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## The Chemokine CCL2 Protects against Methylmercury Neurotoxicity

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[AQ1]

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[AQ4]

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Industrial pollution due to heavy metals such as mercury is a major concern for the environment and public health. Mercury, in particular methylmercury (MeHg), primarily affects brain development and neuronal activity, resulting in neurotoxic effects. As chemokines can modulate brain functions and are involved in neuroinflammatory and neurodegenerative diseases, we tested the possibility that the neurotoxic effect of MeHg may interfere with the chemokine CCL2. We have used an original protocol in young mice using a MeHg-contaminated fish-based diet for 3 months relevant to human MeHg contamination. We observed that MeHg induced in the mice cortex a decrease in CCL2 concentrations, a neuronal cell death, and a microglial activation. KO CCL2 mice fed with a vegetal control food already presented a decrease in cortical neuronal cell density in comparison with wild-type animals under similar diet conditions, suggesting that the presence of CCL2 is required for normal neuronal survival. Moreover, KO CCL2 mice showed a pronounced neuronal cell death in response to MeHg. Using *in vitro* experiments on pure rat cortical neurons in culture, we observed by blockade of the CCL2/CCR2 neurotransmission an increased neuronal cell death in response to MeHg neurotoxicity. Furthermore, we showed that *sox* genes are upregulated in brain of wild-type mice fed with MeHg in contrast to KO CCL2 mice and that CCL2 can blunt *in vitro* the decrease in glutathione levels induced by MeHg. These original findings demonstrate that CCL2 may act as a neuroprotective alarm system in brain deficits due to MeHg intoxication.

[AQ5]

**Key Words:** methylmercury neurotoxicity; chemokines; CCL2/CCR2; fish diet; neuroprotection; microglia; neuronal cell death.

Climate changes, ecological modifications due to pollution, in particular chemical/industrial pollution, bring forth scientific, political, and health issues. Among the most polluting compounds known in the industrial world are heavy metals such as mercury (Hg). Methylmercury (MeHg) is an environmental

poison which has been shown to drastically affect various organs and in particular the brain. Major actions have been taken to reduce the use and emission of mercury in the environment in the 1950s, following the Minamata disaster in Japan that had been caused by a dramatic industrial pollution of the sea by MeHg (Eto, 2000). Nonetheless, such a contamination remains a serious problem in many countries raising a controversial major concern for the environment with implications for public health.

Exposure to MeHg has been shown to disrupt normal brain development and cerebral activity in adult humans as well as in various animal species and to produce neurotoxicity in selective brain areas such as the cerebral cortex and the cerebellum in adults (Auger *et al.*, 2005; Eto, 2000). It was extensively reported that neuronal cells were highly susceptible to MeHg toxicity (Rush *et al.*, 2009). Neuronal cell death by MeHg has been attributed in part to oxidative stress (Ceccatelli *et al.*, 2007; Huang *et al.*, 2008), a mechanism well described in a variety of pathological brain processes including neurodegenerative diseases and neuroinflammation (Huang *et al.*, 2008). However, several of the previous studies carried out to study the toxic effects of MeHg have used high doses of MeHg not directly relevant to current contamination in humans, in particular due to ingestion of contaminated fish (Bourdineaud *et al.*, 2008, 2011).

Chemokines are small proteins (6–10 KDa) that were initially thought to be responsible for the maturation and trafficking of leukocytes, in particular in inflammatory diseases (Ransohoff, 2009). Recently, the possibility has been raised that they are constitutively present in the brain in both glial cells and neurons and might act as neurotransmitters or neuromodulators (Rostene *et al.*, 2007). In particular, the CC chemokine CCL2 (also known as MCP-1: monocyte chemoattractant protein1) has been extensively described in the central nervous system (CNS), where it is located as well as its cognate receptor CCR2 in

neurons in selective brain regions such as the cerebral cortex (Banisadr *et al.*, 2005a,b). A recent report on gene expression profiling has shown a CCL2 messenger RNA modulation in mouse cerebellum following MeHg treatment (Hwang *et al.*, 2011).

Here by means of both *in vivo* and *in vitro* approaches, we report that the chemokine CCL2 can be released by neurons and may prevent cortical neuronal cell death induced by a low contamination with MeHg present in a food pellet diet containing 0.1% of dry flesh from MeHg-contaminated *H. aimara* fish (Bourdineaud *et al.*, 2008). Furthermore, *in vitro* blockade of CCL2/CCR2 neurotransmission or *in vivo* blockade obtained in mice invalidated for the CCL2 gene, demonstrate a higher neurotoxic effect of MeHg. This protective effect of CCL2, via its CCR2 receptor, may be associated with the regulation of oxidative stress and/or CCL2-MeHg chemical interaction. These results are the first to show that chemokines, such as CCL2, may have a crucial role in the neuroprotective mechanisms in response to MeHg contamination.

## MATERIALS AND METHODS

### Ethics Statement

Experiments on mice were performed in accordance with the European Community council directive no. 8616091 EEC. Agreement no. 75-108 (05/02/2001) to WR from Paris Prefecture. The experiments were conducted according to the agreement of the University Pierre et Marie Curie committee no. A75-19-01.

### In vivo Experiments

**Preparation of the mice diets.** Adult 3-week-old male mice of the C57Bl/6 Jico inbred strain (IFFA Credo, Lyon, France) or mice deficient in CCL2 (B6.129S4-Ccl2tm1RoI/J) on the same C57Bl/6 background (Lu *et al.*, 1998) were purchased from Jackson Laboratory (Bar Harbor, ME). Young mice were used because they grow quickly and are more sensitive to MeHg than older animals. In order to be close to human contamination by fish ingestion, a low dose of MeHg contamination was obtained with a food pellet diet containing 0.1% of dry flesh from MeHg-contaminated *H. aimara* fish (aimara groups) (Bourdineaud *et al.*, 2008, 2011). Total Hg content in the aimara groups was  $5.4 \pm 0.5$  ng Hg/g of food pellet (manufactured by Dietex). The control RM1 diet was 100% vegetal (RM1 diet; Dietex, Saint-Gratien, France) and contained  $1.4 \pm 0.2$  ng Hg/g. Another control was carried out in order to avoid a possible effect of the fish flesh itself with noncontaminated farmed salmon flesh ( $1.55 \pm 0.15$  ng Hg/g). Mice were fed for 3 months with the control or MeHg-contaminated diets (Bourdineaud *et al.*, 2008). The amount of polyunsaturated fatty acids  $\omega 3$ ,  $\omega 6$ , and  $\omega 9$  are similar for aimara and salmon flesh and represents less than 0.003% in the diets (Bourdineaud *et al.*, 2008, 2011). The nutritional as well the metal composition of the various diets are given in the supplemental data (Supplementary table 1).

