Mercury-induced toxicity of rat cortical neurons is mediated through N-methyl-D-Aspartate receptors

Fenglian Xu1,2*, Svetlana Farkas1, Simone Kortbeek1, Fang-Xiong Zhang2, Lina Chen2, Gerald W Zamponi2 and Naweed I Syed1

Abstract

Background: Mercury is a well-known neurotoxin implicated in a wide range of neurological or psychiatric disorders including autism spectrum disorders, Alzheimer’s disease, Parkinson’s disease, epilepsy, depression, mood disorders and tremor. Mercury-induced neuronal degeneration is thought to invoke glutamate-mediated excitotoxicity, however, the underlying mechanisms remain poorly understood. Here, we examine the effects of various mercury concentrations (including pathological levels present in human plasma or cerebrospinal fluid) on cultured, rat cortical neurons.

Results: We found that inorganic mercuric chloride (HgCl2 –at 0.025 to 25 μM) not only caused neuronal degeneration but also perturbed neuronal excitability. Whole-cell patch-clamp recordings of pyramidal neurons revealed that HgCl2 not only enhanced the amplitude and frequency of synaptic, inward currents, but also increased spontaneous synaptic potentials followed by sustained membrane depolarization. HgCl2 also triggered sustained, 2–5 fold rises in intracellular calcium concentration ([Ca2+]i). The observed increases in neuronal activity and [Ca2+]i were substantially reduced by the application of MK 801, a non-competitive antagonist of N-Methyl-D-Aspartate (NMDA) receptors. Importantly, our study further shows that a pre-incubation or co-application of MK 801 prevents HgCl2-induced reduction of cell viability and a disruption of β-tubulin.

Conclusions: Collectively, our data show that HgCl2-induced toxic effects on central neurons are triggered by an over-activation of NMDA receptors, leading to cytoskeleton instability.

Keywords: Mercury Chloride, Rat cortical neurons, Toxicity, MK 801, NMDA receptor, Excitotoxicity, Cytoskeleton

Background

Mercury is a potent environmental contaminant that exerts toxic effect on a variety of vital organs in the human body. It exists in three predominant forms: elemental (Hg0), organic (such as methylmercury -MeHg), and inorganic (mercuric chloride - HgCl2) mercury [1]. The major sources of mercury load in humans are food contamination, drug and vaccine preservatives, dental amalgams, or occupational exposure [2]. Both acute and chronic exposure to mercury is also known to cause a variety of neurological or psychiatric disorders [3-6].

The central nervous system (CNS) is one of the most vulnerable organs affected by mercury toxicity. Within the CNS, two of the most often affected areas are the cerebral cortex [7,8] and the cerebellum [9,10]. While the mechanisms underlying mercury-induced toxicity remain largely unknown, several studies have shown toxic effects of organic MeHg on the CNS as it can easily cross the blood–brain barrier and accumulate in the brain at high concentrations [1,11]. MeHg has been reported to interact with a wide range of cellular targets and affect multiple cellular functions. These toxic effects include, but are not limited to, the inhibition of neuronal ion channels [12-14], disruption of presynaptic transmitter release and postsynaptic receptor function [15-17], damage to neuronal cytoskeleton components and DNA structures [8,18,19], and alteration of Na+ /K+ ATPase

* Correspondence: fxu@ucalgary.ca
1 Department of Cell Biology & Anatomy, University of Calgary, Alberta, Canada
2 Physiology & Pharmacology, Hotchkiss Brain Institute, Faculty of Medicine, University of Calgary, Alberta, Canada

© 2012 Xu et al; licensee BioMed Central Ltd. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.
and mitochondrial function [20,21] (also see review [22]). Moreover, a large body of recent evidence has also drawn the link between mercury and glutamate-mediated excitotoxicity. For instance, mercury has been shown to affect several aspects of glutamatergic signaling, including the inhibition of glutamate reuptake in astrocyte [23], inhibition of glutamine synthetase activity [24], and an enhancement of spontaneous glutamate release from neurons [25]. These effects result in an increase in glutamate concentration at the synaptic cleft. MeHg has also been found to impact postsynaptic N-Methyl-D-Aspartate (NMDA) receptors. For instance, Basu et al. (2007) have demonstrated that mercury decreases the number of NMDA receptors in several brain regions including the basal ganglia, cerebellum, brainstem, and occipital cortex of mink [26]. In contrast, Ndountse and Chan (2008) have reported that MeHg increased NMDA receptor expression, which in turn may be responsible for neurotoxicity [27]. Another study by Miyamoto et al. (2001) suggested that the enhanced sensitivity of NMDA receptors might contribute to the vulnerability of developing cortical neurons to MeHg neurotoxicity [28]. Although the mercury-induced toxic effects on the nervous tissue have been well documented, the findings are often conflicting and the underlying mechanisms remain poorly defined.

