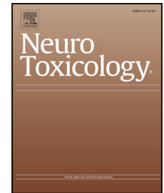
Neurotoxicology 69 (2018) 278–287

Contents lists available at ScienceDirect

Neurotoxicology

journal homepage: www.elsevier.com/locate/neuro



Full Length Article

Neurotransmitter amines and antioxidant agents in neuronal protection against methylmercury-induced cytotoxicity in primary cultures of mice cortical neurons

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ARTICLE INFO

Keywords:
Methylmercury neurotoxicity
Neuroprotection
Dopamine
Serotonin
Antioxidants
Cell viability
Cultured cortical neurons

ABSTRACT

Methylmercury (MeHg) is an environmental toxicant with detrimental effects on the developing brain and adult nervous system. The main mechanisms identified include oxidative stress, changes in intracellular calcium, mitochondrial changes, inhibition of glutamate uptake, of protein synthesis and disruption of microtubules. However, little is known about mechanisms of protection against MeHg neurotoxicity. We found that resveratrol (10 μM) and ascorbic acid (200 μM) protected MeHg-induced cell death in primary cultures of cortical neurons. In this work, we aimed at finding additional targets that may be related to MeHg mode of action in cell toxicity with special emphasis in cell protection. We wonder whether neurotransmitters may affect the MeHg effects on neuronal death. Our findings show that neurons exposed to low MeHg concentrations exhibit less mortality if co-exposed to 10 μM dopamine (DA). However, DA metabolites, HVA (homovanillic acid) and DOPAC (3,4-dihydroxyphenylacetic acid) are not responsible for such protection. Furthermore, both DA D1 and D2 receptors agonists showed a protective effect against MeHg toxicity. It is striking though that DA receptor antagonists SKF83566 (10 μM) and haloperidol (10 μM) did not inhibit DA protection against MeHg. In addition, the protective effect of 10 μM DA against MeHg-induced toxicity was not affected by additional organochlorine pollutants exposure. Our results also demonstrate that cells exposed to MeHg in presence of 100 μM acetylcholine (ACh), show an increase in cell mortality at the "threshold value" of 100 nM MeHg. Finally, norepinephrine (10 μM) and serotonin (20 μM) also had an effect on cell protection. Altogether, we propose to further investigate the additional mechanisms that may be playing an important role in MeHg-induced cytotoxicity.

1. Introduction

Methylmercury (MeHg), a relevant persistent environmental contaminant, is widely recognized as a potent neurotoxicantin humans (WHO, 1990) that affects both the developing and mature central nervous system (CNS) (Karagas et al., 2012). MeHg from natural or anthropogenic sources biomagnifies through the food chain and gives rise to human exposure primarily through consumption of higher trophic level fish and marine mammals [National Research Council (NRC) 2000].

Individual and community studies have contributed with clear evidence indicating that maternal consumption of MeHg can have serious and irreversible effects on the physical and mental development of children, even if the mother exhibits much less or none outward symptoms (Ekino et al., 2007; Grandjean et al., 2010; Harada, 2010; Debes et al., 2016). Nonetheless, the evolution of insights into MeHg neurotoxicity continues to be challenging due to the incomplete documentation about prenatal neurotoxicant exposures and eventual neurodevelopmental deficits (Granjean and Herz, 2011).

In addition to the epidemiological studies, *in vitro* approaches based on cultured cells, isolated mitochondria and tissue slices, as well as *in vivo* studies based mainly on the use of rodents, have helped to understand MeHg-induced excitotoxicity (Farina et al., 2011a). Some of the most proved effects of MeHg include the perturbation of intracellular Ca^{+2} levels, alteration of glutamate homeostasis and oxidative stress probably due to depletion of intracellular antioxidants, the inhibition of critical enzymes and the modulation of the activity of transporter and neurotransmitter or neuromodulator receptor activity

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https://doi.org/10.1016/j.neuro.2018.07.020
Received 28 November 2017; Received in revised form 26 July 2018; Accepted 30 July 2018
Available online 31 July 2018
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(reviewed by Farina et al., 2011a,b).

A neuronal target that has been related to MeHg-induced neurotoxicity is the dopamine (DA) neurochemical homeostasis (for review see Farina et al., 2017). MeHg impairs the canonical metabolism and the transport of DA. Several reports show that MeHg either increases or decreases the levels of DA and its metabolite DOPAC (3,4-dihydroxyphenylacetic acid) (O'Kusky et al., 1988; Dreiem et al., 2009; Agrawal et al., 2015; Tiernan et al., 2015), decreases the density of D1-like receptors in rat cortex and the affinity and density of D2-like receptors in the cortex and caudate-putamen (Coccini et al., 2011; Daré et al., 2003), and decreases the gene expression of dopamine receptors (Drd1a, Drd2 and Drd3) (Zimmer et al., 2011) while it increases the spontaneous release of DA (Faro et al., 2002; Dreiem et al., 2009), and increases DA-mediated locomotor activity (Daré et al., 2003). Early-life MeHg exposure was shown to be a risk factor for loss of dopaminergic function later in life in wildtype worms (Martinez-Finley et al., 2013). Furthermore, a genomic/proteomic study using MeHg- and MPP+-treated dopaminergic neuron cells showed that dopaminergic signaling transduction was the most affected pathway. Indeed, 61 common proteins were changed by MeHg and MPP+ treatment, some of them involved in Parkinson disease (PD) and other neurodegenerative diseases (Shao et al., 2015). Furthermore, epidemiological studies based on the Faroe's Island population led to the conclusion that the intake of whale meat and blubber (both a vehicle for oral MeHg exposure) in adult life were significantly associated with PD (Petersen et al., 2008a), however this association was not seen when considering prenatal MeHg exposure through maternal whale meat ingestion (Petersen et al., 2008b). On the other hand, increased levels of serotonin (5-HT) in rat cerebral cortex after early postnatal MeHg exposure, and decreased levels of 5-HT on the brain extracellular fluid in zebrafish acutely exposed to low-levels of MeHg, which were accompanied by anxiety-like symptoms and increased lipid peroxidation, have been described (O'Kusky et al., 1988; Maximino et al., 2011). It has also been reported that MeHg affects cholinergic neurotransmission. It acts as a competitive inhibitor of muscarinic cholinergic receptors (mAChR) in rat brain and other mammals, while after repeated in vivo MeHg exposure the density of mAChR was affected in a brain-area, gender-, time and developmental period-dependent fashion (Coccini et al., 2000, 2007; Basu et al., 2008).