**Brain measurement of CCL2 and total Hg determination in mice brain.** At the end of the 3-month feeding period, mice were killed by decapitation, and the cortex was taken at three different levels: anterior (from bregma + 2.96 mm to + 1.18 mm corresponding mainly to the frontal cortex), medial (from bregma + 0.86 mm to -2.3 mm corresponding to the sensorimotor cortex), and posterior (from bregma -2.46 mm to -4.36 mm corresponding to the visual cortex) (Franklin and Paxinos, 1997). CCL2 was extracted by sonication for few seconds in 250  $\mu$ l of assay diluent (cat No 51-2641KC; BD Biosciences, Le Pont de Claix, France). Samples were centrifuged at 10,000 rpm for 15 min at 4°C, and the supernatant was collected. CCL2 was measured with an

enzyme-linked immunosorbent assays (ELISA) kit (mouse CCL2 ELISA set, BD OptEIA cat no. 555260; BD Biosciences). Total Hg concentrations in mice tissues and diets were determined by flameless atomic absorption spectrometry (Bourdineaud *et al.*, 2008).

**Neuronal Cell death measurement in mice brain.** For determination of neuronal cell number, 20- $\mu$ m brain sections were cut with a cryostat (Leica CM 3050 S) from bregma -1.46 mm to -2.06 mm according to the atlas of Franklin and Paxinos (1997) and mounted on glass slices. Preliminary experiments using a human anti-HuD antibody (1:8000) (Szabo *et al.*, 1991) and TUNEL determination on sections pretreated for 15 min with paraformaldehyde (PFA) 4% confirm that the cell death we observed was mainly apoptotic and selective for neurons (Banisadr *et al.*, 2005a). Neuron quantification was carried out on nonfixed sections pretreated for 5 min with 70° ethanol. The sections were then dipped in cresyl violet (5g/l) and 0.0006 N acetic acid, washed with distilled water (20 s), and dipped again in 70° ethanol and acetic acid 0.0003 N (5 s), 95° ethanol (5 s), 100° ethanol (5 s), and xylene (2  $\times$  5 min). All sections were mounted with entellan (Electron Microscopy Science, Hatfield, PA; ref 14800). Neuronal cells were measured at different layers of the parietal/sensorimotor cortex with an image analyzer (Mercator; Explora Nova, La Rochelle, France). Two determinations on the same surface (20,000  $\mu$ m<sup>2</sup>) were carried out on each section in the various layers, and mean values calculated for each animal.

**Microglia immunocytochemistry.** For microglia determination, PFA 4% pretreated sections were mounted on glass slices, washed with PBS 1  $\times$  (3  $\times$  5 min), and incubated overnight at room temperature with an anti-Iba1 Rabbit (1:400 dilution) (Wako Pure Chemical Industries Ltd., code no: 019-19741). After incubation, sections were rinsed with PBS 1  $\times$  (3  $\times$  5 min) and incubated at room temperature for 2 h with an Alexa 488-conjugated anti-rabbit antibody (Molecular Probe; Invitrogen, France; ref A11-008) at a 1:200 dilution. The sections were washed with PBS 1  $\times$  (3  $\times$  5 min) and mounted with mountant permafluor (Labovision; Thermo Fisher Microm, France; ref TA-030-FM). Iba-1 fluorescence was determined with a fluorescent microscope (Leica DM400B). Microglia were measured in the parietal/sensorimotor cortex. Two determinations on the same surface (200,000  $\mu$ m<sup>2</sup>) were carried out by image analysis on each section, and mean values calculated for each animal.

**Sod gene expression in mice cortex.** Total RNAs were extracted from 40 mg of fresh parietal/sensorimotor cortex using the Absolutely RNA Miniprep kit (Stratagene, France). First-Strand DNA was synthesized from 5  $\mu$ g total RNA using the Stratascript First-Strand DNA synthesis kit (Stratagene). Specific primer pairs were determined for *sod1* and *sod2* genes. Real-time PCR reaction was performed in a LightCycler (Roche) according to the manufacturer's instructions. PCR amplification, reaction specificity, and gene expression normalized to  $\beta$ -actin were carried out as previously described (Bourdineaud *et al.*, 2008).

### In vitro Experiments

**Primary neuronal cultures.** Rat embryos were collected in cold PBS (1  $\times$ ) from impregnated females at embryonic day 14. Briefly, fetal cortex was dissected in cold PBS (1  $\times$ ) under sterile conditions, and cells were mechanically dissociated by pipetting, in Neurobasal medium (Gibco Invitrogen Corporation, France), collected by centrifugation (800  $\times$  g, 5 min), and gently resuspended in serum-free medium (see below). Cell viability was estimated by Trypan Blue exclusion. Cells were plated (density  $1.10^5$  cells/well [48-well plates] for cell death experiments and  $2.10^5$  cells/well [24-well plates] for glutathione [GSH] determination) on wells previously coated with poly-L-lysine (15  $\mu$ g/ml). Cultures were maintained for 7 days at 37°C in a humidified incubator (95% O<sub>2</sub>/5% CO<sub>2</sub>). At days 1 and 2, aracytine (AraC) was added at 25 and 50  $\mu$ M, respectively, to prevent glial proliferation. The culture serum free medium used was Neurobasal medium (Gibco Invitrogen Corporation) supplemented with 0.5mM L-glutamine, 50  $\mu$ g/ml streptomycin, 50 U/ml penicillin, and B27 complement (Gibco Invitrogen Corporation). Medium was changed 1/3 at days 2, 5, and 7. In the presence of AraC and of Neurobasal medium supplemented with B27, the percentage of GFAP-positive astrocytes was  $0.4 \pm 0.2\%$ , and no Iba-1 microglial-positive cells were detected, suggesting that the cells grown under these conditions represent neuronal cortical cells as confirmed with HuD staining (Szabo *et al.*, 1991). In some

experiments, cortical cells were also prepared without AraC in order to obtain mixed glial and neuronal cell populations (Supplementary figure 1). Cells were stained with a rabbit CCL2 antibody (Torrey Pines Biolabs; cat: TP209) at a final concentration 1/400 in PBS 1× supplemented with 0.05% Triton.

