attitudinal). Symptomatology treatment and physiotherapy care of patients with spinal cord injury requires high financial costs not only for the patient himself and family, but also for society. Statistics indicate the U.S. annual growth of 11000 patients, which represents approximately 1.3 million people with spinal cord injury. Injuries impose heavy economic costs on society, particularly as they often affect young population and severely limit their productivity and quality of life. The economic costs for health care of 25 years old patient are around \$3 mil U.S. In the European Union there are about five hundred thousand people affected by SCI. The incidence of SCI in France is roughly 20 cases per million habitants per year or 934 cases per year [1]. In Europe the most commonly injured age group is 16 years to 34 years (43%), followed by 31 years old to 45 years old (28%) [2]. Traumatic SCI can result from several different causes such

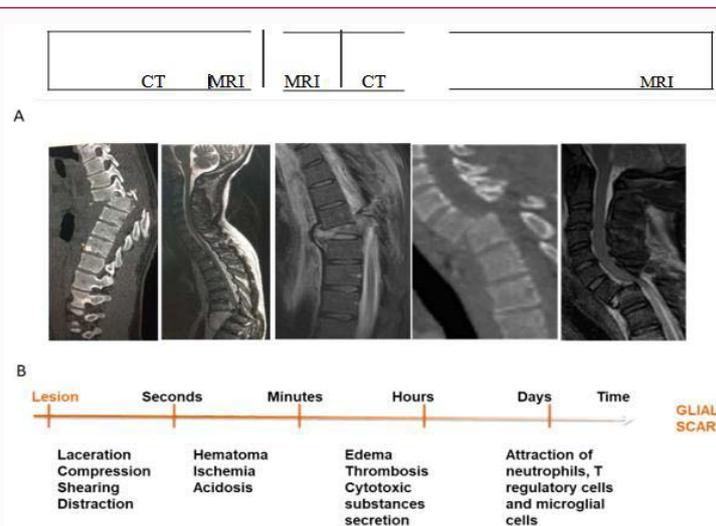


Figure 1: A) Pictures of human spinal cord injuries i.e. in the following order, CT and MRI of dorsal dislocation, then MRI of dorsal fracture with significant displacement of the posterior wall, then CT and MRI cervical dislocation. B) Primary lesion and its secondary extension over time.

as road traffic crashes, falls, and violence. Nowadays, the increased incidence of trauma may be related to popular sports such as ice hockey, American football, and rugby, horse riding and diving. SCI can be induced also by non-traumatic causes such as, communicable diseases: tuberculosis, Human Immunodeficiency Virus (HIV), or non-communicable diseases: osteoarthritis leading to spinal stenosis, cardiovascular disease, nutritional deficiencies, neural tube defects, vitamin B12 deficiency and complication of medical care. The neurological outcomes depends on the range of damaged neuronal populations at the injury site, the level of disconnection of ascending and descending neuronal pathways, the secondary damage (edema, inflammation, ischemia) and the activation of regenerative processes (endogenous production of tropic factors, revascularization) [3]. Each patient's experience is unique in terms of the site of the injury and its severity. Thus, how a spinal cord injury impacts a person's life is highly individualized. Injuries to the upper segments of the spine are most devastating, with the losing functions in the arms and legs, as well as bowel, bladder, chest, abdominal, and diaphragm. While, the Injuries occurring in the lower segments of the spine are mainly limiting movement and sensation in the lower body. The complexity of the nervous system, the varied nature of SCI, and the severity of the loss of function present real and significant hurdles to be overcome to reach total functional restoration.

Therapeutic Strategies

Currently there is no effective therapy available for patients with SCI. The new challenge facing researchers is to develop effective strategies to prevent further tissue loss, maintain the health of living cells and replace cells that have died, grow axons and reestablish synapses that restore the neural circuits required for functional recovery. A key factor for effective therapy is the elucidation of the distinct phases of SCI and the cellular and molecular events underlying them. The most promising outcomes were obtained during the treatment of acute SCI (hours to days after injury) which is directly linked to the trauma and results in neurodegeneration with cell death at the lesion site. Diverse groups of cells and molecules from the nervous, immune, and vascular systems are involved. Most participating cells reside in the spinal cord, but others are transmitted to the site of injury from the circulatory system. Thus, after primary

trauma, cellular and molecular inflammatory cascades are set-up causing activation of resident microglia and astrocytes, infiltration of innate immune cells as lymphocytes and monocytes. The local release of cytokines and chemokines by microglia, macrophages and neurons induces a particular environment that can be either neurotoxic or neurotrophic. During acute phase, macrophages phagocyte cell debris and glial scar protects healthy tissue [3]. Chronic inflammatory processes (weeks after trauma) lead to aberrant tissue remodeling and nerve tissue dysfunction. Various cellular and molecular events designed to heal the injury can paradoxically lead to further neuronal injury or death. The site of injury may spread to adjacent areas of the spinal cord, sometimes extending four spinal segments above and below the initial site. The affected area of injury markedly expands, becomes filled with immune cells, and a "scar" is formed [3].

