Project Periodic Report:

Publishable Summary

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Periodic report  1st □  2nd □  3rd □  4th □  5th □

Period covered  From 01/01/2014 to 31/12/2014

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NOX Enzymes as Mediators of Inflammation-
Triggered Neurodegeneration: Modulating NOX Enzymes as Novel Therapies

The global aim of NEURINOX is to develop novel therapeutic targets for the treatment of inflammatory neurodegenerative diseases (ND). The NEURINOX project focuses on NOX, based on the fact that NOX enzymes are key mediators of neuroinflammatory processes and that modulation of NOX activity represents a powerful target for the development of therapies against neurodegenerative diseases.

To achieve this aim, the NEURINOX project is implementing a multi-disciplinary research programme to attain the following specific scientific and translational objectives:

- To understand how, when and in which cells NOX enzymes control the neuroinflammatory component of ND, and to elucidate their role in the progression of neuroinflammation and ND.
- To validate NOX enzymes as drug targets for neuroinflammatory disorders in animal models and in human samples from patients affected by multiple sclerosis (MS), Amyotrophic lateral sclerosis (ALS) and mesial temporal lobe epilepsy (MTLE).
- To demonstrate the validity of this approach in other rare autoimmune peripheral neuropathies (APN), such as recurrent Guillain Barré Syndrome (rGBS) and chronic inflammatory demyelinating polyneuropathy (CIDP).
- To develop small molecules NOX modulators as therapeutics developed in collaboration with SME partners.

NEURINOX is carrying out the following four main activities focusing on several diseases with strong neuroinflammatory components (ALS, MTLE-HS, MS and other autoimmune neuropathies).

Altogether, this approach should lead to identification and validation of novel drugs for the treatment of the neuroinflammatory aspect of ND and to slow down their development.

General project progress after three years

During the first year: NEURINOX partners initiated a fruitful collaboration through meetings and telephone conferences. Standardised procedures were established and several tools were developed and shared within the consortium: These included in vitro and in vivo models of neuroinflammation, methods of ROS detection and development of new specific inhibitors/activators of NOX enzymes.

In the second period of the project, much progress was made in the identification and characterisation of small molecules modulating NOX activity (both inhibitors and enhancers) (iii) the confirmation of the key role of NOX in genetically modified mice and (iii) importantly in terms of impact, oxidative biomarkers were identified for ALS and MS patients. The pre-clinical evaluation of phenothiazine compounds in an ALS mouse model did not show the expected efficacy. Therefore, the planned clinical trial evaluating the efficacy and safety of a phenothiazine compound in ALS patients was later cancelled and it was decided to instead deepen the studies made on ALS patients and enlarge studies to other neurological diseases like Parkinson, CIDP and Huntington.

Most findings initiated during the first two years were consolidated during the third period leading to manuscript submissions and publications in high ranked journals. The following chapter describes the specific progress and results obtained per type of research activity in the third project year.
Specific project progress in the third project period

The Neurinox project is advancing on different fronts: preclinical models of neuroinflammation, identification, validation and development of drugs targeting NOX and clinical studies.

Animal models

Models of neuroinflammation: Different models of neuroinflammation have been used and developed in the consortium. These include:

(i) Rodent models autoimmune neuroinflammation (peripheral neuritis, and multiple sclerosis), which showed increased severity when NOX2 was deleted and responded favorably to NOX2 agonists;

(ii) Mouse models of aggregation-induced neuroinflammation (ALS, prion disease, idiopathic basal ganglia calcification -a rare disease condition characterized by abnormal deposits of calcium in the brain, cerebellar degeneration and temporal lobe epilepsy). Development of these neurodegenerative pathologies is invariably associated with a massive increase of NOX2 expression in CNS tissue. We detected that NOX2 expression was mostly restricted to microglia and represents a hallmark of microgliosis. These pathologies were modelled in NOX2 deficient mice showing a modest improvement of disease in prion infected mice, but no benefit in ALS, while small molecules NOX inhibitors may show a slight benefit on some read-outs. Interestingly NOX2 inhibition leads to decreased tissue oxidation. Identification of affected pathways by proteomics and transcriptomics is ongoing.