**Cell exposure.** After 7 days of culture, MeHg (methylmercury chloride; Sigma-Aldrich, St Louis, Mo ref: 442534-5G-A) was added at various concentrations (50nM–5μM) for 1, 2, 3, and 5 days depending on the experiment. For 5 days exposure to MeHg, medium was changed 1/3 at day 2. In order to block the *in vitro* effects of CCL2, a blocking rabbit anti-CCL2 antibody (Torrey Pines Biolabs cat TP209, East Orange, NJ) was added in the incubation medium at days 1 and 3 (for 5 days MeHg treatment) at 0.5 μg/ml. We previously observed that this antibody totally trapped CCL2 leading to an undetectable level of the chemokine in the incubation medium (data not shown). A nonpeptide CCR2 receptor antagonist, INCB3344, was also added at 10μM (Brodmerkel *et al.*, 2005) at the same times as above. In some experiments, CCL2 (rat CCL2, ref: 400-12; Preprotech Inc., Rocky Hill, NJ) was added in the culture medium at concentrations ranging from 1 to 50nM at days 1 and 3.

**Cell death counting.** At the end of the experiments, neuronal cells were washed with PBS supplemented with Triton X 100 (0.1%) and fixed with filtered 4% PFA for 5 min. Cells were washed again with PBS. DAPI (4,6-diamidino-2-phenylindole) solution (Chimie, ref 32671; Sigma-Aldrich) was applied on cells at 0.1 μl/ml for 10 min. Condensed nuclei were considered as apoptotic and counted among the whole cell population labeled with a blue homogeneous staining. Measurement was carried out on 50 cells/area with an inverted Nikon Diaphot 300 epifluorescence microscope.

**GSH measurement in neuronal cells.** Cells were suspended in 150 μl ice-cold PBS for protein analysis and GSH measurement. Protein levels were analyzed using bovine serum albumin as external standard. For GSH analysis, a 100 μl aliquot of the PBS suspension was diluted twice with ice-cold 5% perchloric acid containing 1mM EDTA to precipitate protein and centrifuged for 1 min at 12,000 rpm. The total cellular GSH levels were determined by the enzymatic recycling method reported by Tietze (1969) using the supernatant fraction.

**Statistical analysis.** Data are expressed as means ± SEM. All *in vitro* experiments were carried out at least in triplicate. The number of animals for the *in vivo* experiments is mentioned in the results. One-way ANOVA was performed followed by Student Newman-Keuls test or by a Kruskal-Wallis test. Student-*t*-test was used when only two groups were compared. A two-way ANOVA at two levels with unequal numbers of samples was used when more than one treatment was studied, followed by a Student Newman-Keuls test (Harvey, 1966; Winer, 1971). The threshold of significance was  $p < 0.05$ . Statistical software was used (SigmaStat version 3.5; Jandel Scientific, San Jose, CA).

## RESULTS

### Impact of Fish-Contaminated Diets on Brain Hg Bioaccumulation and CCL2 Content in Mouse Cortex

We recently demonstrated (Bourdineaud *et al.*, 2008, 2011) that we could mimic human food contamination with MeHg by giving to mice food pellets at a concentration as little as 5 ng/g Hg (containing less than 2 ng/g MeHg) brought by dry fish flesh supplementation obtained from contaminated aimara fish. Male C57Bl/6 mice were thus contaminated for 3 months with such diet and compared with control mice provided 100% vegetal RM1 or farmed salmon diets. Aimara diet induced a 10-fold increase in total Hg content in the brain ( $0.0312 \pm 0.006$  vs.  $0.0026 \pm 0.0003$  μg Hg/g dry weight in RM1 and farmed salmon diets; means ± SEM,  $n = 8$ /group). The highest amounts of total Hg with the aimara diet were found in the kidneys ( $0.907 \pm 0.063$  μg Hg/g dry weight; means ± SEM,  $n = 8$ /group) and in the hairs

**TABLE 1**  
Effect of MeHg Contamination *In Vivo* on CCL2 Concentration in Mice Cortex

	Concentration of CCL2 (pg/mg protein)		
	Frontal cortex	Sensorimotor cortex	Visual cortex
Control RM1	4.36 ± 0.7	4.06 ± 0.7	4.46 ± 0.67
Control salmon	4.44 ± 0.8	4.51 ± 0.8	4.85 ± 0.7
MeHg aimara	3.37 ± 0.46	2.14 ± 0.1*	2.99 ± 0.24*

*Note.* Mice were fed for 3 months with pellets prepared from MeHg-contaminated aimara fish (Aimara group) or salmon flesh (control salmon) and compared with mice fed with 100% vegetal RM1 food (control group),  $n = 24$  for each group. MeHg treatment induced a decrease in CCL2 content in the mouse cortex. A Student *t*-test was used for comparison with each control group. Values indicate means ± SEM.

\* $p < 0.05$  versus respective controls

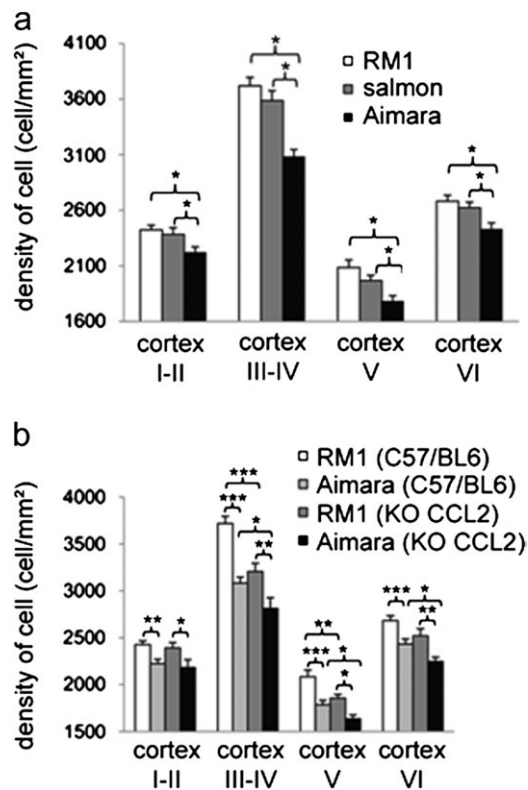
( $0.47 \pm 0.06$  μg Hg/g dry weight; means ± SEM,  $n = 8$ /group). In mice submitted to such Hg contamination, a significant decrease in CCL2 content was observed in the cortex, in particular in the sensorimotor and visual cortex (Table 1).