In contrast to MeHg, much less is known about the toxic effect of inorganic HgCl2, despite the fact that it can easily cross the blood–brain barrier and accumulate in the brain at much higher concentrations [29,30]. Moreover, HgCl2 is a more reactive form of mercury as it interacts with many macromolecules and exhibits a long latency of neurotoxicity [24,31]. For instance, HgCl2 is known to be five times as potent as MeHg in blocking L-type Ca2+ channels in hippocampal neurons [32]. It is also a more potent inhibitor of glutamate reuptake and glutamine synthetase activity than MeHg [24]. However, little is known about its actions on the NMDA receptors. In the present study, we first assessed the impact of inorganic HgCl2 on neuronal viability and network integrity in both developing and mature rat cortical neurons in cell culture. Next, we investigated the cellular mechanisms underlying HgCl2-induced toxic effect by studying the involvement of glutamatergic NMDA receptor activity. Our results show that HgCl2 disturbs neuronal network structure and induces cell apoptosis in cortical neurons. These actions involve a degradation of β-tubulin, an important component of the neuronal cytoskeleton, and these effects were evoked by NMDA-receptor function. Inhibition of NMDA-receptor activity by MK 801 not only inhibited HgCl2-induced increase in synaptic currents and intracellular Ca2+, but also prevented HgCl2-induced disassembling of cytoskeleton protein and disruption of neuronal network integrity.

**Results**

**HgCl2 inhibits neuronal outgrowth and induces degeneration of both developing and mature cortical neurons in culture**

To examine how mercury may impact neuronal outgrowth and network formation between central neurons, we first investigated the effects of HgCl2 on neurite initiation and network formation during early outgrowth. To this end, rat neonatal cortical neurons were cultured either in the absence (control) or presence of HgCl2 at the concentrations of 25 nM, 100 nM, and 25 μM for 3 days. Neuronal viability, neurite outgrowth and network formation were evaluated 3 days later. The choices of these experimental doses were based on the studies showing that in the Minamata Bay exposure in Japan, the mercury concentration in the brains of patients was found to be around 1.7 μM to 26 μM, whereas in non-exposed controls its levels averaged around 30 nM [33,34]. Additionally, several studies have revealed that mercury levels in brain tissues are 2–10 fold higher in patients with dental amalgam fillings, Alzheimer’s disease, autism spectrum disorders, epilepsy or hydrocephalus [35–38]. Figure 1A shows a typical culture dish grown under control conditions (no mercury) yielding a healthy population of both neurons and glial cells (indicated by a circle) with extensive neurite outgrowth and network formation (indicated by an arrow). Although cells developed extensive neurites and neuronal network in the presence of both 25 nM and 100 nM HgCl2 (Figure 1B & C, indicated by arrows), it is interesting to note that HgCl2, at 100 nM, appeared to cause cell body damage, abnormal neurite outgrowth, or degeneration of some neurites (Figure 1C, indicated by an asterisk and an arrow head). Cells cultured in the presence of 25 μM of HgCl2 however failed to develop neurite processes and hence were devoid of network connectivity (Figure 1D, indicated by an arrow head and asterisks). To quantify the effect of HgCl2 on cdead cell assay /dead cell assay (LIVEDEAD® Viability/Cytotoxicity Kit, Invitrogen, see Materials and Methods). Specifically, cells were maintained in culture for 3 days either in the absence or presence of HgCl2 at concentrations as described above. On day 3, neurons were incubated and stained with green-fluorescent calcein-AM for 30 mins to indicate intracellular esterase activity (live cells), and red-fluorescent ethidium homodimer-I to determine the damage to plasma membrane integrity (dead cells) (Figure 1E & F). Fluorescent labeling of live and dead cells was visualized using a confocal microscope (LSM-510) and images were acquired under the same confocal parameter settings using a 10x or 20x objective. The number of live (green) and dead (red) cells in the above culture conditions was counted using imageJ software. Specifically, neurons were counted based on a
randomized selection of three areas of 1 mm$^2$ under each culture condition. The mean value of cell viability reflected by the percentage of live cells was calculated and compared. Figure 2A showed that in four experiments cell viability under control conditions was 89.2 ± 3.5% (n = 4). There was however no significant difference between 25 nM (90.7 ± 3.7%, n = 4, $P = 0.352$) and 100 nM (89.6 ± 3.0%, n = 4, $P = 0.914$) of HgCl$_2$. HgCl$_2$ used at the concentration of 25 μM did, however, significantly (11.4 ± 1.5%, n = 4, $P < 0.0001$) compromised cell viability compared to control (Figure 2A).