Genetically modified mice: Two novel conditional Ncf1 mutant mice were generated. They will allow identifying the role of NOX2-derived ROS in different subsets of cells during the neuroinflammatory process, including microglia, T cells or neutrophils. They are now ready to be shared between partners to address the questions specific to each disease model.

Drug discovery

Redoxis and GenKyoTex, the two SMEs, have progressed on the optimisation of NOX activators and NOX1 inhibitors respectively. A number of molecules with drug-like characteristics and high potency in in vitro assays are now available. NOX2 enhancers have been used in above-mentioned models of autoimmune inflammation and ex vivo on T cells carrying the Ncf1 mutation.

Patient studies

A number of prospective clinical studies involving MS and ALS patients have been performed and are currently ongoing. This involves genetic studies around NOX related genes and identified ROS regulators, measurement of oxidative biomarkers in cerebrospinal fluid (CSF) samples of MS patients and controls and follow-up of patients. NOX2 activity was measured in blood from ALS, MS and CIPD patients showing that (i) NOX2 activity is increased following therapeutic challenges for CIPD and MS patients and (ii) a strong correlation between survival of ALS patients and ROS production was observed (Fig. 1). The mechanisms underlying these observations are currently analysed using EBV-immortalised lymphocytes from the ALS patients carrying different ALS-causing mutations. In MS patients, different isoprostanes were measured in cerebrospinal fluid and plasma of patients with different forms of MS and before and after treatment with the glucocorticoid drug Methylprednisolone and natalizumab, a therapeutic humanised monoclonal antibody Figure 2. Our data show that specific isoprostanes are surrogate biomarkers of MS progression.
Survival of ALS patients

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<th>% survival</th>
<th>Low NOX2 activity</th>
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\[ p < 0.05 \]

Figure 1 – NOX2 activity

NOX2 activity was measured using the Phagoburst™ assay in whole blood from 83 patients and 83 healthy controls. No difference was observed between controls and patients. However, inside the patient groups, individuals with NOX2 activity under the median of the group (low NOX2 activity) showed significant increased survival compared to patients with NOX2 activity above the median (high NOX2 activity).

Figure 2 - Natalizumab treatment

Natalizumab treatment decreases PGF2α in CSF of sporadic and primary progressive MS patients. Results are shown for the specific F2-IP 5-series: 5(R)-iPF2α (n = 12) before (baseline) and after treatment with natalizumab (60 weeks). Data show results for individual subjects and mean ± SEM. Open circle symbols represent values at or below detection limit. Dotted line represents the average limit of detection (LOD) for each product. Statistical significance was determined by Wilcoxon matched-pairs signed rank test.

Conclusions to date regarding the role of NOX in ND and impact on future research

Most evidence points towards NOX2 as an essential regulator of neuroinflammation. Primarily in the circulation, NOX2-derived ROS are essential to control T-cell activation. Upon NOX2 dysfunction, T cells remain activated and are causative of autoimmune demyelinating diseases. Secondly, NOX2 is associated with the activation state of microglial cells in the phagocytes of the CNS. In physiological CNS, NOX2 is barely detectable. After CNS insult, microglial NOX2 is induced controlling an active state in which microglial cells display a protective phenotype (alternative activation) in order to clear debris and support neuronal survival. However, if the inflammatory stimulus persists, constant elevated levels of NOX2-derived ROS contribute to neuronal dysfunction. High expression of NOX2 is thus a common hallmark of ND.

The findings on the correlation of disease severity of both ALS and MS with NOX2 activity and oxidation status are respectively encouraging us to broaden our scope to other neurodegenerative diseases, such as Parkinson’s, chronic inflammatory peripheral neurites, and Huntington’s disease. The pathways involved in low/high oxidation status and severity will be addressed using both preclinical and clinical material. In addition, the role of other CNS NOX isoforms NOX1 and NOX4 will be evaluated in neuroinflammation.