### Consequence of MeHg-Contaminated Diet on Neuronal Cell Death in the Mouse Cortex

Cell counting carried out in the different layers of the sensorimotor cortex demonstrated a significant loss of neuronal cells following 3 months diet with aimara-contaminated food (Fig. 1a). Quantitative measurement by image analysis showed that this decrease affected all layers of the cortex from the surface (cortex I–II) till the deep layer (cortex VI) (Fig. 1a). No effect was observed with the non-Hg-contaminated fish flesh obtained from farmed salmon diet. Interestingly, as shown in Figure 1b, a loss in neuronal cell density was already observed in KO CCL2 mice in comparison with the wild-type animals fed with the control RM1 diet, in particular in the deep layers of the cortex. Furthermore, quantitative measurements and statistical analysis using a two ways ANOVA (Harvey, 1966; Winer, 1971) demonstrated a significant difference between groups but no significant interaction between the diet and the presence or absence of CCL2. However, KO CCL2 mice subjected to the aimara-contaminated food showed a significant higher neuronal cell death in the cortical deeper layers in response to MeHg contamination. These *in vivo* data suggested that CCL2 was not directly involved in the mechanism of neurotoxicity induced by MeHg but had a neuroprotective effect against MeHg contamination (Fig. 1b).

### Consequence of MeHg-Contaminated Diet on Microglial Activation and Sod Gene Expression in the Mouse Cortex

As shown in Figure 2, aimara food diet in wild-type mice induced an increase in the number of microglial cells as stained



[AQ10] **FIG. 1.** Effect of MeHg-contaminated diet on neuronal cell death in the mouse cortex. Quantitative measurement by image analysis showing the loss of neurons in various layers of the sensorimotor cortex (a) in wild-type mice fed with the contaminated aimara fish food (■). No effect of salmon diet was observed (■). (b) A more pronounced effect can be seen in KO CCL2 animals in particular in the deeper layers of the cortex.  $n = 24/\text{group}$  for control vegetal RM1 food, salmon, and aimara groups and  $n = 17$  for KO CCL2. A two-way ANOVA at two levels with unequal numbers was used for comparison (Harvey, 1966). Values indicate means. Error bars indicate SEM.  $*p < 0.05$  was considered as significant.  $**p < 0.01$ ;  $***p < 0.001$ .

with Iba-1 in the sensorimotor cortex. In contrast, the salmon diet had no effect on microglial activation (Figs. 2a–c). KO CCL2 mice presented a slight deficit in microglial cell number under control RM1 diet and did not show any significant increase in microglia under aimara food diet (Fig. 2d). Exposure of wild-type mice to aimara food diet for 3 months demonstrated a twofold upregulation in the expression of the superoxide dismutase-encoding genes *sod1* (cytoplasmic Sod enzyme) and *sod2* (mitochondrial Sod enzyme) (Table 2). In contrast, in the brain of KO CCL2 mice, the aimara diet did not trigger this increased expression of both *sod* genes. Moreover, in mice fed with the control RM1 diet, KO CCL2 brain displayed *sod* gene expression 60% higher than that in wild-type brain, indicating an increased oxidative level in the absence of CCL2. In addition, KO CCL2 mice fed with the aimara-containing diet demonstrated a significant decrease in the expression level of the *sod2* gene as compared with wild-type brain.

### Dose- and Time-Dependent Neurotoxic Effects of MeHg In Vitro

In order to further study the neuroprotective effect of CCL2 in response to MeHg exposure, *in vitro* experiments were carried out. Pure rat cortical neurons in primary cultures were grown with various concentrations of MeHg (50nM–5 $\mu$ M) and over different durations from 1 to 5 days. Neuronal cells determined with a specific neuronal marker HuD (Szabo *et al.*, 1991) expressed CCL2 (Figs. 3a and 3b). Neuronal cells in culture were considered as apoptotic when presenting nuclei condensation when counterstained with DAPI as compared with the whole cell population (Fig. 3c). Increasing concentrations of MeHg induced a dose-dependent apoptotic neuronal cell death starting at 500nM following 1-day incubation with MeHg (Fig. 4a). Interestingly, the same concentration of MeHg, which was slightly neurotoxic after 1 day, was able to induce the same level of apoptosis (40–50%) than 1 $\mu$ M MeHg after 2 days of treatment. After 5 days, 80% of the cells died when exposed to 500nM MeHg, similarly to 5 $\mu$ M MeHg after 24 h of treatment (Fig. 4b). In complementary experiments, we observed that when primary cultures were grown in the absence of aracytine (AraC) which allowed glial proliferation and thus provided a neuron-glia co-culture model, only a slight significant neurotoxic effect of 500nM MeHg could be observed starting at day 5 (Supplementary figure 1). Therefore, the following *in vitro* experiments were carried out on pure rat cortical neurons in culture.

### Neuroprotective Effect of the Chemokine CCL2 against MeHg Neurotoxicity

In order to study the role of CCL2, three different experiments were performed. First, we trapped the chemokine present in the culture medium by means of a blocking anti-CCL2 antibody resulting in an undetectable level of CCL2 in the incubation medium (data not shown). Under these conditions, an increase in neuronal cell death was observed as soon as 24 h. When the anti-CCL2 antibody was added in the presence of MeHg, more apoptotic cell death was observed (Fig. 5a). Second, we used a selective nonpeptide receptor antagonist to block the binding of CCL2 on its main receptor CCR2 (Brodmerkel *et al.*, 2005). Similar to what we observed with the blocking CCL2 antibody, blockade of CCR2 increased neuronal cell apoptosis and the neurotoxic effect of MeHg was significantly enhanced in the presence of the CCR2 antagonist after 5 days in culture (Fig. 5b). Third, instead of blunting the CCL2 effect, increasing concentrations of CCL2 were introduced into the incubation medium (Fig. 5c). Under our experimental conditions, the basal neuronal cell death represents around 20%. Addition of MeHg in the incubation medium induced a twofold increase in neuronal cell death (40–50%). A dose-dependent effect with a U-shaped curve was observed with an increased neuroprotective effect against 500nM MeHg incubation for 2 days with low concentrations of CCL2 (1–10nM), with a peak