In addition to the study of MeHg toxicity using several model systems, the effectiveness of different agents to protect or reverse MeHg effects has been investigated for at least the past fifteen years. For instance, Farina et al. (2003) demonstrated that *in vivo* exposure to MeHg causes a dose-dependent decrease in glutamate uptake and that the organoselenium drug ebselen, which did not affect the uptake per se, reverted this effect. Protection by ebselen was confirmed a few years later by Roos et al. (2009). Also, these authors showed that guanosine and diphenyl diselenide could protect cortical rat brain slices from MeHg-induced reactive oxygen species (ROS) generation. Farina et al. (2005) extended their research by studying the possible protective effects of *Polygala paniculata* extract against methylmercury (MeHg)-induced neurotoxicity in adult mice. They found that MeHg exposure significantly inhibited glutathione peroxidase and increased the levels of thiobarbituric acid reactive substances in the cerebral cortex and cerebellum; these effects as well as the behavioral interference in the MeHg exposed animals being prevented by administration of *Polygala* extract. Following this line of research, the same group examined the potential protective effects of three compounds isolated from *Polygala* species against MeHg- and mercuric chloride ($HgCl_2$)-induced disruption of mitochondrial-enriched fractions from mouse brains (Franco et al., 2007). Among the isolated compounds, they found that only quercetin, which did not display chelating effects on MeHg or $HgCl_2$, prevented mercurial-induced glutathione oxidation. However, another work showed controversial results when studying the beneficial effects of quercetin against the neurotoxicity induced by MeHg in Swiss female mice (Martins et al., 2009). They found that MeHg plus quercetin elicited a higher cerebellar lipid peroxidation than MeHg or quercetin alone. These authors suggest that under *in vivo* conditions quercetin and MeHg cause additive pro-oxidative effects toward the mice cerebellum and that such phenomenon is associated with the observed motor deficit. New compounds continued to be studied aiming to find protection against MeHg deleterious effects and to further understand the mechanisms involved in MeHg cytotoxicity. Among these compounds are probucol (Farina et al., 2009), the sex steroid 17β-estradiol (Daré et al., 2000; Malagutti et al., 2009), the flavonoid quercetin (Wagner et al., 2010), the carotenoid lycopene (Qu et al., 2013), the flavonoid chrysin, found mainly in passion fruit (Manzolli et al., 2015), tea polyphenols (Liu et al., 2011, 2014), vitamin K (Sakaue et al., 2011) and vitamin B3 also known as niacin (Silva de Paula et al., 2016). All these studies proved that protection against MeHg is mainly attributed to the scavenging activities of these compounds and their ability to restore glutathione peroxidase levels which is in agreement with the widely accepted mechanisms of MeHg-mediated neurotoxicity: oxidative stress and mitochondrial dysfunction (Qu et al., 2013). Nonetheless, there is yet much to discover about the mechanisms of protection against MeHg neurotoxicity and more importantly, how all these effects (MeHg plus protective agents), affect or correlate with other important molecules present in the neural cells (Branco et al., 2017).

Besides MeHg, another group of environmental pollutants was investigated: Persistent Organic Pollutants (POPs). POPs refer to organic compounds which remain in the surroundings and persist in living beings, with extensive half-lives in ecological systems and biota. Those mostly lipophilic compounds, which concentrate in adipose tissue, gradually accumulate in the bodies of predator animals along the food chain and exhibit toxic effects in living organisms (La Merrill et al., 2013). POPs comprise aliphatic and aromatic compounds with at least one chlorine substitute. This chlorine substitute contributes to the organochlorines' lipophilic character, increasing their uptake and storage in fatty tissue including the brain (Covaci et al., 2004; Dang et al., 2016; Arrebola et al., 2013; Roncati et al., 2016). There is evidence for an interaction between MeHg and polychlorinated biphenyls (PCBs), also present in marine food, (Piedrafita et al., 2008; Rice, 2008; Cauli et al., 2013). In two epidemiological studies, the interaction was revealed when the cohorts were divided by categories with respect to MeHg and PCB exposure, where the neurological effects of PCBs were stronger at the highest methylmercury exposure (Rice, 2008). In another study, PCBs and seven organochlorine compounds showed a negative association with glutamate levels in human cord blood, this association being weakened at high mercury exposure (Palou-Serra et al., 2014).

In this work, we aimed at finding additional targets that may be related to MeHg mode of action in cell toxicity with special emphasis in cell protection by using primary cultured cortical neurons. We evaluated the effects of molecules already present in the nervous cells, such as neurotransmitters, in order to increase our knowledge about how these molecules interact with MeHg.

2. Materials and methods

2.1. Materials

Pregnant NMRI mice (16th gestational day) were obtained from Charles River/Iffa Credo (Saint Germain-sur-l'Arbreste, France). Plastic multi-well culture plates were purchased from Nunc (Rockilde, Denmark). Fetal bovine serum (FBS) was from Gibco (Invitrogen, Barcelona, Spain). Dulbecco's modified Eagle's minimum essential medium (DMEM) was obtained from Biochrom (Berlin, Germany). Isoflurane (FORANE) was from Abbot Laboratories (Madrid, Spain). Dimethyl sulfoxide (DMSO), poly-D-lysine, trypsin, soybean trypsin inhibitor, bovine serum albumin (BSA), DNAse, HEPES, 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), methylmercury(II) chloride, acetylcholine chloride, dopamine hydrochloride, raclopride, dieldrin, hexachlorobenzene, apomorphine,

norepinephrine, serotonin, homovanillic acid, 3,4-dihydroxyphenylacetic acid (DOPAC), haloperidol, resveratrol and ascorbic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). PCB153 (35602), 4,4'-DDE (C12041000) and PCB138 (C200138001) were from Dr. EhrenstorferGmbH (Augsburg, Germany). SKF38393 hydrobromide and SKF83566 hydrobromide were from Tocris (Bristol, UK). Monochlorobimane was from Molecular Probes (Thermo Fischer Sci.). Any other reagents were also purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Neuronal cell cultures

Primary cultures of cortical neurons were prepared from cerebral cortices of 16th gestational day mice fetuses as previously described (Briz et al., 2010; Regueiro et al., 2015). Pregnant animals were anesthetized with isoflurane, killed by cervical dislocation, and the fetuses were extracted. Cortices were dissected with forceps and mechanically minced, and cells were then dissociated by mild trypsinization 0.02% (w/v) at 37 °C for 10 min followed by trituration in a DNAse solution 0.004% (w/v) containing soybean trypsin inhibitor 0.05% (w/v). The cells were then suspended in DMEM containing 31 mM glucose, and 0.2 mM glutamine supplemented with p-aminobenzoate, insulin, penicillin, and 10% fetal bovine serum. The cell suspension (1.5×10^6 cells per milliliter) was seeded in 6-, 24- or 96-well plates precoated with poly-D-lysine and incubated in a humidified 5% CO2/95% air atmosphere at 37 °C A mixture of 5 μM 5-fluoro-2'-deoxyuridine and 20 μM uridine was added at 24–48 h in culture to prevent glial proliferation. Animals were handled in compliance with protocols approved by the Generalitat de Catalunya (DAAM 323/14), Spain, following European Union Guidelines. All efforts were carried out to reduce the number of animals and minimize their suffering.

