EUROPEAN SYNCHROTRON RADIATION FACILITY

INSTALLATION EUROPEENNE DE RAYONNEMENT SYNCHROTRON

ESRF

Experiment Report Form

The double page inside this form is to be filled in by all users or groups of users who have had access to beam time for measurements at the ESRF.

Once completed, the report should be submitted electronically to the User Office via the User Portal:
https://wwws.esrf.fr/misapps/SMISWebClient/protected/welcome.do

Deadlines for submission of Experimental Reports

Experimental reports must be submitted within the period of 3 months after the end of the experiment.

Experiment Report supporting a new proposal ("relevant report")

If you are submitting a proposal for a new project, or to continue a project for which you have previously been allocated beam time, you must submit a report on each of your previous measurement(s):

- even on those carried out close to the proposal submission deadline (it can be a *"preliminary report"),*
- even for experiments whose scientific area is different form the scientific area of the new proposal,
- carried out on CRG beamlines.

You must then register the report(s) as "relevant report(s)" in the new application form for beam time.

Deadlines for submitting a report supporting a new proposal

- 1st March Proposal Round - **5th March**
- 10th September Proposal Round - **13th September**

The Review Committees reserve the right to reject new proposals from groups who have not reported on the use of beam time allocated previously.

Reports on experiments relating to long term projects

Proposers awarded beam time for a long term project are required to submit an interim report at the end of each year, irrespective of the number of shifts of beam time they have used.

Published papers

All users must give proper credit to ESRF staff members and proper mention to ESRF facilities which were essential for the results described in any ensuing publication. Further, they are obliged to send to the Joint ESRF/ ILL library the complete reference and the abstract of all papers appearing in print, and resulting from the use of the ESRF.

Should you wish to make more general comments on the experiment, please note them on the User Evaluation Form, and send both the Report and the Evaluation Form to the User Office.

Instructions for preparing your Report

- fill in a separate form for each project or series of measurements.
- type your report in English.
- include the experiment number to which the report refers.
- make sure that the text, tables and figures fit into the space available.
- if your work is published or is in press, you may prefer to paste in the abstract, and add full reference details. If the abstract is in a language other than English, please include an English translation.

ESRF	**Experiment title:** **Assessing the role of astrocytes in manganese developmental neurotoxicity**	**Experiment number**: LS-3142
Beamline: ID21	**Date of experiment**: from: 15/02/2023 to: 20/02/2023	**Date of report**: 27/02/2023
Shifts: 15	**Local contact(s)**: Luis Colocho	*Received at ESRF:*

Names and affiliations of applicants (* indicates experimentalists):

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Preliminary report (experiment scheduled close to the proposal submission deadline, 10 days):

Background. Manganese (Mn) is both an essential nutrient and a neurotoxic element. While Mn neurotoxicity due to occupational exposures has been known for many years, the developmental neurotoxicity of Mn has only recently begun to be explored. There is growing concern about early life exposure to Mn and developmental neurotoxicity. Mn in drinking water has been associated with lower cognitive performance at all stages of development, including intelligence quotient IQ, and attention-deficit hyperactivity disorders. The mechanisms underlying Mn neurodevelopmental toxicity are still poorly understood but seem to involve glial cells. Neuroinflammation has been studied as a key mechanism in Mn neurotoxicity where astrogliosis can play a central role.

Aim. In this experiment, we have tested the hypothesis that astrocytes can protect against Mn neurotoxicity by decreasing neuronal Mn content. Our samples have consisted in primary rat hippocampal neurons cultured alone, or in co-culture with astrocytes. We have compared Mn concentrations and subcellular distributions in: 1) neurons cultured alone exposed to low levels of Mn; 2) neurons co-cultured with astrocytes exposed to low levels of Mn; 3) neurons cultured alone exposed to high levels of Mn; 4) neurons co-cultured with astrocytes exposed to high levels of Mn.

Methods. Living cells, neurons and astrocytes, were labelled for Golgi apparatus with Cell-Light Golgi GFP (green fluorescent protein) and with Hoechst 33342 (blue fluorescence) for nucleus localization (Figure 1A). Samples were flash-frozen in liquid ethane using a Vitrobot system (FEI). Cryogenic fluorescence microscopy was performed to locate Golgi-apparatus and nucleus in neurons and astrocytes using a Leica cryo-fluorescence microscope recently implemented at the University of Bordeaux. Samples were gently freeze-dried, this method was prefered to fully cryogenic analysis as it greatly facilitated the identification of the cells whith no elemental redistribution at the beam size scale (700 nm). The position of the cells of interest and the morphology was recorded by bright field optical microscopy before XRF imaging at ESRF, on ID21 beamline.

SXRF Imaging. At the beginning of the experiment we encounterd some difficulties. We started to work at 9.8 keV to image Zn too, an element which homesotasis and quantitative distribution could be altered by Mn exposure, but the photon flux was too reduced at this energy to detect the elemental content in cells. So we decreased the energy of the incoming beam to 7.4 keV to have a higher flux (around 10 times higher). We succesfully imaged Mn in neurons and astrocytes, using a dwell time of 100 ms, and a pixel size of 500 nm and a beam size of 700 nm.

Result. During this beamtime we could image and quantify neurons and astrocytes exposed for 24h to 0, 250 µM and 500 µM $MnCl_2$. We could analyze a high number of cells, ~12, for each condition. We could clearly

identify for the first time in primary hippocampal neurons and astrocytes that Mn was mainly located in the Golgi apparatus in both cell types. For example, in cells exposed to 250 µM $MnCl_2$, Mn accumulates in the Golgi apparatus (Figure 1A and D, red arrows), but not only, Mn was also detected in hot-spots along the dendrites (Figure 1B, white arrow).