We next sought to determine whether the established cortical neuronal network at early stages of cell culture could be affected by shorter exposure (< 24 hrs) to HgCl$_2$ at different concentrations. To do this, cells were maintained in culture for 4 days and allowed to exhibit neurite outgrowth and network connectivity. Neurons were then cultured in medium containing either no HgCl$_2$ (as control) or HgCl$_2$ at 25 nM, 100 nM or 25 μM for 16–24 hrs. To evaluate the effects of mercury on neuronal network and cell viability, neurons were again stained with calcein-AM (green), whereas dead cells with compromised membranes were stained with ethidium homodimer-1 (red). Scale bars, 20 μm.

**Figure 1** HgCl$_2$ exposure perturbs neurite outgrowth and compromises neuronal viability in cultured cortical neurons. Rat cortical neurons were grown either in the absence (A, control) or presence of HgCl$_2$ at 25 nM (B), 100 nM (C), and 25 μM (D) for 3 days. Phase contrast images were taken on day 3. A, Cortical culture under control conditions exhibited healthy population of neurons and glial cells (indicated by a circle), exhibiting extensive neurite outgrowth and network formulation (indicated by an arrow). B, Cells cultured in the presence of 25 nM HgCl$_2$ also developed extensive neurite processes and formed extensive network. C, Neuronal populations cultured in the presence of 100 nM of HgCl$_2$ underwent apoptotic cell death (indicated by an asterisk) and exhibited collapse or retraction of neurites (indicated by an arrow head). D, HgCl$_2$ at 25 μM induced cell death (indicated by asterisks) and inhibited neurite extension and network formation (indicated by an arrow). E & F shows representative confocal images of live/dead assay of cortical neurons cultured either in the absence (E) or presence (F) of 25 μM of HgCl$_2$ for 3 days. Live cell bodies and their neurites were stained with calcein-AM (green), whereas dead cells with compromised membranes were stained with ethidium homodimer-1 (red). Scale bars, 20 μm.
$P = 0.072$) and 100 nM (94.1 ± 3.8%, $n = 4, P = 0.254$) did not affect the established network and cell survival as compared with control (96.9 ± 7.1%, $n = 4$). However, a shorter (16–24 hrs) exposure of cells to 25 μM of HgCl$_2$ not only remarkably disrupted the neuronal physical connectivity, but also significantly reduced the cell viability (24.9 ± 2.8%, $n = 4, P < 0.001$) (Figure 2B).