2.3. Cell treatments

Cells were seeded in 96-well plates for viability and enzymatic assays and in 24-well plates for immunocytochemistry assays. Cultured cortical neurons (CCN) were exposed to increasing concentrations of MeHg (0–600 nM) and/or the suspected protective agents by adding concentrated solutions of these compounds directly to the culture medium at 24 h after seeding (day in vitro (div) 1), and kept in the same medium up to 5–7 div. MeHg exposure was simultaneous to the treatment with the different compounds unless otherwise indicated, a protocol previously used in long-term exposure paradigms (Farina et al., 2009; Caballero et al., 2017). MeHg was dissolved in deionized water. MeHg solutions were handled in compliance of safety measures for toxic chemicals. Resveratrol, dopamine (DA), homovanillic acid (HVA), 3,4-dihydroxyphenylacetic acid (DOPAC), SKF38393, apomorfine, SKF83566, raclopride, haloperidol and organochlorine pollutants (OCP) were dissolved in dimethylsulfoxide (DMSO), whose concentration did not exceed 0.1% in the culture medium. Ascorbic acid (AA) and acetylcholine (ACh) were dissolved in deionized water.

2.4. Cell viability assays

Cell viability was first assessed by visual inspection under phase-contrast microscopy and then by the MTT assay (Mossman, 1983), which is based on the reduction by viable cells of 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) to a formazan product via a group of nonspecific mitochondrial dehydrogenases as previously described (Regueiro et al., 2015). Cell viability was expressed as a percentage of the controls. Immunocytochemistry was used to get a qualitative image of cells exposed to the variety of compounds. For immunocytochemistry, cells were seeded in 24-well plates. After discarding the solutions of the plate, 4% paraformaldehyde (PFA) was added for 10–15 min to fix the cells. PFA was removed from the plate and cells were washed three times for 5–10 min with PBS. Cell membranes were permeabilized with 0.15% v/v triton-PBS for 15 min to allow antibodies to enter. Blocking unspecific binding sites was done by incubating cells with 1% w/v BSA-PBS for 1 h. Primary antibodies were diluted in 0.1% w/v BSA-PBS, 1:500 rabbit polyclonal anti-Tau (Sigma T-6402) and 1:1000 mouse monoclonal anti-Glial Fibrillary Acidic Protein (GFAP, Sigma G3893), and added to the plate. Incubation of the cells with the primary antibodies was done in agitation at 4 °C overnight. On the following day, cells were washed three times with PBS for 5–10 min. Secondary antibodies were diluted in 0.1% w/v BSA-PBS, 1:1000 green goat anti-rabbit IgG (Alexa-488 Molecular Probes, A11008) and 1:1000 red goat anti-mouse IgG (Alexa Fluor 594 Molecular Probes, A11032). Cells were incubated with secondary antibodies at room temperature for 1 h protected from light. Next, cells were washed once with PBS for 5–10 min and incubated with 5 μM bisbenzimide (nuclear dye) in PBS for 5–10 min and, as final step, washed with PBS for 5–10 min. Photographs were taken using a Leica DMI 4000B fluorescence microscope. For D1 and D2 receptor detection the primary antibodies were diluted in 0.1% w/v BSA-PBS, 1:1000 rabbit polyclonal anti-D1R (Abcam, ab20066) and 1:1000 goat polyclonal anti-D2R (Abcam, ab 30743). Tyrosine hydroxylase (TyrH) positive neurons were detected using the same procedure with rabbit polyclonal anti-TyrH primary antibody (1:1000 dilution; Abcam, ab 112) and 1:1000 green goat anti-rabbit IgG (Alexa-488 Molecular Probes, A11008).

2.5. Determination of reduced glutathione (GSH) in a cell-free sample

In this assay, monochlorobimane is converted into a fluorescent compound after forming an adduct with GSH, this reaction being catalyzed by glutathione S-transferase. Solutions of methylmercury and DA in PBS were incubated overnight at 37 °C. Thereafter, GSH and glutathione *S*-transferase were added and the solutions were incubated for 2 h at 37 °C. Monochlorobimane was added for 60 min and fluorescence was read at Ex394/Em490 nm.

2.6. Statistical analysis

Variables are reported as mean values ± SEM calculated for independent experiments, each performed in triplicate. Data were analyzed using GraphPad Prism v.4 for Windows. Two-way ANOVA followed by post-hoc comparison Bonferroni's tests were used for comparing all groups studied.

3. Results

3.1. MeHg-induced cell death is protected by dopamine, norepinephrine and serotonin

In an attempt to investigate how different neurotransmitters might play a role in MeHg-induced cytotoxicity and especially under the consideration that MeHg may be involved in Parkinson's disease (Petersen et al., 2008a,b; Shao et al., 2015), dopamine was the first neurotransmitter tested in presence of MeHg. As shown in Fig. 1A, 10 μM dopamine significantly protected cells from low concentrations of MeHg-induced toxicity. Since dopamine is metabolized into homovanillic acid (HVA) by different breakdown pathways, HVA and the intermediary metabolite DOPAC were also tested. Neither HVA nor DOPAC were able to protect cells from MeHg toxicity (Fig. 1B). Dopamine protective effect was also proven by immunohistochemistry (Fig. 1C–h vs Fig. 1C–d).

From the results obtained by dopamine and their metabolites we sought to investigate other monoamine neurotransmitters such as norepinephrine and serotonin. Fig. 2 shows that 10 μM norepinephrine and 20 μM serotonin were able to significantly protect cells from 100 nM MeHg-induced toxicity, however they did not protect against higher MeHg concentrations. Comparison of the effects of these monoamines,