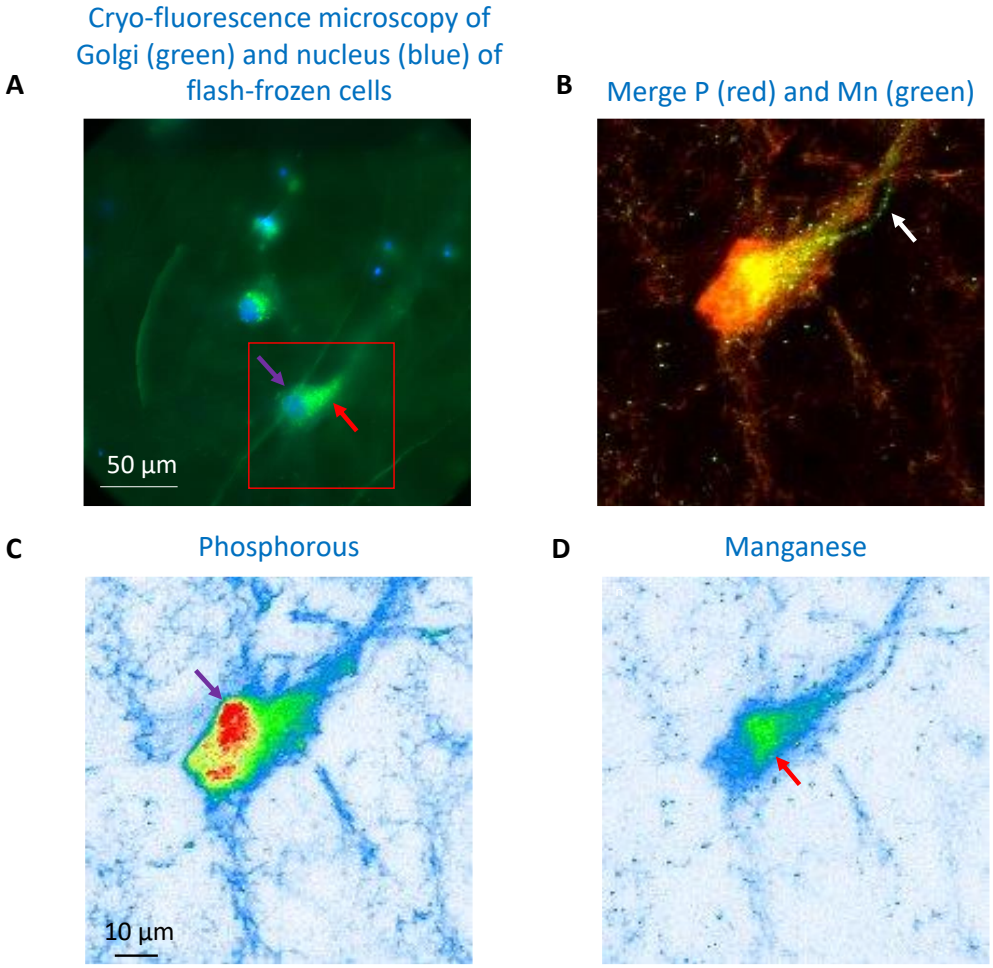

Figure 1. Synchrotron X-ray fluorescence imaging of a single primary rat hippocampal neuron after exposure to 250 µM $MnCl_2$. (A) Merged image of the Golgi apparatus (green, red arrow) and the nucleus (blue, purple arrow) obtained by cryo-fluorescence microscopy on flash-frozen cells. (B) Merged image of P and Mn SXRF maps highlighting Mn hot-spots along dendrites (white arrow). (C) SXRF imaging of phosphorous, an element mainly located in the nucleus (purple arrow). (D) SXRF imaging of manganese showing that this element accumulates in the Golgi apparatus (red arrow). Comparing the cryo-fluorescence images and SXRF maps we can conclude that Mn is mainly located in the Golgi apparatus, and present as well along dendrites, suggesting a synaptic localization to be further investigated.

The AXO standard was used to calibrate the setup and to express element content in ng/mm^2. Mn quantification will now be performed carefully in each region of interest, to compare Mn content in neurons and astrocytes.

Conclusion. The experiment was very successful thanks to ID21 setup. Our first hypothesis was verified experimentally, we could identify the Golgi apparatus as the main site of Mn accumulation in primary hippocampal neurons and astrocytes, for the first time in these cells. This result is of paramount importance to understand the mechanisms of Mn-neurotoxicity, it reveals the Golgi apparatus as a key organelle in Mn-detoxification and toxicity. At low Mn exposure, the Golgi apparatus may serve as a detoxification pathway, while at higher exposure, Golgi apparatus functions could be overwhelmed and altered, such as vesicular trafficking, a critical cellular pathway in neurodegeneration, also leading to redistribution of Mn into synaptic compartments, as suggested by the presence of Mn in hotspots along the dendrites. We now need to quantify the data to explore the hypothesis that astrocytes may protect neurons by decreasing Mn content in neurons, and to study potential quantitative changes in other elements (P, S, Cl, K, Ca, Fe) due to Mn-exposure.

Perspectives. It would be very interesting to identify whether the Mn hot-spots along dendrites are located within synaptic compartments.