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# Methylmercury disrupts the balance between phosphorylated and non-phosphorylated cofilin in primary cultures of mice cerebellar granule cells A proteomic study

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ABSTRACT

Methylmercury is an environmental contaminant that is particularly toxic to the developing central nervous system; cerebellar granule neurons are especially vulnerable. Here, primary cultures of cerebellar granule cells (CGCs) were continuously exposed to methylmercury for up to 16 days *in vitro* (div). LC50 values were 508 ± 199, 345 ± 47, and 243 ± 45 nM after exposure for 6, 11, and 16 div, respectively. Proteins from cultured mouse CGCs were separated by 2DE. Seventy-one protein spots were identified by MALDI-TOF PMF and MALDI-TOF/TOF sequencing. Prolonged exposure to a subcytotoxic concentration of methylmercury significantly increased non-phosphorylated cofilin both in cell protein extracts (1.4-fold; $p<0.01$) and in mitochondrial-enriched fractions (1.7-fold; $p<0.01$). The decrease in P-cofilin induced by methylmercury was concentration-dependent and occurred after different exposure times. The percentage of P-cofilin relative to total cofilin significantly decreased to 49 ± 13% vs. control cells after exposure to 300 nM methylmercury for 5 div. The balance between the phosphorylated and non-phosphorylated form of cofilin regulates actin dynamics and facilitates actin filament turnover. Filamentous actin dynamics and reorganization are responsible of neuron shape change, migration, polarity formation, regulation of synaptic structures and function, and cell apoptosis. An alteration of the complex regulation of the cofilin phosphorylation/dephosphorylation pathway could be envisaged as an underlying mechanism compatible with reported signs of methylmercury-induced neurotoxicity.



## Introduction

Methylmercury is an environmental contaminant which affects the nervous system. The neurotoxic effects of methylmercury are widely known, since two important episodes took place in Japan and Iraq as a result of people ingesting contaminated food (Harada, 1995; Ekino et al., 2007). More recently, concern has grown regarding the deleterious effects of methylmercury consumed through a fish-rich diet, as methylmercury poses a threat to neural development (Castoldi et al., 2001; Clarkson et al., 2003; Strain et al., 2008). Several governmental organizations have issued specific advice on fish consumption for the population at risk (child-bearing women and infants) (www.cfsan.fda.gov; www.ec.europa.eu; www.foodstandards.gov.au).

Clinically, methylmercury-intoxicated people are characterized by neurological dysfunction that induces cerebellar-based ataxia, generalized extremity weakness, and sensory disturbances including speech, vision, and hearing impairment (Clarkson et al., 2003; Sanfeliu et al., 2003; Ekino et al., 2007). All these effects correlate with the loss of neurons from several areas of the brain, among them the cerebellar granule cell (CGC) layer (Korogi et al., 1994; Ekino et al., 2007). In addition to cerebellar neurodegeneration, abnormal migration of neurons in the cerebellum and microtubule formation deficits were observed during neural development and in affected newborn babies (Choi et al., 1978; Castoldi et al., 2000). In mammals, CGCs are the most abundant neurons in the central nervous system and are responsible for excitatory neurotransmission in the cerebellum. They are generated in the embryonic rhombencephalon and migrate from the external to the innermost layer of the cerebellum during development. These cells have *in vivo* a phenotype of glutamatergic neurons that is preserved when they are grown in culture (Sonnewald et al., 2004; Suñol et al., 2008). Primary cultures of CGCs are therefore of great interest in the study of methylmercury neurotoxicity.

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The neurotoxic mechanisms of methylmercury have not yet been fully elucidated. However, a lot of studies have provided several putative mechanisms involving oxidative stress, amino acid transport, calcium homeostasis, neurotransmitter receptors, and microtubule dynamics, among others. Studies using cultured CGCs have revealed that short-term exposure to methylmercury induces both apoptotic and necrotic cell death (Castoldi et al., 2000; Daré et al., 2000), produces reactive oxygen species, and impairs mitochondrial function and intracellular $Ca^{2+}$ homeostasis (Daré et al., 2000; Gassó et al., 2001; Yuan and Atchison, 2007; Kaur et al., 2007). It also disrupts glutamatergic and GABAergic neurotransmission (Fonfría et al., 2001, 2005; Herden et al., 2008). Acute neurotoxic effects of methylmercury on CGCs have been studied using conventional biochemical, neurochemical, and molecular approaches. At present, there is a need for research that considers exposure to lower concentrations of methylmercury for prolonged periods of time. Such studies would be more representative for the lower exposure levels associated with chronic human exposure. Furthermore, as far as we know, no studies address the neurotoxicity of methylmercury using global proteomic approaches; except for a preliminary report from our laboratory (Vendrell et al., 2007). Several papers have addressed the analysis of protein expression in the whole brain or in brain regions (Lubec et al., 2003; Yang et al., 2004). Some other work has addressed the study of the proteome of specific neural cells (Yu et al., 2004; Yang et al., 2005). However, protein profile maps for cultured mouse CGCs are still lacking.

The objective of this study was to detect changes in the proteome profile of CGCs after long-term exposure to low concentrations of methylmercury by using primary cultures of mouse CGCs. Thus, we sought to identify cellular targets that could be early markers of methylmercury neurotoxicity. At the same time, we provide a minimal description of the 2DE protein map on these cells as a tool that is necessary for our current and future studies.