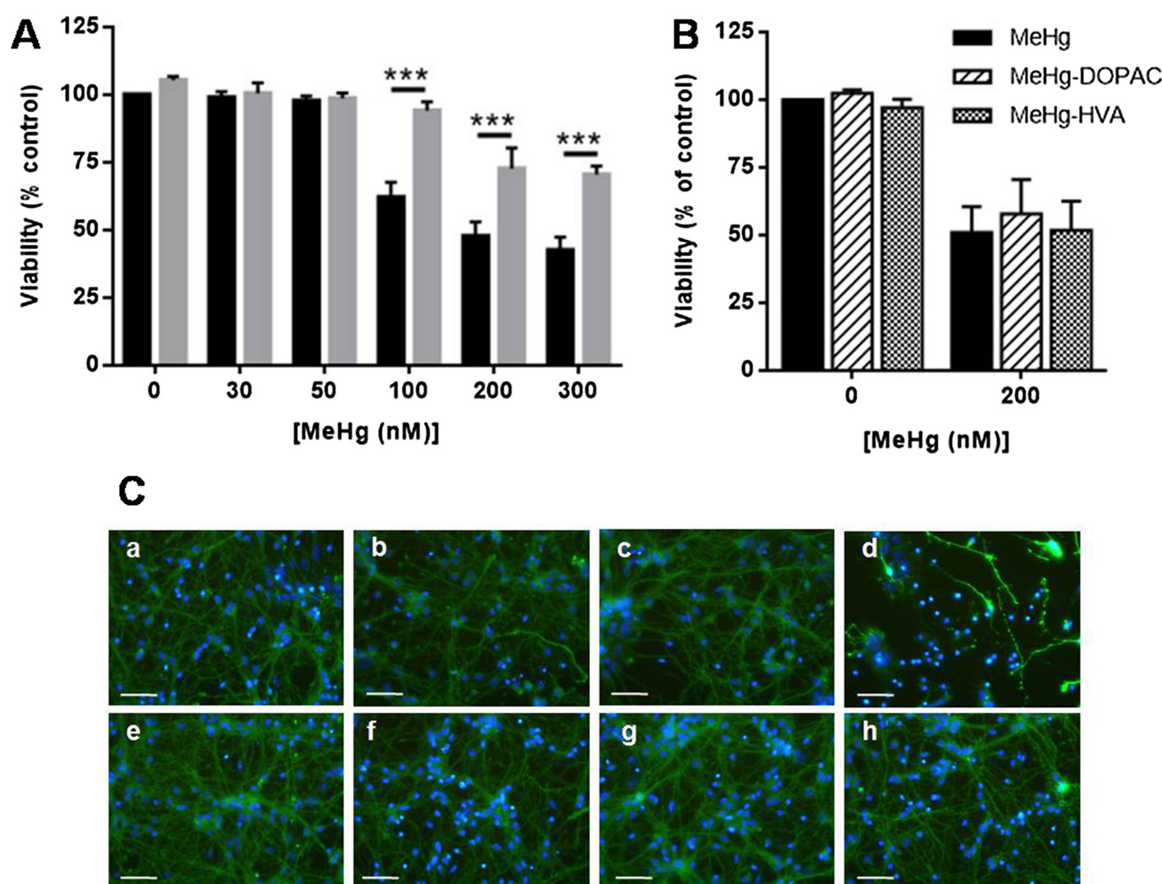

Fig. 1. Dopamine decreases MeHg-induced toxicity in CCN. Cells were exposed to MeHg from div 1 to div 7. Cell viability was evaluated at div 7 by the reduction of MTT. A) Cells were exposed to MeHg only (black columns) or MeHg plus 10 μM dopamine (grey columns). B) Cells were exposed to MeHg only or MeHg plus 5 μM of each dopamine metabolites DOPAC and homovanillic acid (HVA). Results are expressed as percent of survival cells compared to control values. Data are expressed as mean ± SEM (N = 3–6 independent experiments). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs cells exposed to MeHg only, Bonferroni's test after two-way ANOVA. C) Images representing the different treatments: a–d) 0, 30, 50, 100 nM MeHg only; e–h) 0, 30, 50, 100 nM MeHg plus dopamine. Immunohistochemical labeling of CCN by Tau (green cells) and nuclei were stained by DAPI (blue). The scale bar represents 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

where DA was the most effective, prompted us to further deep on possible mechanisms of neuroprotection by DA.

3.2. Dopamine does not chelate MeHg

We thought about the possibility that DA could be forming a chemical complex with MeHg that would prevent the action of the latter in the cells. We determined reduced GSH in a cell-free sample in the absence and the presence of MeHg or MeHg plus DA by using the monochlorobimane assay. A linear fluorescence response was found for the formation of the GS-monochlorobimane adduct (Fig. 3). Based on the complex formation of methylmercury with GSH through the binding

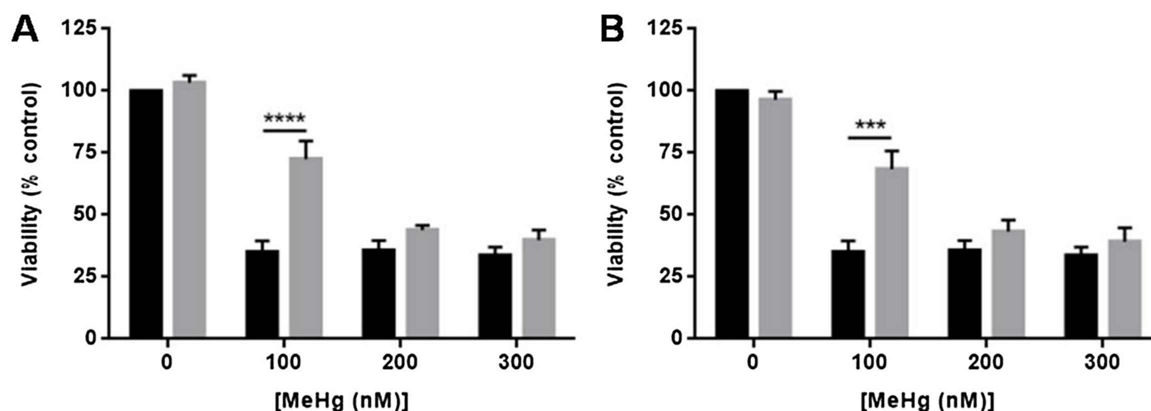

Fig. 2. Norepinephrine and serotonin decrease MeHg-induced toxicity in CCN. Cells were exposed to MeHg from day div 1 to div 7. Cell viability was evaluated at div 7 by the reduction of MTT. A) Cells were exposed to MeHg only (black columns) or MeHg plus 10 μM norepinephrine (grey columns). B) Cells were exposed to MeHg only or MeHg plus 20 μM serotonin. Results are expressed as percent of survival cells compared to control values. Data are expressed as mean ± SEM (N = 3 independent experiments). ***$p < 0.001$, ****$p < 0.0001$ vs cells exposed to MeHg only, Bonferroni's test after two-way ANOVA.

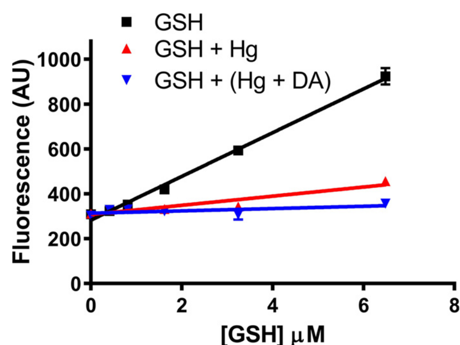

Fig. 3. Fluorescence response of the GS-monochlorobimane adduct (■, black line). MeHg (27 µM) was incubated overnight at 37 °C without/with DA (13 µM) (▲ red line, and ▼ blue line, respectively). Thereafter, GSH (0, 4–6, 5 µM) was incubated with MeHg or MeHg + DA for 2 h. Reduced GSH was determined after reaction with monochlorobimane, catalyzed by Glutation-*S*-transferasa. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to and the inactivation of the SH group in the cysteine moiety, we hypothesized that the formation of the adduct MeHg-SG through an S bonding would diminish the amount of reduced GSH in a cell-free sample containing GSH and MeHg. Fig. 3 also shows that MeHg (▲, red line) and MeHg plus DA (▼, blue line) reduced the formation of the GS-monochlorobimane adduct (■, black line) on the same extension, thus indicating that DA did not avoid the effect of MeHg on GSH in a cell-free system. These results do not support a chemical chelating interaction between MeHg and dopamine.