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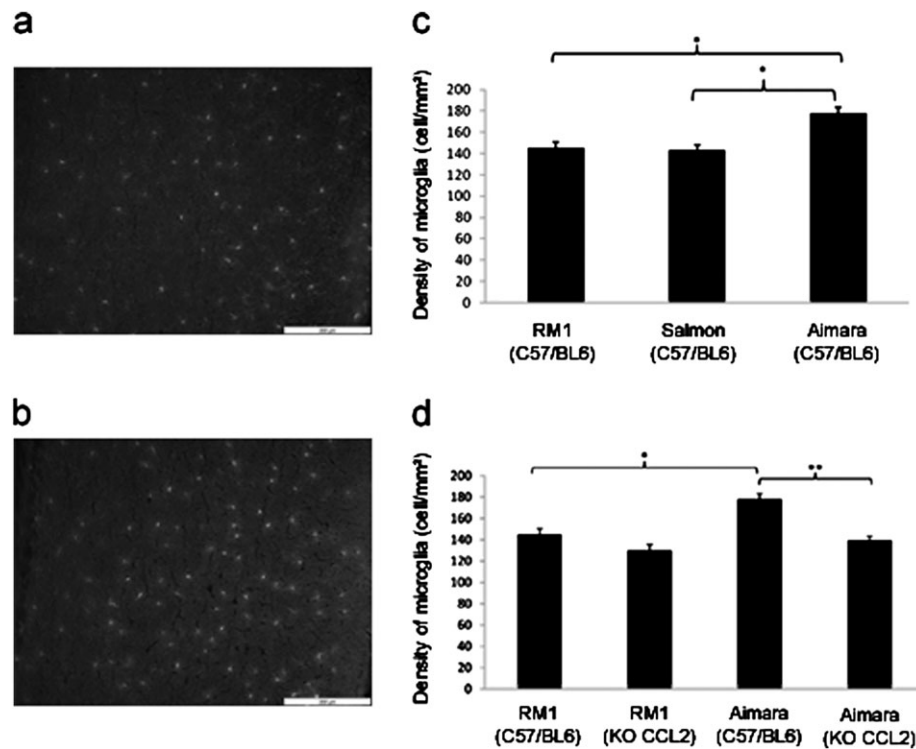
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**FIG. 2.** Effect of MeHg-contaminated diet on microglial activation in the mouse cortex. (a and b) Microphotographs taken at the level of the mouse sensorimotor cortex stained with an anti-Iba-1 rabbit antibody and a second Alexa-488 fluorescent antibody in order to visualize microglial cells. (a) Control mice fed for 3 months with vegetal RM1 food. (b) Mice fed for 3 months with contaminated aimara fish. (c) Quantification of the increase in microglial number in aimara fed wild-type mice as compared with animals fed with RM1 or salmon diet,  $n = 17/\text{group}$ . (d) Number of microglial cells in wild-type (C57/BL6) mice versus KO CCL2 under control RM1 and aimara diets,  $n = 17$ . Values are expressed as number of microglial cells/mm<sup>2</sup> and indicate means. Error bars indicate SEM. \* $p < 0.05$  was considered as significant. \*\* $p < 0.01$ . Scale bars: 200  $\mu\text{m}$ .

at 10nM followed by a progressive and reversed effect at 50nM (Fig. 5c). Ten nanomolars of CCL2 was thus used in the following experiment.

#### CCL2 Blunts the Inhibition in GSH Levels Induced by MeHg

MeHg neurotoxicity has been correlated to the production of reactive oxygen species (ROS) inducing a decrease in energy balance, in particular in the production of GSH, an antioxidant agent (Ceccatelli *et al.*, 2007; Stringari *et al.*, 2008). We confirmed these data in our *in vitro* model showing that 500nM MeHg for 2 days produced a significant decrease in GSH concentration (Table 3). It can be noticed that CCL2 by itself at 10nM did not modify the amount of GSH but was able to counteract the decreased level of GSH observed after MeHg application (Table 3).

#### DISCUSSION

The first major finding of the present work is that the chemokine CCL2 has a neuroprotective effect against MeHg neurotoxicity. Previous reports had suggested that chemokines, known to play an essential role in the immune system, may be activated in neuroinflammatory processes such as multiple

sclerosis, stroke, and HIV encephalitis presumably through leukocyte attraction and migration into the CNS (Ransohoff, 2009). It has been demonstrated that these chemokines may be produced by various glial cells in the CNS (Ransohoff, 2009). However, more recently, we and others observed that under normal conditions in which the blood brain barrier is intact selective neurons in the brain were also able to synthesize CCL2 and to express CCR2 which play a modulatory role in neurotransmission (Banisadr *et al.*, 2005a,b; Rostene *et al.*, 2007). The present *in vitro* experiments confirm this observation because neurons from mice and rat cortex are able to synthesize CCL2. Furthermore, evidence has demonstrated a fast expression and a protective effect of several chemokines such as CCL2 in response to neuronal damage (Bruno *et al.*, 2000; Eugenin *et al.*, 2003; Schreiber *et al.*, 2001).

Here, we observed that CCL2 is able to protect neurons against MeHg-induced neuronal cell death. To support the physiological neuroprotective role of CCL2 in MeHg neurotoxicity, we carried out a series of *in vivo* and *in vitro* experiments. An original aspect of the present work is the experimental *in vivo* paradigm used to study the effects of MeHg intoxication. We have transferred and mimicked in wild-type and transgenic mice the quantity of MeHg and the mode of contamination observed in the Wayana Amerindians living in the French Guiana (Bourdineaud *et al.*,

**TABLE 2**  
**Superoxide Dismutase *sod1*- and *sod2*-Encoding Gene**  
**Expression in Brains from Wild-Type or KO CCL2 Mice Fed**  
**with Fish-Containing Diets for 3 Months**

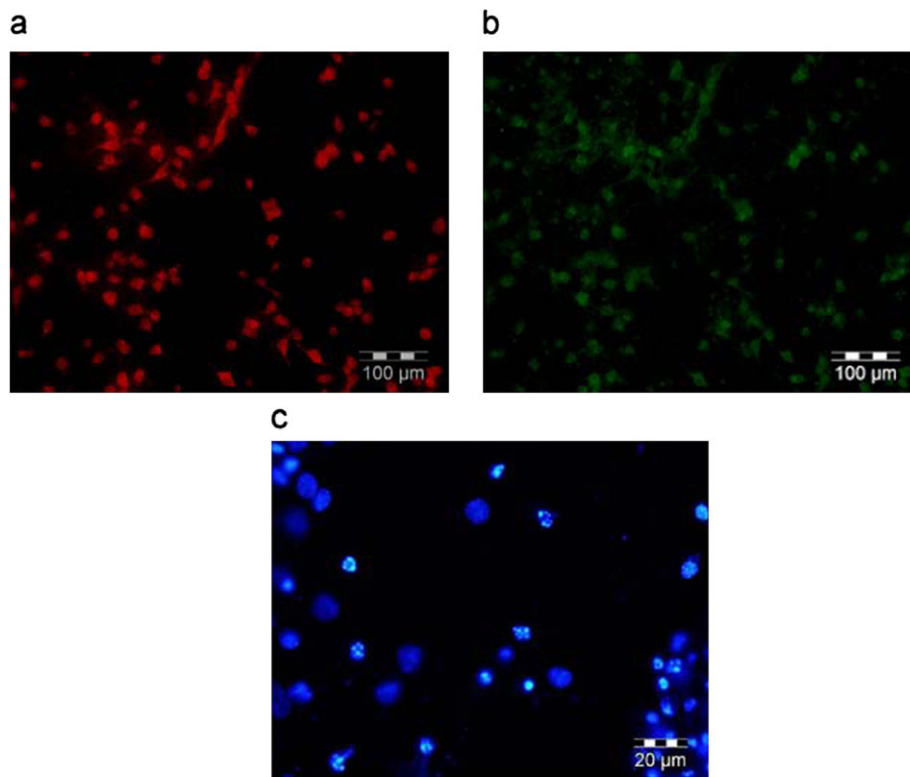
Gene	Wild-type mice (C57/BL6)			KO CCL2 mice			
	Control	RM1	Aimara	Control	RM1	Aimara	Ratio A/C
<i>Sod1</i>	28.3 ± 7.9		*56.7 ± 4.5	*2.0	44 ± 8	44 ± 14	1.0
<i>Sod2</i>	14.6 ± 2.9		*37.4 ± 4.7	*2.6	°24 ± 2	§18.5 ± 3	0.76

