

Subcellular distribution of Mn in neurons assessed by synchrotron X-ray Microprobe

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INTRODUCTION Mn-enhanced magnetic resonance imaging (MEMRI) uses Mn^{2+} as contrast agent to study the activity of specific neural network and to enhance neuroarchitecture^{1,2}. Indeed, Mn^{2+} can enter and leave neurons through voltage-gated calcium channel³. Among rodent brain structures, the Mn^{2+} accumulation is particularly strong in the hippocampus where it seems to distribute from the dentate gyrus (DG) to the CA3 pyramidal neurons. To get further insights in Mn toxicity – a major drawback of the technique – and in the transport mechanisms involved in MEMRI, one needs to identify the sub-cellular compartments targeted by Mn. In this study, we characterize the Mn accumulation and sub-cellular distribution in neuroblastoma cells (N2A, a model used to study neurotoxicity) and hippocampal neurons.

MATERIELS AND METHODS *Cells preparation:* N2A (mouse neuroblastoma) were maintained in DMEM supplemented with 10% FBS, 1% NEAA and 100 units/mL penicillin and streptomycin. The primary hippocampal neuronal culture was prepared from neonatal mice (E=18.5). Hippocampus were isolated, chopped and cultivated in DMEM, 10% FBS and 1% PS. After 2h, the culture medium was replaced by Neurobasal, glutamax 1X and B27 50X. Cells were grown at 36.5°C in a humidified atmosphere with 5% CO₂. N2A were split in 60mm dishes every 3-4 days. Eventually, cells were placed on silicon nitride windows in view of synchrotron imaging. To facilitate hippocampal neurons (HN) adhesion, poly-L-lysine coating was added to the silicon nitride window. Differentiation of N2A cells was induced by retinoic acid. N2A differentiate in neuron-like cells producing tubuline and acetylcholine esterase. *Mn administration:* Differentiated N2A were cultured during 5 days before adding Mn in the culture medium. Six Mn concentrations (between 0 and 3000µM) were evaluated. HN were grown 2 days before adding MnCl₂ (either 20 or 100µM). *MRI:* 24h after Mn administration, cells were rinsed twice, trypsinated and centrifuged. The cell pellet was lysed to release Mn (Protocol summarized in Fig. 1A). Cells were studied by a 7T MRI system (Bruker). T1 measurements were obtained using an Inversion Recovery sequence. Mn concentration was determined using $1/T1Mn = 1/T1_0 + r1[Mn]$, where $r1$ was $6.77mM^{-1}.s^{-1}$ and $T1_0$ was 2.87s (Fig. 1B). *Synchrotron imaging:* Cells grown on silicon nitride windows were rinsed, quickly cryofixed at -160°C in N₂-chilled isopentane, and lyophilised prior to synchrotron imaging. The X-ray nanoprobe facility of ESRF was used for multi-elemental X-ray fluorescence (XRF) mapping. The samples were raster-scanned through the focused X-ray beam (150 nm spotsize) with a stepsize of 200 nm (Fig. 2).

RESULTS MRI results show that Mn concentration in N2A increases with the Mn concentration in the culture medium up to a concentration in the medium of 2mM (Fig. 1B). IC₅₀ for N2A was 900µM (data not shown). Fig. 2 shows representative distributions of phosphorus (P), Mn, and iron (Fe) within differentiated N2A cells and HN, grown with and without Mn. In control cells, P is located in the nucleus. Control N2A cells exhibit a diffuse distribution of Mn and Fe mainly located in the perinuclear region. In control HN, Mn is barely detectable and Fe has a granular distribution found in axons and cytoplasm. When exposed to a non toxic concentration of Mn (not shown), Mn is found in the perinuclear region of N2A cells and in the nucleus in HN. Exposing N2A and HN to a toxic dose of Mn (1mM and 100µM respectively) alter the sub-cellular distributions of Fe and Mn: Fe is redistributed towards Mn-rich regions. The average Mn concentration derived from the full x-ray fluorescence map of cells, were: 3 ng/cm² for N2A control, 148 ng/cm² for N2A exposed to 1mM Mn, 0.5 ng/cm² for control HN and 17 ng/cm² for HN added 100µM Mn. Assuming a density of 1 and an average cell thickness of 2 µm, these concentrations can be converted to 0.3 µM, 1.6 µM, 48 nM and 155 nM respectively.

DISCUSSION This study demonstrates that very low Mn concentrations may be detected by MRI (femtomolar) on bulk samples. Although this has been determined in optimal conditions, i.e. free Mn from cell lysate solutions, it seems reasonable to think that, even with Mn stored in cellular structure in tissue, MEMRI sensitivity allows the use of low Mn concentrations. Our results show that cellular Mn localisation change with cellular type and with Mn concentrations. Moreover, at toxic concentrations, Mn interferes with Fe in agreement with previous report⁴. It has been reported that Mn is localised in golgi apparatus of dopaminergic cells (PC12)⁵. In this study, we observed a perinuclear Mn localisation in N2A (non dopaminergic cells) and putative Mn nuclear localisation in HN (mixed catecholamines). *In vivo*, it has been observed that Mn accumulates in the hippocampus, in the olfactory bulb, and in the cerebellum¹. A similarity between these regions is the abundance of gabaergic interneurons. As a perspective, it would thus be interesting to evaluate the cellular distribution of Mn in gabaergic neurons using the same technique.

REFERENCES ¹Aoki et al. Neuroimage 2004. ²Pautler et al Toxicol. Appl. Pharmacol 2004. ³Narita et al. Brain Res. 1990. ⁴Erikson et al. Neurotoxicology 2006. ⁵Carmona et al. ACS Chemical Neurosciences 2010