3.3. Cell death induced by coexposure to MeHg and POPs is protected by dopamine

Due to the protecting effect of 10 µM DA, we wanted to investigate whether the same viability protecting effect could also be observed if cells were exposed not only to MeHg but additionally to a POP mixture. The concentration of the five organochlorine compounds used in this work were based on the INMA Project Valencia mother-infant cohort study (Vizcaino et al., 2010; Guxens et al., 2012; Childhood and environment, a study involving several mother-child cohorts in Spain with the aim to investigate the impact of the environmental pollutants on children). 4.4’-DDE, β-HCH, HCB, PCB-138 and PCB-153 were used as mixture, since these toxicants were detected in high percentage of the population cohort (78–100%). Concentrations were used at 10 or 100 times the geometric mean (GM) values of concentrations detected in cord blood of the INMA project and are summarized in Table 1. These concentrations cover the range of concentrations found on these samples (highest range values were 14–52 fold those of mean values). Cells were exposed consequently for 8–9 days from div1 to MeHg concentrations ranging from 0 to 300 nM and the mixture of POPs at 10x GM and 100× GM concentrations. No statistically significant toxic effects of the POPs mixture at 10× or 100× GM, nor statistically significant interactive cytotoxic effects between the different treatment

Table 1
Organochlorine toxicant concentrations found in samples of cord blood in the INMA project (references: Guxens et al., 2012; Vizcaino et al., 2010).

OCP compound	Geometric Mean (GM) Values ng/ml	100× GM Values ng/ml (nM)	10× GM Values nM
4,4’-DDE	0.82	82 (258)	25.8
HCB	0.29	29 (102)	10.2
β-HCH	0.12	12 (41)	4.1
δ-HCH	0.023	2.3 (7.9)	0.79
PCB-138	0.1	10 (28)	2.8
PCB-153	0.13	13 (36)	3.6

conditions were found, as illustrated in Fig. 4A. Thereafter, CCN were exposed for 5–6 days to the 100× GM POPs mixture plus MeHg at different concentrations (0–300 nM) in absence or presence of 10 µM DA. Fig. 4B shows that 10 µM DA significantly protected cells exposed to both MeHg and POP mixture.

3.4. Does dopamine protect through activation of DA receptors?

Dopamine can interact with different receptors, therefore D1-like and D2-like receptors were investigated as possible mediators for dopamine effect in CCN. The presence of these receptors in cultured cortical neurons was proven by immunohistological labeling (Fig. 5A). In addition, the presence of dopaminergic neurons was evaluated by anti-TyrH staining (Fig. 5B). The results show that the number of dopaminergic neurons in the CCN culture was scarce.

Fig. 6 shows the results obtained when D1 and D2-like dopamine receptors were treated by the dopamine agonists SKF-38393 and apomorphine (Fig. 6A and B, respectively) or the antagonists SKF-83566 and haloperidol (Fig. 6C and D, respectively). When cells were treated with agonists no dopamine was added to the culture. However, when cells were treated with dopamine antagonists, dopamine was also added to the culture. When the D1 agonist SKF-38393 was applied together with MeHg cell viability was maintained until div 7 as depicted in Fig. 6A. Cell viability was also maintained when D2-like dopamine agonist apomorphine was applied (Fig. 6B). These results suggest that DA receptors could mediate the protectant effect of DA against MeHg-induced toxicity. Finally, cell viability was significantly protected against MeHg-induced toxicity by DA plus either the D1-like dopamine antagonist SKF-83566 (Fig. 6C) and the D2-like dopamine antagonist haloperidol (Fig. 6D).

3.5. Methylmercury-induced cytotoxicity is avoided by resveratrol and ascorbic acid

The fact that DA antagonists did not inhibit the protective effect of DA weakened the conclusion that DA was acting exclusively on dopaminergic receptors. An action of DA on cellular redox status was envisaged based on its scavenging effects on reactive oxygen species (Yen and Hsieh, 1997). There are much more data in the literature on the cytotoxic effect of methylmercury and its protection by antioxidants in cultured cerebellar granule cells (CGC) than in cultured cortical neurons (CCN). Furthermore, we have previously reported that toxicity induced by prolonged exposure to MeHg was significantly higher in CGC than in CCN, this toxicity being rescued by the antioxidant probucol (Caballero et al., 2017). Thus, we consider of interest to show the protection by natural antioxidants against MeHg-induced toxicity in CCN. The protective effect of resveratrol (10 µM) and ascorbic acid (200 µM) against MeHg-induced toxicity was determined in cultures of cortical neurons (CCN) by the addition of different MeHg concentrations (0–500 nM) from div 1 to div 7 together with the corresponding antioxidant (Fig. 7). Fig. 7B shows that resveratrol prevented the cellular death induced by MeHg in the range of 100–300 nM MeHg concentrations. Likewise, Fig. 7A shows the protective effect of ascorbic acid although in this case the antioxidant was able to reduce cell mortality at a higher range including 500 nM MeHg. In fact, it seems as if ascorbic acid were stimulating cell viability since it is higher in the lower MeHg concentrations as well. Fig. 7C shows tau-immunostaining representative of the different treatments at 0, 30, 50 and 100 nM MeHg depicting the protective effect of both antioxidants. It is striking though, that in the absence of antioxidant, 100 nM MeHg might be more toxic to neurons than the MTT analysis can determine as depicted by tau labeling (Fig. 7C_d). Also, the complete breakdown of the neurite extension was recovered by ascorbic acid (Fig. 7C_h and resveratrol (Fig. 7C_l).

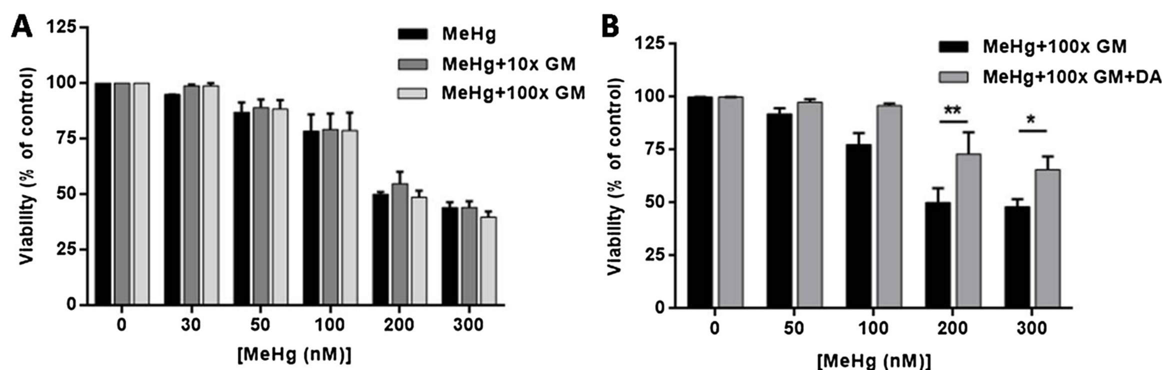

Fig. 4. A) POP mixtures do not modify MeHg-induced toxicity in CCN. Cells were exposed to MeHg and POPs mixture at 10× or 100× GM from div 1 and cell viability was evaluated at div 7 by the reduction of MTT. B) Dopamine decrease MeHg-POPs mixture-induced toxicity in CCN. Cells were exposed to MeHg-POPs only (black columns) or MeHg-POPs plus 10 μM dopamine (grey columns). Results are expressed as percent of survival cells compared to control values. Data are expressed as mean ± SEM (N = 3–6 independent experiments). *$p < 0.05$, **$p < 0.01$ vs cells exposed to MeHg only, Bonferroni's test after two-way ANOVA.