## Materials and methods

### *Neuronal cell culture*

Primary cultures of CGCs were obtained from 7-day-old NMRI mice (Iffa Credo; St. Germain-sur-l'Arbreste, France) (Fonfría et al., 2001; 2005; Babot et al., 2005). In brief, the cerebellum tissue was trypsinized at 37 °C, followed by trituration in DNase solution (0.004% wt./wt.) containing a trypsin inhibitor from soybeans (0.05% wt./vol.). Cells were suspended in Dulbecco's modified Eagle medium (DMEM) (25 mM KCl, 31 mM glucose and 0.2 mM glutamine), supplemented with *p*-aminobenzoate, insulin, penicillin and 10% foetal calf serum. The cell suspension ($1.5\times10^6$ cells/mL) was seeded in 96-well plates (viability studies), 24-well plates (cell proteome and Western blot studies), or 6-well plates (mitochondrial proteome), precoated with poly-D-lysine and incubated for several days in a humidified 5% $CO_2$/95% air atmosphere at 37 °C. Plates were also coated with poly-L-lysine in some experiments. Methylmercury had similar effects in both coatings; however the background of the two-dimensional gels was higher in poly-L. A mixture of 5 μM 5-fluoro-2′-deoxyuridine and 20 μM uridine was added after 24–48 hours in culture to prevent glial proliferation. Granule cells do not divide in culture and differentiate into mature neurons during the culture period. Animals were handled in compliance with protocol DMAiH 3809 approved by the Generalitat of Catalonia, Spain, following EU guidelines.

### *Methylmercury treatment*

Cultured CGCs were exposed to methylmercury by adding a concentrated aqueous solution of the neurotoxic agent directly to the culture medium in the well. Control cells were treated with the same amount of water (0.6%). Methylmercury was added after the antimitotic agent at 1 or 2 days *in vitro* (div), when cells were firmly attached to the bottom and produced neurites. No differences were observed in the cultures exposed to methylmercury at 1 or 2 div, provided the cultures were exposed for the same period of time (e.g., cell viability values were $21.1\pm0.7\%$ and $22.6\pm0.5\%$ of the control when the cells were exposed for 10 days to 600 nM methylmercury beginning at div 1 and at div 2, respectively). The culture medium was not changed during the culture period, as required for the survival of these cells. Cells were harvested at the end of the exposure, as indicated in Sample preparation. Methylmercury solutions were handled in compliance with safety measures for toxic chemicals. Excess solutions were disposed of appropriately. When determining intracellular glutamate content, cells were harvested in 100 mM Tris containing 2.5 mM EDTA, homogenized and centrifuged at $14{,}000\times g$. Supernatants were analysed by HPLC with fluorimetric detection, as described elsewhere (Babot et al., 2005).

### *Cell viability*

Cell viability was determined by measuring the reduction of 3-(4,5-methylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) to a coloured formazan salt by mitochondrial reducing activity, as described previously (Babot et al., 2005; Vendrell et al., 2007). Briefly, at the end of the exposure period, the cultures were rinsed and incubated for 15 min at 37 °C in a solution of MTT (0.25 mg/mL) dissolved in HEPES buffer solution at 37 °C. After washing off the excess MTT, the cells were digested overnight with 5% SDS at 37 °C in darkness, and the coloured formazan salt was measured at 560 nm in a spectrophotometer plate reader.

### *Proteomics*

*Sample preparation.* Primary cultures were rinsed in a prewarmed phosphate-buffered saline solution (PBS: 138 mM NaCl, 2.7 mM KCl, 8 mM $Na_2HPO_4$, 15 mM $KH_2PO_4$ at pH 7.4). Cells were harvested with a PBS solution containing a protease inhibitor cocktail (1% vol./vol.; Sigma-Aldrich, St. Louis, MO, USA) and stored at −80 °C until used. Samples were sonicated with 3 ultrasonic bursts of 30 sec each in the presence of NP-40 detergent (0.5% vol./vol.), with the samples always kept on ice. Then, total protein extracts were precipitated with trichloroacetic acid (10%) and rinsed in acetone (Quero et al., 2004). After this, the protein pellets were resuspended with a rehydration buffer (2% CHAPS, 0.5% ampholyte buffer, 7 M urea, 2 M thiourea, 100 μg/mL of bromophenol blue, and 1.2% DeStreak as a reducing agent). The protein content was quantified using an RC DC Biorad Kit (Bio-Rad Laboratories; Hercules, CA, USA).

To obtain mitochondrial-enriched fractions, primary cultures of CGCs grown for 13 div ($18\times10^6$ cells) were rinsed in prewarmed PBS solution. Cells were harvested with an homogenization buffer (20 mM HEPES, 320 mM sucrose, 1 mM EGTA, 5 mM dithiothreitol, and 1% protease inhibitor cocktail), homogenized in a Dounce Teflon-glass homogenizer (10 passes; 450 rpm), and submitted to differential centrifugations. Supernatants obtained after centrifugation at $800\times g$ for 10 min were recentrifuged at $10{,}000\times g$ for 10 min, at 4 °C. The final pellet containing the mitochondrial-enriched fractions was stored at −80 °C until used. The pellet was lysed with lysis buffer (40 mM Tris, 7 M urea, 2 M thiourea, and 4% CHAPS) and proteins were precipitated using a commercial kit (2D Clean Up Kit, GE Healthcare, Uppsala, Sweden). The protein pellets were resuspended with the rehydration buffer for 2DE analysis. The purity of the mitochondrial-enriched fractions was confirmed by determining the activity of succinate dehydrogenase (SDH, a mitochondrial enzyme localized in the complex II of the respiratory chain) and of lactate dehydrogenase (LDH, a cytosolic enzyme) using standard spectrophotometric procedures (Hatefi,