In order to provide visibility to the public, industry community and peers as well as to the scientific community on the work progress made within NEURINOX as well as the work plan till the end of the project, a symposium is being organized on 23 and 24 September 2015 at MBC in Turin, Italy. For more information, please visit the project public website at http://www.neurinox.eu/page/news-and-events.php.
This event will be the opportunity to also bring forward to the EC, scientific and industry communities the need for future research/funding and to follow-up with the stakeholders interested in the NEURINOX research till the end of the project.

Description of the expected final results and potential impact and use

The NEURINOX consortium is the first concerted effort to gain a comprehensive view of the implication of ROS-generating NOX enzymes in neuroinflammation. NEURINOX contributes to better understand brain dysfunction and more particularly the link between neuroinflammation NOX enzymes and aims at identifying new therapeutic targets for neurodegeneration. A successful demonstration of the benefits of NOX modulating drugs in ALS and MS animal models, and in ALS pre-clinical trials can validate novel high potential therapeutic targets for ALS and also other neurodegenerative diseases. Final expected results are the following:

- A better understanding of common mechanisms of brain diseases, and in particular oxidative stress-mediated neurodegeneration and the link between the different NOX isoforms and neuroinflammation and how their activities control the inflammatory process in neurodegenerative disease.
- Novel oxidation biomarkers, molecular pathways, genes and SNPs correlating with NOX activity and ND for neurodegenerative and autoimmune-mediated neuroinflammation.
- Small molecules (NOX inhibitors and NOX activators), validated by NEURINOX partners in animal models.
- Validation of NOX as viable targets for the development of therapeutics for ALS and potentially many other neuroinflammatory neurodegenerative diseases.

With these results, Neurinox will have the following impacts:

- **Impact on better understanding of brain function.** The Neurinox results will represent a major breakthrough in the understanding and therapeutic approaches of the role of NOX in neuroinflammation in major diseases such as ALS, MS and MTLE-HS. It will also help identifying more general mechanisms that play a role in other ND disorders, hence providing the general understanding of neuroinflammation.
- **Impact on better management of neuroinflammatory and subsequent neurodegenerative diseases.** Costs of ND are huge to society and a breakthrough in the treatment of ND will allow great economic gains. By exploring a new therapeutic approach in ALS and NOX as a new therapeutic target in many other ND, Neurinox contributes to the improvement in clinical management of ND and hence to a reduction of health care costs.
• **Impact on public health.** NOX-mediated therapeutics may be used for slowing progression of neurodegenerative diseases, which are so far untreatable.

• **Impact on ND research and for structuring European research efforts.** Neurinox will bring together international experts in neuroinflammation and related areas and also seek collaboration with other European initiatives in the area of ND research, thus contributing to structuring European research efforts.

• **Impact on competitiveness of European industry.** Neurinox aims at creating new knowledge and translating it into novel therapeutic targets through the involvement of a number of SMEs who are well positioned to derive new therapeutic products from the project results. Through industrial collaboration, the proposed work will increase the competitiveness and boost the innovative capacity of European health-related enterprises, which is a global priority of the FP7 HEALTH programme.

**NEURINOX partners**

The NEURINOX consortium is composed of the following organisations:

• University of Geneva (UNIGE)
• University of Zürich (UZH)
• GenKyoTex SA (GKT)
• Redoxis AB (RDX)
• ARTTIC (ART)
• University of Torino (UT)
• Karolinska Institute (KI)
• Joseph Fourier University (UJF)
• SynapCell (SYN)
• Biomedical Research Foundation Academy of Athens (BRFAA)
• University of Athens (UOA)
• University of Sydney (USYD)
• NEURIX (NEURIX)
• Victor Chang Cardiac Research Institute Limited (VCCRI)

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