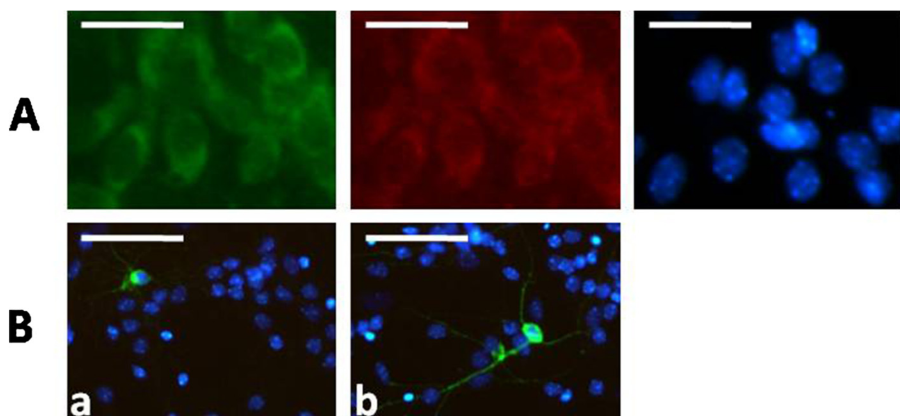

Fig. 5. A) Immunohistochemical labeling of CCN by Anti-Dopamine Receptor D1 (green cells), Anti-Dopamine Receptor D2 (red cells), nuclei were stained by DAPI (blue). The scale bar represents 25 μm. B) Immunohistochemical labeling of CCN by anti-TyrH (green cells) and cells nuclei (blue). TyrH positive neurons were detected in presence a) or absence b) of dopamine. The scale bar represents 100 μm. Cell fixation was performed at div 7. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.6. Acetylcholine increases MeHg-induced toxicity in CCN

In further neurotransmitters testing it was found that concentrations up to 100 μM ACh did not change cell survival (data not shown), which is why in the next experiments 100 μM were used. Acetylcholine proved to slightly, although significantly increased cell death in the presence of low concentration (100–200 μM) of MeHg (Fig. 8).

4. Discussion

Our group has previously shown that prolonged nanomolar exposure (≥5 div) to MeHg in CCN primary cultures was significantly associated with increased cell death (Caballero et al., 2017). The concentrations of MeHg used in this work (0–500 nM) cover the range of concentrations found in general population (mean and percentile 75 values of Hg levels in the cord blood in the cohort INMA-Valencia (Spain) correspond to 43 nM and 198 nM MeHg, respectively; Palou-Serra et al., 2014). Taking into account the relationships between cord blood and maternal blood (1,6 fold; Sakamoto et al., 2008) and between brain and blood (6 times; Cernichiari et al., 2007) in humans, the concentrations of MeHg used in this work might likely represent actual exposure scenarios for the nervous system.

A hallmark of MeHg intoxication in Minamata disease is cerebellar ataxia caused by damage to the layer of granule cells in cerebellum. Furthermore, damage to the occipital and temporal lobes of the cortex has also been reported in acute poisoning cases (Ekino et al., 2007; Harada, 2010). In addition, the fetal exposure to MeHg has been reportedly linked to deficits in early child development (Grandjean et al., 2010) that remained detectable through young adult age in the Faroe's birth cohort (Debes et al., 2016), which might involve cortical areas. The cortex receives inputs from aminergic neurons (cholinergic, dopaminergic, noradrenergic and serotonergic). Monoamine neurotransmission systems (ACh, 5HT, DA, NE) are characterized by the fact that their neurons are of long, deeply branched axons, whose cell bodies are concentrated mainly in areas of the midbrain, brainstem and basal brain. Dopamine terminals synapse on dendritic spines and shafts of pyramidal cells in the prefrontal cortex (Carr et al., 1999) and provide direct input to both excitatory and inhibitory cells in the monkey entorhinal cortex (Erickson et al., 2000).

The results of this work show that dopamine has a significant protective effect. It is not clear how this mechanism is working out, since neither SKF-83566 nor haloperidol (D1- and D2-receptor antagonists) inhibited the protectant effect of DA against MeHg-induced cell death. This suggests that dopamine may be acting independently of D1 or D2 receptors. Nonetheless, it is also possible that dopamine being present was sufficient enough to compete with the antagonists and still protect cells from MeHg toxicity. Although the association between MeHg exposure and PD has not yet been established, a significant amount of data suggests that exposure to mercury can have substantial impact on the normal functioning of the dopamine system and consequently on PD development (Caudle et al., 2012; Shao et al., 2015). In this regard, it has been shown that MeHg alters DA metabolic profile in undifferentiated PC12 cells and shunts DA metabolism along the alternative reductive metabolic pathway (Tiernan et al., 2015). The consequence of impaired DA metabolism contributes to accumulation of 3,4-dihydroxyphenylaldehyde (DOPAL), a toxic DA metabolic intermediate. In the present work, if DA metabolism was affected, this change was not enough to reduce cell viability. In this sense, Mohamed Moosa et al. (2014) showed that following exposure to 6-OHDA (oxidopamine or 6-hydroxydopamine), animals that were exposed to MeHg prenatally had a significantly lower antioxidant capacity than that of controls. 6-OHDA is a neurotoxic synthetic organic compound that