Unlike peripheral nerves, the mature mammalian central nervous system has a limited regenerative capacity due to the inflammatory response, inhibitory molecules and scar tissue. Different molecules and therapies have been used by many laboratories to preserve healthy tissue, stimulate and reactivate spared tissue and to promote neuronal survival and axonal outgrowth. Injury to the spinal cord leads to an acute inflammatory response [4], that is primordial to preserve tissue homeostasis and therefore in parallel regulates inflammation by the production of anti-inflammatory molecules [5]. One of the approved clinical treatments for SCI is administration of methyl prednisolone in order to modulate inflammation. However, high-dose of this drug is often associated with severe immuno suppression and side effects, such as pulmonary or urinary tract infections [6,7]. Among different mono-therapies, more complex-cellular therapy has several advantages targeting multiple aims: to bridge cavities or cysts, to replace dead cells, to create favorable environment to allow axonal regeneration [5-7]. Transplantation of olfactory unsheathing cells, embryonic stem cells, adult stem cells (MSCs, NPCs), or activated macrophages [6] have been studied to answer to these aims. Molecular therapy is used to protect neurons from secondary process, to promote axon growth and to enhance conduction. Different types of molecules are used as erythropoietin and minocycline (neuroprotective effect), growth factors (BDNF, GDNF, NGF, NT-3), chondroitinase ABC to eliminate Chondroitin Sulfate Proteoglycans (CSPG) with the major component NG2 which inhibit the regeneration of damaged

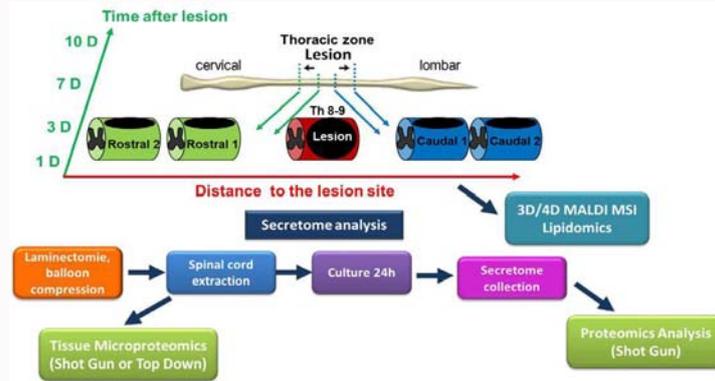


Figure 2: Workflow of the global spatial temporal OMICs study on rat SCI.

axons [8,9], nogo-A is one of several neurite growth inhibitory molecules. There by, Nogo neutralizing antibodies or blockers of the post-receptors components RhoA are used to improve long-distance axon regeneration and sprouting [10]. Rho pathway is important to control the neuronal response after CNS injury. Drug called cethrin that blocks activation of Rho is actually in phase I/IIa clinical trials [11]. However among all these molecules and therapies currently used to ameliorate neuroprotection, neurite outgrowth or to reduce inflammation none of them allow a total understanding of the inflammation mechanism in the entire spinal cord to target specific segment at appropriate time for SCI treatment. A lack in the understanding of the molecular cross-talk occurring between cells at the lesion site and in the adjacent segments needs to be investigated. Such study could uncover molecular targets taking into account both spatial and temporal information.

Molecular Investigation of SCI

Such investigation could be performed by a proteomic approach which could be linked to cellular and physiological studies as well as to a global Regeneration-Activated Gene (RAG) investigation. Mass Spectrometry (MS) plays a central role among proteomic approaches. Several developments allow fast identification and relative quantification through label free quantification methods to thousands of proteins allowing now to identify proteins of lower abundance as cytokines and chemokines. MS is highly used in neuroscience to discover biomarker candidates and also to study the differential expression of proteins at any given time in a proteome of pathological tissue and then compared with the pattern of healthy one [12]. Several groups studied SCI using 2-D separation followed by MS/MS to identify proteins after SCI [13-15]. For example, Ding et al. [15], have shown the up-regulation of 30 protein spot for whole tissue five days after SCI that are involved in stress response, lipid and protein degeneration and neural survival and regeneration. They have shown also the expression of 11-zinc finger protein and glypican which may be involved in the neurite and regeneration inhibition. Kang et al. [14], demonstrated by analyzing tissues from the lesion site 24 hours after injury an over-expression of 39 proteins such as neurofilament light chain, annexin 5, peripherin, apolipoprotein A and an 21 proteins with an under-expression Yan et al. [13], described by the analysis of tissue from the lesion site 31 proteins such as Heat Shock Protein (HSP70-1B) over-expressed at 1 day after SCI, septin-7 up-regulated up to day 5 after SCI. However even if in-gel based proteomic study was highly used this approach has inherent limitations such as low reproducibility, poor representation