Finally, we sought to determine whether longer-term exposure (>7 days) of cells to HgCl$_2$ could affect fully developed neurons and their network. To test this, cortical cells were first allowed to grow in culture for 10 days and establish outgrowth and extensive networks. Cells were subsequently treated with HgCl$_2$ at 25 nM, 100 nM and 25 μM and maintained in culture for another 7 days. Neuronal viability and network formation were examined at day 17. We found that at this stage, the cortical neurons exhibited extremely dynamic neuronal outgrowth pattern, and the active growth of glial cells formed enormous layers in the culture dish. The extensive background labeling of glial cell layers made the survival assay, particularly the counting of live cells, very difficult. We therefore opted to evaluate the effect of HgCl$_2$ on neuronal viability using phase contrast images. Specifically, cortical neurons were cultured on glass cover slips with a grid (Bellco Glass, aid for counting). Phase contrast images were taken before exposure to HgCl$_2$ on day 10 and after an additional 7-day exposure to HgCl$_2$ (i.e., on day 17). The average numbers of neurons were calculated based on a randomized selection of five different areas of 0.33 mm$^2$ in each dish. The mean numbers of survived neurons were compared between day 10 and day 17. Neurons were identified based on their size, shape, arrangement and number of processes. Our results showed that 7-day exposure to HgCl$_2$ at all concentrations examined, caused a significant reduction in the rate of neuronal survival. Specifically, control cultures had on average 113 ± 8.5 neurons/0.33 mm$^2$ on day 10 and it did not change after 7 days in culture (108.2 ± 18.3, $n = 5, P = 0.842$). Cultures exposed to 25 nM HgCl$_2$ for 7 days had a substantial reduction in the number of neurons (33.6 ± 6.9, $n = 5, P = 0.005$) as compared with that before
HgCl₂ exposure on day 10 (102 ± 10.2, n = 5). Similarly, HgCl₂ at 100 nM (from 122.2 ± 18.7 to 30.6 ± 4.7, n = 5, ΔP = 0.003) and 25 μM (from 101.8 ± 13.1 to 25.6 ± 3.2, n = 5, ΔP = 0.002) caused significant decrease in cell viability (Figure 2C). These data indicated that chronic exposure to HgCl₂ at concentrations, as low as 25 nM to 100 nM, caused detrimental effects on central neurons even at a more matured developing stage. It is, however, important to note that because the mercury-induced effects are not neuron-type specific, the significant reduction in the number of neurons at all concentrations examined might also involve a secondary toxic effect involving the glial cells. Specifically, mercury toxicity to glial cells may prevent astrocytes from forming a firm “bottom layer” which plays an essential role for neuronal outgrowth and survival under our culture condition. The toxic impact of mercury on glial cells may have thus exasperated neuronal loss in our culture condition. However, our experiments together with previous studies warn against the potential effects of chronic low-dose mercury exposure [39,40].

Based on the fact that HgCl₂ used at 25 μM consistently caused neuronal damage to both developing and more mature neurons during short-term and long-term exposure, we next investigated the mechanisms underlying the toxic effect of HgCl₂ on cortical neurons using the concentration of 25 μM in all subsequent experiments, unless stated otherwise.

HgCl₂-induced cell damage is associated with loss of cytoskeleton structures

The architecture and survival of the central neurons rely significantly upon their cytoskeleton components, specifically the actin filament (F-actin) and microtubules such as β-tubulin. Any disturbance of these dynamic structures or their polymerization/ddepolymerization status due to environmental or genetic factors could compromise cell survival [41-43]. Based on our observations that HgCl₂ induces cell damage and retraction of neuronal processes, we hypothesized that HgCl₂ toxicity may involve disruption of neuronal cytoskeleton proteins such as the F-actin and β-tubulin. To test this hypothesis, cortical neurons were first cultured for four to ten days, which allowed for the development of neurite outgrowth and network connectivity. Cells were then exposed to culture medium containing either no HgCl₂ (as control) or HgCl₂ at 25 μM for 24 hrs. The preparations were then fixed with 4% paraformaldehyde and stained with anti-β-tubulin antibody and rhodamine-phalloidin (to label F-actin). As shown in Figure 3, the pyramidal cortical neurons in control condition (A) exhibited bigger cell bodies with multiple healthy processes and extensive network. In contrast, neurons treated with HgCl₂ (B) appeared to be much smaller in size with degraded neurite and compromised network. Accordingly, a strong fluorescent labeling of cell bodies and neurites with both β-tubulin (A) and F-actin (C) antibodies were observed in control neurons. The cells exposed to HgCl₂ at 25 μM, however, remarkably lost the fluorescent intensity of β-tubulin (B) although there was no apparent change in the intensity of F-actin staining (D). These results clearly demonstrated that the β-tubulin protein is most likely affected by HgCl₂-induced toxicity in the CNS. The reduction of overall intensity of β-tubulin is likely due to HgCl₂-induced degeneration of neurons rather than cell death as the level of F-actin intensity remained unaffected by HgCl₂. One would need to be cautious, however, that these experiments may not preclude the possibility that mercury exposure might also change the conformation of β-tubulin altering access of the primary antibody to the epitope. Future investigation and analysis using western blot techniques are still required for further validation of these data.