[AQ12] *Note.* *Sod1* and *sod2* gene expression was measured by real-time PCR. Data are given as relative gene expression (mean ± SEM,  $n = 5$ ).  $\beta$ -actin was the reference gene. Asterisks indicate a significant differential gene expression in brain of wild-type mice fed the aimara diet compared with those fed the control diet, as determined with the Mann-Whitney  $U$ -test, \* $p < 0.05$ . The circle indicates a significant differential gene expression in brain of KO CCL2 mice fed the control diet compared with wild-type mice fed the same diet, as determined with the Mann-Whitney  $U$ -test, ° $p < 0.05$ . The symbol § indicates a significant differential gene expression in brain of KO CCL2 mice fed the aimara diet compared with wild-type mice fed the same diet, as determined with the Mann-Whitney  $U$ -test, § $p < 0.05$ . Ratio A/C: ratio of the mean relative expression observed in tissues from mice fed the aimara diet over that of the control diet

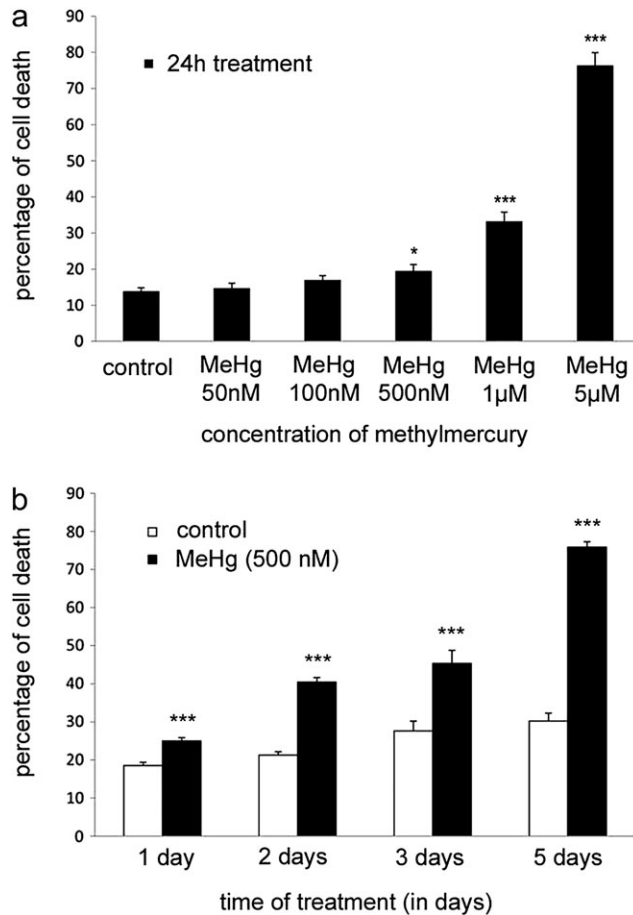
405 2008, 2011). These Amerindian populations are contaminated after consumption of such carnivorous fish as aimara due to climatic and environmental conditions as illicit gold mining.

More than 85% of Wayana Amerindians present a hair Hg concentration (12  $\mu\text{g/g}$ ) exceeding the safety limit set up by the World Health Organization (10  $\mu\text{g/g}$ ). We previously found that the aimara fish diet used in the present study, which represents 0.1% of fish flesh in food corresponding to 5 ng/g of Hg, was able to produce a 10-fold increase in Hg concentration in the brain as also reported here, resulting in altered mitochondrial protein concentrations and ATP synthesis (Bourdineaud *et al.*, 2008, 2011). Others have mainly used high micromolar concentrations of MeHg either in the drinking water (Stringari *et al.*, 2008) or by gavage (Huang *et al.*, 2008), which do not represent the main contamination observed in humans with food via fish consumption. Indeed, the toxic methylated form of Hg, MeHg, represents the major form of Hg in fish (Maury-Brachet *et al.*, 2005).

The main adverse health effects of MeHg observed in humans are by far neurological disorders affecting behavior and motricity (Auger *et al.*, 2005; Eto, 2000). We previously reported that feeding wild-type mice with such a low dose of MeHg-contaminated aimara food induced an increase in anxiety levels (Bourdineaud *et al.*, 2008) without affecting locomotor activity (data not shown). We report here that such diet induces a decrease in cortical CCL2 level (Table 1). The decrease in CCL2 is more pronounced in the sensorimotor and visual cortex than in the frontal cortex. It was reported by Eto (2000) in human brain of Minamata disease that the effect of MeHg was not uniformly