of low abundant proteins, difficulty with highly acid of basic protein, over size or hydrophobic proteins and the co-migration of different protein in the same spot renders inaccurate the quantification. Other studies analyze Cerebrospinal Fluid (CSF) from rat or human to highlight biomarkers. CSF analyses allow the creation of real time molecular picture after SCI. Lubieniecka et al. [16]. Studied CSF from rat in order to find biomarkers of injury severity after rat SCI. They identified 10 potential biomarkers by LC-MS/MS, and validated 3 of them by Western-blot: 14-3-3 protein zeta/delta (Ywhaz), Inter-alpha-trypsin Inhibitor Heavy Chain H4 (Itih4), and Glutathione Peroxidase 3 (Gpx3). In 2014, Sengupta et al. [17] proceeded with proteomics of CSF derived from secondary phase of SCI in human at two time periods (1 day to 8 days and 15 days to 60 days) after injury.

Spatio-Temporal Proteomic Studies of SCI

Spatio-temporal proteomic approach performed on rat SCI after balloon compression has been recently performed by our group [18] (Figure 2). We combined a global proteomic analysis with 3D molecular mass spectrometry imaging study, time course analysis of immune cells infiltration and cytokine microarrays quantification. The whole spectrum of the data has allowed us to depict a complete scheme along the spinal cord axis of the cellular and molecular sequel of events occurring through the time course of inflammatory process and abortive regeneration. Specific markers for each spinal cord segment at different time points (3, 7 and 10 days) contributing to the biochemical-pathophysiological processes were observed. Surprisingly, segments below the lesion site (caudal segments) host a robust inflammatory process accompanied by the local synthesis of neuroprotective and regenerative molecules [18]. We demonstrate that the caudal segment adjacent to the lesion site possesses, at least temporarily, all the intrinsic components/features that may allow axonal regeneration. Such a caudal-to-rostral altered regenerative potential is likely hampered by inhibitory signals such as glycans that are abundantly detected or even secreted at the lesion site. Among the proteins from rostral and lesion segments, some are related to chemokines, cytokines or to neurogenesis factors. In contrast, proteins from caudal segments are more related to neurocan, aggrecan, brevican but also to RhoA pathway and to immunoglobulin's [18]. The Conditioned Media (CM) from each spinal segment were used *in vitro*, for culturing microglial BV2 cell lines and DRGs explants, showing a lesion site-dependent impact on microglia activation and DRGs neurite outgrowth. In addition, while naive BV2 cells exhibited insignificant staining for CX3CR1 receptor, the level of CX3CR1 was strongly enhanced in some BV2 cells after their stimulation by CM

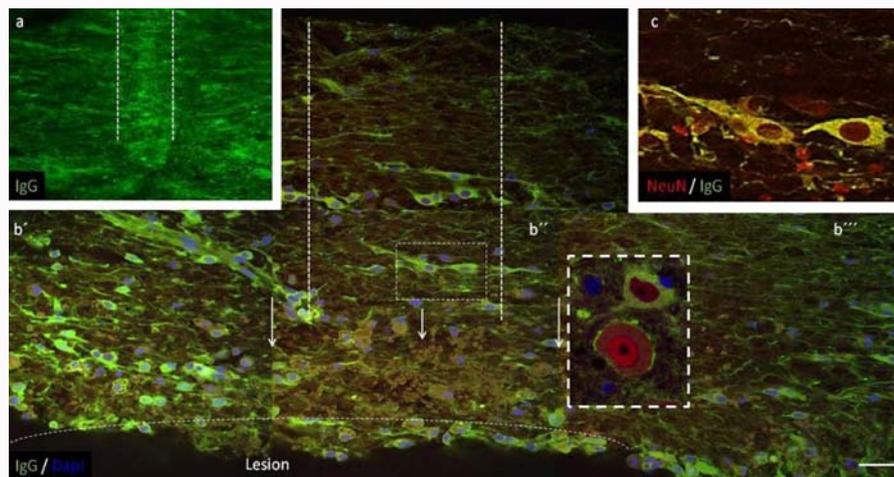


Figure 3: Intra spinal delivery of anti-CD-20 antibody in rats surviving 3 days. (a) Identification of anti-CD-20 delivery tract within the spinal cord tissue labeled with goat-anti rat IgG (green). Note that there is no visible depletion of IgG labeling. (b, b', b'', b''') composite of four detailed figures showing the region adjacent to the lesion, where delivery of CD-20 was performed (outlined by two bars) and is accompanied with infiltrated erythrocytes (arrows) and neurons expressing IgG. At the injection site as well in the rostro-caudal axis neurons and their processes expressing IgG were detected. (c) Detail of boxed area from b shows double labeling of anti-IgG and anti NeuN confirmed neuronal expression of IgGs. Scale bar =30µm.