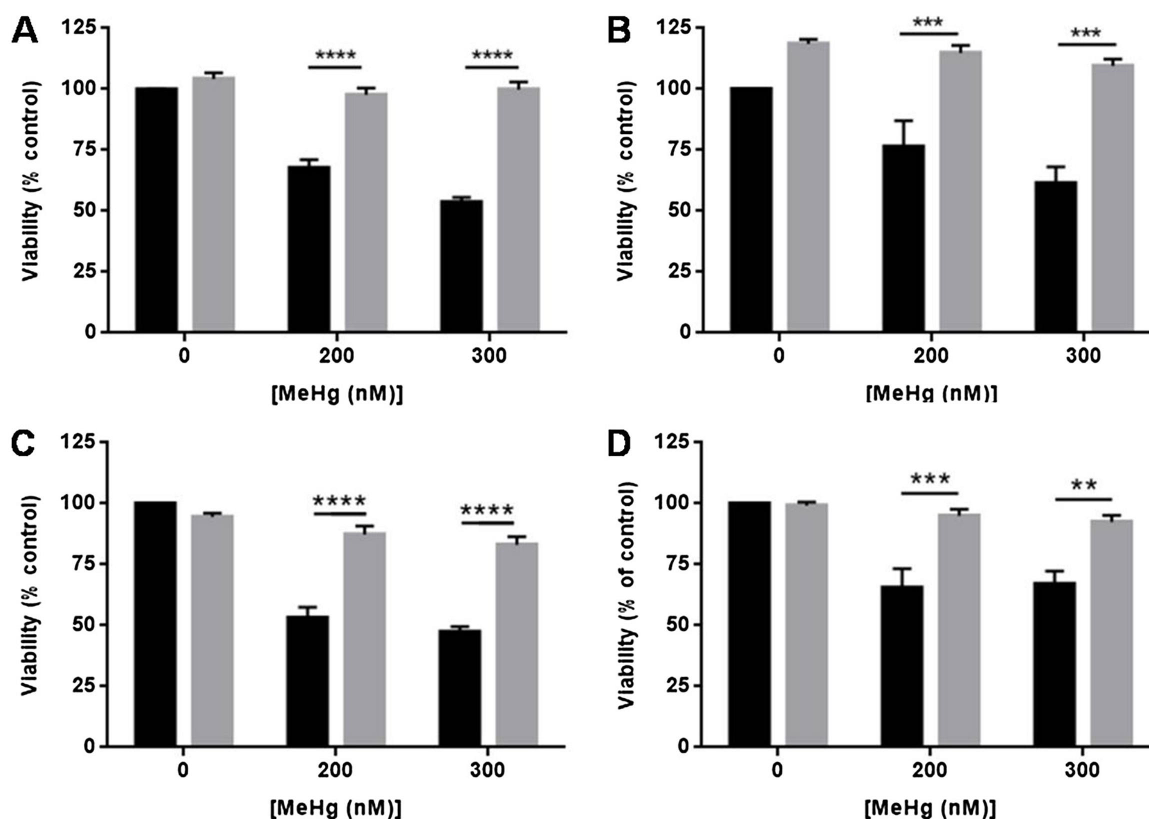

Fig. 6. Effects of dopamine agonists and antagonists against MeHg-induced cytotoxicity. A) Cells were exposed to MeHg only (black columns) or MeHg plus 10 μM D1-like dopamine receptor agonist SKF-38393 (grey columns). B) Cells were exposed to MeHg only (black columns) or MeHg plus 10 μM D2-like dopamine receptor agonist apomorphine (grey columns). C) Cells were exposed to MeHg only (black columns) or MeHg plus 10 μM D1-like dopamine receptor antagonist SKF-83566 and 10 μM dopamine (grey columns). D) Cells were exposed to MeHg only (black columns) or MeHg plus 0.5 μM D2-like dopamine receptor antagonist haloperidol and 10 μM dopamine (grey columns). Results are expressed as percent of survival cells compared to control values. Data are expressed as mean ± SEM (N = 3 independent experiments). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ vs cells exposed to MeHg only, Bonferroni's test after two-way ANOVA.

selectively destroys dopaminergic and noradrenergic neurons in the brain. Therefore, dopamine and norepinephrine might be connected to the cell antioxidant capacity. Other report suggested that dopaminergic pathways are sensible to MeHg toxicity and this would result in the interference with cholinergic mechanisms leading to neuromuscular dysfunction (Sharma et al., 1982), one of the known symptoms after MeHg intoxication. Nonetheless, results are still unclear probably due to the use of a great diversity of models and different treatments.

On the other hand, we also show that dopamine D1 and D2 receptor agonists protect cells against MeHg toxicity. Therefore, based on our results we hypothesize that either the stimulation of only one of the two dopamine receptors is enough to elicit the protective effect or it is possible that DA could be acting in a different target to reduce MeHg toxicity. A different target could include the reactive oxygen species. The antioxidant and free radical scavenging effects of dopamine and norepinephrine have been reported (Yen and Hsieh, 1997). They found that the antioxidant and scavenging activities of dopamine were slightly higher than those of the reference antioxidant alpha-tocopherol, and higher than that of norepinephrine. Structural analysis of catecholamine neurotransmitters indicates that these molecules can exhibit antioxidant activity due to the presence of a catechol moiety (Jodko-Piórecka and Litwinienko, 2015). When applied at concentrations below 30 μM, catecholamines such as dopamine and norepinephrine, promote long-term survival of the dopaminergic neurons. Their protective effect against cell death is connected with decreasing concentrations of intracellular ROS (Troadec et al., 2001) and does not depend on the activation of catecholamine receptors (Noh et al., 1999; Troadec et al., 2001). It is worth to note that the concentration of dopamine used in this work is close to reported physiological concentrations in rat cortex (1,8 μM in basal extracellular rat cortex, which increase 4–6 fold after a depolarizing or chemical challenge; Romero et al., 1998; Díaz-Mataix et al., 2005). Our results also show that common human-exposed organochlorine compounds do not modify the pattern of MeHg-induced cytotoxicity in cultured cortical neurons, neither counteract the protectant effect of DA.

In this work, our results show that cell death in CCN primary cultures after exposure to MeHg was diminished by co-treatment with the antioxidants ascorbic acid and resveratrol. These results are in agreement with previous work where it was demonstrated that treatment and supplement with antioxidants prevents, or at least diminishes, the toxicological effect of MeHg (Farina et al., 2009; Zhang et al., 2009; Wagner et al., 2010; Qu et al., 2013; Manzolli et al., 2015; Liu et al., 2014). In *in vitro* and *in vivo* experiments, resveratrol displays a wide range of beneficial effects on human diseases but the mechanisms by which resveratrol exerts its action have not yet been clarified. Tellone et al. (2015) reviewed the beneficial effects of resveratrol on several human neurodegenerative diseases as Alzheimer (AD), Huntington (HD), Parkinson (PD), and amyotrophic lateral sclerosis (ALS) trying to highlight the mechanisms by which the polyphenol exerts its specific activity. During the analysis of gene expression derived from four microarrays related to ALS, PD and AD, Recabarren and Alarcón (2017) found that resveratrol is one of the possible drugs as a common treatment for these three pathologies. Ascorbic acid has also been studied in relation to neurodegenerative diseases. A recent review has proposed that ascorbic acid acts mainly by decreasing oxidative stress and reducing the formation of protein aggregates, which may contribute to the reduction of cognitive and/or motor impairments observed in neurodegenerative processes (Moretti et al., 2017). In addition, the role