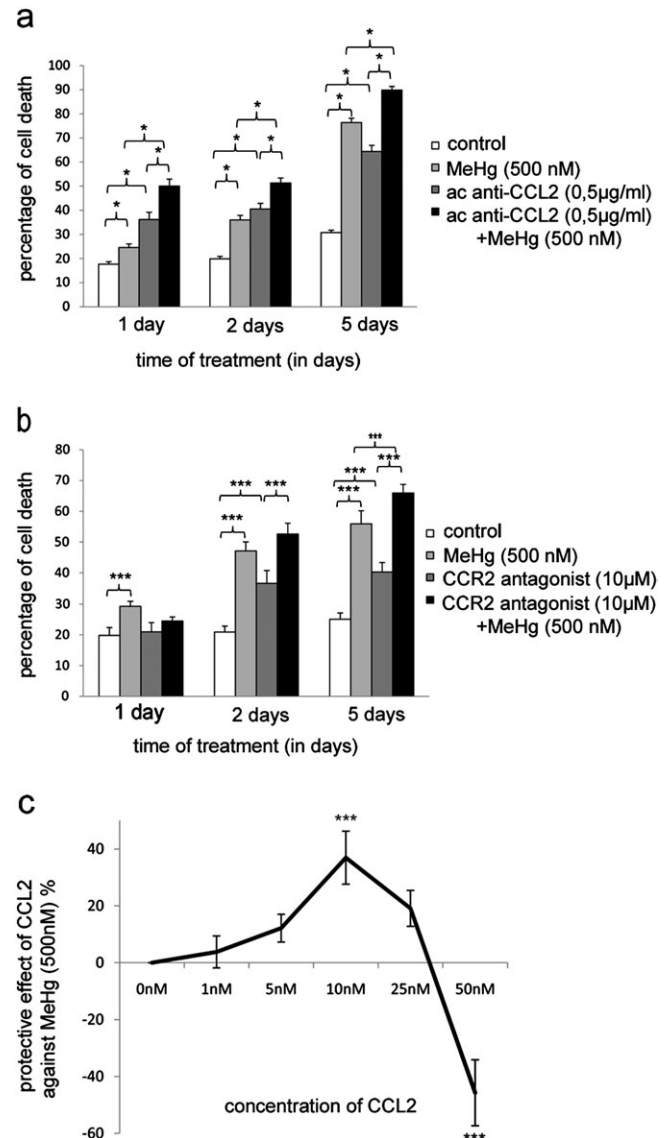
**FIG. 3.** Immunocytochemistry of the cells grown in primary cultures. (a) Cortical neuronal cells stained with a human anti-HuD antibody selective for neuronal phenotype. (b) Cortical neuronal cells stained with a rabbit anti-CCL2 antibody. (c) Apoptotic neuronal cortical cells presenting a DNA condensation with DAPI staining.



**FIG. 4.** Dose- and time-dependent neurotoxic effect of MeHg *in vitro*. (a) Dose-dependent effect of MeHg on cortical neuronal cell death following 1-day MeHg exposure. (b) Time-dependent effect of 500nM MeHg on cortical neuronal cell death. Open bars represent cell death observed in control cultures without MeHg. Experiments were carried out in triplicate.  $n = 12/\text{point}$  for (a);  $n = 36/\text{point}$  for (b). Values indicate means. Error bars indicate SEM.  $p < 0.05$  was considered as significant. \* $p < 0.05$ ; \*\*\* $p < 0.001$ .

observed in all brain regions mainly affecting structures in which neurons are known to be more vulnerable to toxic insults as in the cortex. Variations in Hg content may thus explain the differences observed in the various cortical regions in the effect of MeHg on the decrease in CCL2 levels.

This decrease in CCL2 content, probably resulting from both neurons and glial cells (Banisadr *et al.*, 2005a; Lawrence *et al.*, 2006), may be linked to less neuronal protection. Indeed, we made an interesting observation showing that KO CCL2 mice fed with the control food RM1 present a significant reduction in neuronal cell density in the cortex in comparison with wild-type animals under similar diet conditions, suggesting that the presence of CCL2 is required for normal neuronal survival even in the absence of MeHg. The lack of statistical interaction between the diet and the presence or not of CCL2 suggests that CCL2 is not directly involved in the mechanism by which MeHg induced neuronal cell death. In other words, by decreasing CCL2 or in the absence of CCL2, neurons become



**FIG. 5.** Role of CCL2 in the effects of MeHg (a) Blockade of CCL2 in the incubation medium by a blocking anti-CCL2 antibody induced an increase in neuronal cell death alone and following MeHg incubation ( $n = 18/\text{point}$ ). (b) Blockade of the CCL2 receptor, CCR2, similarly induced an increase in apoptotic neuronal cell death alone and following MeHg incubation. (c) Administration of increasing concentrations of CCL2 in the incubation medium during 2 days produced a dose-dependent protective effect on MeHg-induced neuronal cell death with a peak at 10nM CCL2 ( $47.63 \pm 2.45\%$  vs.  $32.57 \pm 1.01\%$  cell death for MeHg and MeHg + CCL2, respectively,  $p < 0.001$  vs. MeHg), whereas a high concentration (50nM) induced apoptosis ( $41.53 \pm 2.68\%$  vs.  $61.57 \pm 2.63\%$  cell death for MeHg and MeHg + CCL2, respectively,  $p < 0.001$  vs. MeHg;  $n = 18/\text{point}$ ). Experiments were carried out in triplicate. Values indicate means. Error bars indicate SEM. An ANOVA test was used for comparison.  $p < 0.05$  was considered as significant. \* $p < 0.05$ ; \*\* $p < 0.01$ ; and \*\*\* $p < 0.001$ .

more susceptible to the neurotoxic effect of MeHg. Indeed, as shown in Figure 1b, KO CCL2 mice show a significant higher decrease in neuronal cell density in the parietal/sensorimotor cortex in response to MeHg.



TABLE 3  
Inhibition by CCL2 of MeHg-Induced GSH Depletion in  
Cortical Neurons in Culture

	Control	MeHg (500nM)	MeHg (500nM) + CCL2 (10nM)	CCL2 (10nM)
Concentration of GSH (nmol/mg protein)	26.98 ± 1.56	20.09 ± 1.82 *	27 ± 2.1**	26,25 ± 1.84

Note. MeHg (500nM) induced a decrease in GSH levels. This effect was counteracted by 10nM CCL2, which by itself had no effect on GSH ( $n = 12$ ). Values indicate means ± SEM. A two-way ANOVA was used for comparison (Harvey, 1966; Winer, 1971).

[AQ11] \* $p < 0.05$  versus control. \*\* $p < 0.05$  versus MeHg.

455 MeHg has been shown to produce neuronal cell death  
*in vitro* on cerebellar granular cells (Dare *et al.*, 2000; Sakaue  
*et al.*, 2005), hippocampal cells (Falluel-Morel *et al.*, 2007),  
and cultured neural progenitor cells (Ceccatelli *et al.*, 2007;  
460 Tamm *et al.*, 2006; Xu *et al.*, 2010). It was reported that under  
various conditions MeHg caused both necrosis and apoptosis  
(Ceccatelli *et al.*, 2010). We recently showed that MeHg can  
induce *in vitro* cortical neuronal cell death via a caspase-  
dependent apoptosis and suppression of the Rho-family protein  
465 Rac1, resulting in axonal degeneration preceding the apoptotic  
process (Fujimura *et al.*, 2009). The present *in vitro* results  
carried out on pure neuronal cultures, though not analyzing the  
morphological apoptotic process itself, are in agreement with  
these data because the neurotoxic effects of MeHg are obtained  
470 with similar nanomolar concentrations of MeHg (Fujimura  
*et al.*, 2009). Our results not only show a dose-dependent effect  
of MeHg on neuronal cell death but also a time-dependent effect.  
A dose of MeHg that does not induce cell death at 1 day of culture  
can become as deleterious as a much higher concentration after  
475 2–5 days of treatment. Such effect can be seen in the human  
contamination by MeHg with food intake, in particular fish  
consumption, with a body accumulation of MeHg becoming  
deleterious with time (Bourdineaud *et al.*, 2008, 2011).