collected from SCI. The molecular data might correlate with different polarization of activated microglia and macrophages along the rostro-caudal axis following acute injury. This was partially confirmed *in vivo* by mapping CX3CR1 receptor distribution, revealing higher expression in the rostral segment, with potential neuroprotective action [18,19]. We also established that when considering different time course of the SCI, the cytokines production between rostral and caudal segments appear different by means of qualitative and quantitative properties. At three days, cytokines allowing attracting T regulator lymphocytes have been detected in rostral but not in caudal segments which is line with the immunocytochemical detection of these cells only in rostral segment. These cells expressing CCL20 were found with a certain delay, at 7 days in caudal segments. These data clearly established a discrepancy in nature of cells and cytokines production between rostral and caudal segments [18,19]. Taken together these data suggest that regionalization in terms of inflammatory and neurotrophic responses may occur between rostral and caudal segments in acute SCI.

RhoA Inhibitor Therapeutic Strategy

We thus decided to evaluate each of the specific component found in caudal segment that can hampered the neurogenesis process. For this purpose, we assessed in a rat SCI model the *in vivo* impact of a sustained RhoA inhibitor treatment administered *in situ* via a functionalized alginate scaffold. The clinical therapeutic effects were detected only at the early post-injury time points and after overall survival were accompanied by a dramatic increase of synaptic contacts on outgrowing neuritis. In order to decipher the underlying molecular mechanisms involved in such a process, an *in vitro* neuroproteomic-systems biology platform was developed in which the pan-proteomic profile of the Dorsal Root Ganglia (DRG) cell line ND7/23 DRG was assessed in a large array of culture conditions using RhoA inhibitors and/or conditioned media obtained from SCI *ex-vivo* derived spinal cord slices. A fine mapping of the spatio-temporal molecular events after the treatment in SCI was performed. The data obtained allow a better understanding of the mechanisms induced on both sides of the lesion site. Results notably showed a time-dependent alteration of the transcription factors profile along with the synthesis of growth con-

related factors (receptors, ligands, and signaling pathways) in treated DRG cells [20]. Furthermore, we demonstrate the inflammatory response process involvement via immunoglobulin's by binding to their FcγII/III receptors on the DRG cells upon neurite outgrowth initiation and thus modulating the neurite outgrowth process. Of interest, our data indicate that the treatment at late time points led to an inhibition of several factors implicated in neurogenesis reflecting a global inhibition status as observed *in vivo* at 2 weeks following SCI. We demonstrate that RhoA inhibitor treatment is useful to initiate the neurogenesis process at acute early time points which necessitates the need for a second treatment intervention at chronic time points to facilitate and enhance synaptic connections and promote neuronal reconnections.

The IgG involvement in SCI

The last major point that we have evidenced is the presence of immunoglobulin's following SCI at early stages of lesion. We focus our interest on IgGs through spatio-temporal proteomic analyses along the spinal cord after SCI in secretome in order to determine the time window of their production, their function and their cellular origin in the pathology. Proteomic study of secreted factors demonstrates that inflammatory response starts early, at lesion site, only 12h after injury, and is characterized by accumulation of IgGs, cytokines and activation of Rho-Rock pathway. This inflammation spreads to caudal 1 segment, located just below the lesion, between 12 and 24h after injury, leading to inhibition of neurite outgrowth. Afterwards, a clear segmentation appears between rostral and caudal segments of the lesion. The IgGs which have been detected even no SCI occurs in spinal cord, followed the time course and the spatial repartition with presence of IgG1 and IgG2 subclasses (a, b, c). IgG1 is clearly mostly abundant at 12h and switch in time course to IgG2a at 24h then remain predominant during 3, 7 and 10 days after SCI. RhoA inhibitor treatment influence the IgG switching to IgG2c. We have established that IgGs are found in neurons and astrocytes (neural origin) by co-localization immunofluorescence study with specific anti-IgG, anti-NeuN and anti-GFAP antibodies. While *in vitro* studies have revealed the neural origin of IgGs, it is necessary to clarify this also *in vivo*. Treatment with anti-CD20, did not affect the

neural detection of these IgG [18, 20] (Figure 3). Therefore, our work demonstrates that acute inflammation after SCI is a key phenomenon, and any therapeutic tool has to be used as early as the inflammation spreading is initiated. These tentative results on immunoglobulin production in CNS offer a new avenue to study immune response in CNS and neuro inflammatory diseases.

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