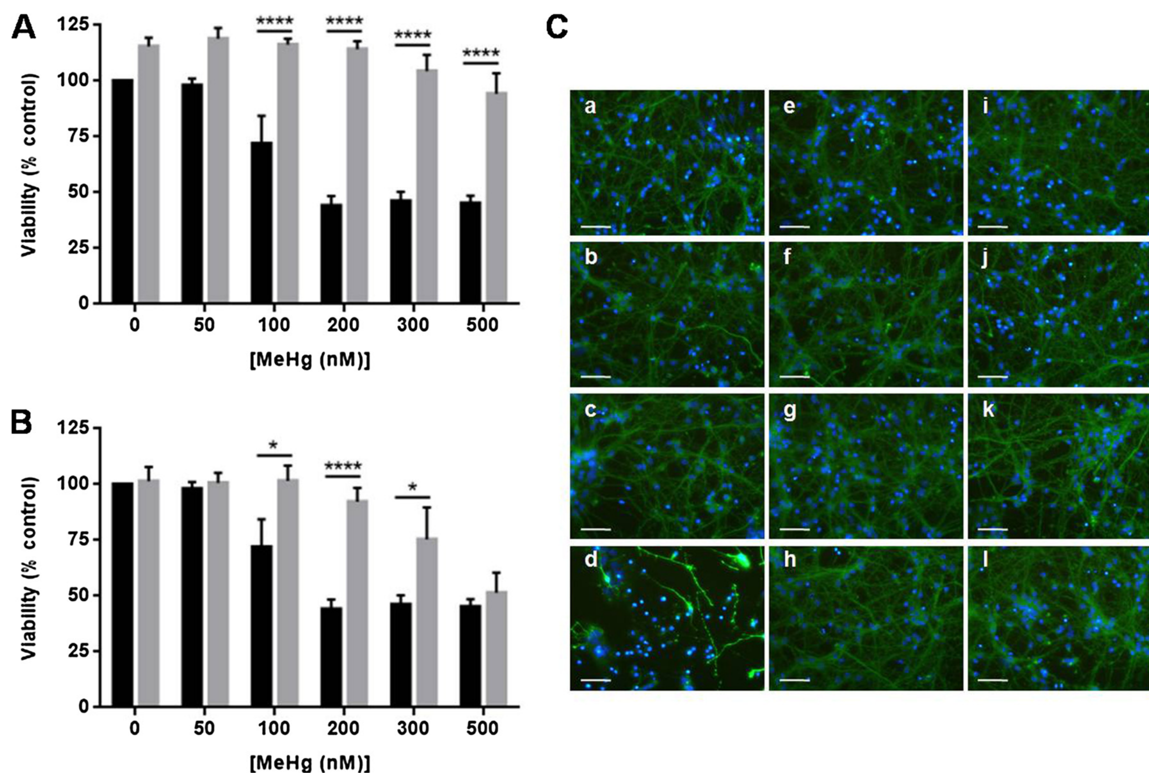

Fig. 7. Antioxidants decrease MeHg-induced toxicity in primary cultures of cortical neurons (CCN). Cells were exposed to MeHg from day in vitro (div) 1 to div 7. Cell viability was evaluated at div 7 by the reduction of MTT. A) Cells were exposed to MeHg only (black columns) or MeHg plus 200 μM ascorbic acid (grey columns). B) Cells were exposed to MeHg only (black columns) or MeHg plus 10 μM resveratrol (grey columns). Results are expressed as percent of survival cells compared to control values. Data are expressed as mean ± SEM (N = 4–8 independent experiments). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ vs cells exposed to MeHg only, Bonferroni's test after two-way ANOVA. C) Images representing the different treatments: a–d) 0, 30, 50, 100 nM MeHg only; e–h) 0, 30, 50, 100 nM MeHg plus 200 μM ascorbic acid; i–l) 0, 30, 50, 100 nM MeHg plus 10 μM resveratrol. Immunohistochemical labeling of CCN by Tau (green cells), GFAP (red cells) and nuclei were stained by DAPI (blue). The scale bar represents 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

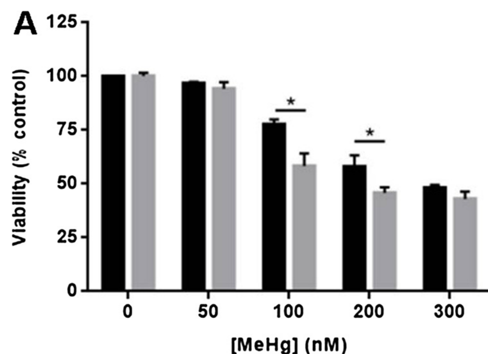

Fig. 8. Acetylcholine increases MeHg-induced toxicity in CCN. Cells were exposed to MeHg from div 1 to div 7. Cell viability was evaluated at div 7 by the reduction of MTT. A) Cells were exposed to MeHg only (black columns) or MeHg plus 100 μM acetylcholine (grey columns). Data are expressed as mean ± SEM (N = 3 independent experiments). *$p < 0.05$ vs cells exposed to MeHg only, Bonferroni's test after two-way ANOVA.

of vitamin C on neurodegenerative diseases including AD, PD, HD, ALS, as well as psychiatric disorders has also been reviewed (Kocot et al., 2017). Therefore, it would be useful to further investigate the effects of resveratrol and ascorbic acid during MeHg exposure, although their mechanisms of action might be completely different as proposed by Sutachan et al. (2012).

We also show that serotonin was as effective as norepinephrine in protecting CCN MeHg-induced cell death. There are some reports proving that serotonin has antioxidant properties (Muñoz-Castañeda et al., 2006; Azouzi et al., 2017). On the contrary, our results show that ACh increases cell death. To our knowledge, ACh has never been considered an antioxidant compound which brings the first difference with catecholamine neurotransmitters and serotonin. It has been already proved that MeHg disrupts calcium homeostasis in a number of neuronal models and contributes to MeHg-induced cell death, impaired synaptic function and disruption of neuronal development (Limke et al., 2004a). A possible explanation for ACh-induced increase of toxicity is linked to the increase of intracellular calcium through nicotinic and muscarinic receptors (Limke et al., 2004a,b). In this sense, Atchison (2005) proposed muscarinic receptors are particularly vulnerable to MeHg since they contain a conserved pair of extracellular cysteine residues that are crucial in agonist and antagonist binding to the receptor. It has also been reported that muscarinic receptors are involved in MeHg-induced cytotoxicity in cultured cerebellar granule cells (Limke et al., 2004b).

Therefore, we hypothesize that the modification of thiol groups may be altering muscarinic receptor activity and together with the addition of ACh concentration these compounds might be promoting an intracellular calcium imbalance and therefore, an increase of cell death.

Conflict of interest

The authors have declared no conflict of interest.

Acknowledgements

This research was supported by the Spanish projects PI13/01252 and PI15/00033 from the Instituto de Salud Carlos III and SAF 2016-

77703-C2-2-R from MINECO, cofinanced with European Social Funds "A Way to build Europe", and the 2017/SGR/106 and CERCA Programme from the Generalitat of Catalunya. N.O. is recipient of a CSIC JAE-Doc contract from the "Junta para la Ampliación de Estudios" Program cofinanced with European Social Funds. The technical assistance of Elisenda Martí is acknowledged.

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