We have used different approaches to demonstrate that the  
480 lack of CCL2 potentiates the neurotoxic effect of MeHg. We first  
observed an increase in neuronal cell death, which is stronger in  
the presence of MeHg when CCL2 is blocked with either an  
antibody against the chemokine resulting in an undetectable level  
of free CCL2 in the incubation medium or with a nonpeptide  
antagonist known to block the binding of CCL2 to its receptor  
485 CCR2. This suggests that CCL2 via CCR2 may be protective  
against basal or MeHg-induced neuronal cell death. Such  
neuroprotective effect of CCL2 has been already reported  
*in vitro* on *N*-methyl-D-aspartate-induced apoptosis (Bruno *et al.*,  
2000; Eugenin *et al.*, 2003). In regard to the data shown in  
490 Figures 5a and 5b, we cannot exclude the possibility that MeHg  
may induce cell death on a population of neurons that are  
different from a population of neurons undergoing cell death in

response to blockade of CCL2 neurotransmission. Second, the  
*U*-shaped curve on the protective effect of CCL2 observed with  
increasing concentrations of CCL2 (Fig. 5c) suggests opposing  
495 functions depending on the chemokine concentration. Such  
process named “hormesis” relates to adaptative responses to  
compensate disruption in homeostasis by overshooting homeo-  
static feedback controls and was recently suggested to play a role  
in the effects of MeHg (Helmcke and Aschner, 2010). 500

It has been shown that neurons respond to MeHg with ROS  
production, representing a key mechanism by which MeHg causes  
cell damage (Aschner *et al.*, 2007; Yee and Choi, 1996). Brain is  
highly vulnerable to oxidative stress due to its high rate of  
505 metabolism, a low level of oxidative defense mechanisms and  
a high production of ROS resulting in neuronal cell death (Aschner  
*et al.*, 2007). Several reports demonstrated that GSH, which has  
important functions as antioxidant and on detoxification of  
xenobiotics, is a potential molecular target for MeHg neurotoxicity  
(Ceccatelli *et al.*, 2007, 2010; Kaur *et al.*, 2006; Stringari *et al.*, 510  
2008). The beneficial effect of GSH is attributed to its capacity to  
form conjugates with MeHg, facilitating cell efflux of these  
organometallic molecules (Stringari *et al.*, 2008).

Interestingly, the presence of CCL2 is able to counteract the  
depletion in GSH induced by MeHg, suggesting a possible  
515 mechanism by which low concentrations of CCL2 protects  
neuronal cells to the neurotoxic effect of MeHg. Indeed,  
maintenance of adequate GSH levels was shown to protect  
against MeHg-induced oxidative stress in primary neuronal cell  
cultures (Ceccatelli *et al.*, 2010). Though the connection between  
520 CCL2 signaling via CCR2 involving various kinases and GSH is  
not well documented, it has been reported in relation to the  
present work that intracellular GSH redox status modulates the  
expression of CCL2 through redox-sensitive transcription  
factors in a rat model of lung disease (Desai *et al.*, 1999). 525

The present results showing an upregulation of *sod* genes  
following MeHg exposure further demonstrate the implication of  
the oxidative stress in the deleterious effect of MeHg. The fact  
that the aimara fed KO CCL2 mice do not present such  
upregulation of *sod* genes confirms the protective effect of the  
530 chemokine. Furthermore, the observation that KO CCL2 mice  
fed with the control RM1 diet displays *sod* gene expression 60%  
higher than that in wild-type brain is indicative that the oxidative  
level increased in the absence of CCL2. Finally, the twofold  
decrease in the expression level of the *sod2* gene as compared  
535 with wild-type brain in KO CCL2 mice fed with the aimara-  
containing diet demonstrates that CCL2 production is needed to  
stimulate the *sod* gene expression in response to MeHg.

The decrease in brain CCL2 content observed *in vivo* in the  
aimara fed mice also reveals another possible mechanism for  
540 MeHg neurotoxicity. The CCL2 molecule has four cysteins in  
its primary structure, which represent a target for MeHg that  
binds in the same manner to GSH and free cysteins (De Melo  
Reis *et al.*, 2007). According to the respective brain concen-  
545 tration of CCL2 and of Hg, the chemokine can be partially  
neutralized by MeHg.

Finally, we observed that MeHg exposure not only induces neuronal cell death but also increases the number of microglial cells in the sensoricortex of wild-type mice. This increase in microglial cell number is not observed in KO CCL2 mice. It may be due to the known effect of CCL2 on microglial migration, which is hampered in the absence of CCL2 (Meucci, 2010). Although it was not the aim of the present work, future experiments would be of interest to study the impact of microglia in brain responses to MeHg neurotoxicity. Indeed, it was recently shown *in vitro* that microglia may be affected by MeHg (Garg and Chang, 2006; Ni *et al.*, 2011). It has been extensively described that microglia as well as astrocytes can synthesize and release CCL2 (Lawrence *et al.*, 2006; Meucci, 2010). The present *in vivo* and *in vitro* results suggest that the activation of glial cells such as microglia following MeHg contamination may prevent neuronal apoptosis by releasing CCL2 and other protective molecules as shown *in vitro* in the Supplementary figure 1. However, when the glial protection is no more active with time under long-term MeHg toxicity, primarily due to a decrease in detoxification process (Bourdineaud *et al.*, 2008) or to the decrease in the neuroprotective effect of CCL2 (decrease in CCL2 content, absence of CCL2, and/or too much CCL2 released; Fig. 5c), the neurotoxic effect of MeHg can be observed, as shown in the present study following 3 months of low contamination with MeHg.

Taken together, these original data bring new ideas and orientations on the possible implication of the chemokines such as CCL2 in the physiopathology of brain diseases due to heavy metal intoxication. The MeHg pollution in the Minamata Bay in Japan (Eto, 2000) brought light to industrial MeHg contamination and the human health concern on the impact of adverse effects of MeHg contamination through daily fish, seafood, or marine mammal consumption (Myers *et al.*, 2007). Therefore, the present work is highly relevant to better understand the basic neurobiological mechanisms involved in brain MeHg intoxication inducing neurological deficits.

#### SUPPLEMENTARY DATA

Supplementary data are available online at <http://toxsci.oxfordjournals.org/>.

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