HgCl₂ triggers sustained intracellular Ca²⁺ rise in cortical neurons, which is blocked by NMDA receptor antagonists

Recent studies have linked the degeneration of cytoskeleton structures to glutamatergic excitotoxicity. Specifically, over stimulation of NMDA receptors and the subsequent overloading with Ca²⁺ have been shown to cause rapid disruption of established cytoskeleton components or inhibition of the assembly of new cytoskeleton elements such as microtubules, F-actin and neurofilament proteins in a variety of preparations [44-46]. Moreover, NMDA receptors have been shown to play a pivotal role in mercury-induced neurotoxicity [19,28]. We next sought to determine whether HgCl₂ affects cortical Ca²⁺ homeostasis through NMDA receptors. To this end, Ca²⁺ ratiometric experiments using Fura-2 AM were performed on cortical neurons. Neurons were maintained in culture for about five to ten days and subsequently loaded with 5 μM of Fura-2 AM for 30 mins at 37°C. Cells were then washed with normal saline containing free Mg²⁺ and 3 μM of glycine in order to activate NMDA receptors at the resting level. Ca²⁺ images and the ratio of Fura-2 fluorescence intensity at 340 nm and 380 nm (reflection of intracellular Ca²⁺ levels) were acquired prior to and post exposure to HgCl₂ at 25 μM. Representative traces of four different cells are shown in Figure 4A (red, green, black and blue traces). As shown in Figure 4A, intracellular Ca²⁺ transients were recorded before HgCl₂ (indicated by an arrow). After local application of HgCl₂, the amplitude and frequency of calcium transients were augmented. After 5 to 10 mins of HgCl₂ exposure, sustained rises in intracellular Ca²⁺ were observed in almost all cells examined (Figure 4A). The ratio of Fura-2 fluorescence
intensity increased by 2.26 ± 0.2 times (n = 8) after 10 mins of HgCl₂ application and 4.55 ± 1.44 (n = 11) times compared to control levels after 15 mins of HgCl₂ application (Figure 4C, two columns on the left panel). Next, we examined the involvement of NMDA receptor activity in HgCl₂-induced rise in intracellular Ca²⁺. To do this, the basal Ca²⁺ levels were first monitored for 5 mins and again intracellular Ca²⁺ transients were recorded in control conditions (Figure 4B, indicated by an arrow). The cells were then exposed to 10 μM of the NMDA receptor antagonist MK 801 for 5 to 10 mins. Note that upon application of MK 801, the intracellular Ca²⁺ transients were almost completely abolished indicating an involvement of tonic NMDA receptor activity in normal Ca²⁺ oscillations. It is also important to note that subsequent application of HgCl₂ (25 μM) in the continued presence of MK 801, failed to induce rises in Ca²⁺. In MK 801 treated neurons, the fluorescence ratio in the continued presence of MK 801 was 1.09 ± 0.13 (n = 11) times after 10 mins of HgCl₂ exposure and 1.37 ± 0.33 times (n = 8) after 15 mins of HgCl₂ exposure (Figure 4C, two columns on the right panel). The rise in intracellular Ca²⁺ in the presence of MK 801 was significantly smaller than that of MK 801 (Figure 4C). These results indicate that NMDA receptor activity may play a crucial role in HgCl₂-induced
alteration of intracellular Ca\(^{2+}\) homeostasis, which might contribute to HgCl\(_2\)-induced disturbance of cytoskeleton network.

It is important to note that HgCl\(_2\)-induced sustained elevations of \([\text{Ca}^{2+}]_i\), occurred simultaneously in groups of cells that were measured as shown in Figure 4A. This led us to propose that HgCl\(_2\) may initially affect the synaptic activity of cortical neurons by acting through their NMDA receptors. These changes in neuronal excitability may in turn alter the status of dynamic or balanced excitability within a network or their neighboring cells.

**HgCl\(_2\) increased synaptic inward currents and cause sustained membrane depolarization in pyramidal cortical neurons through NMDA receptors**

NMDA receptor-mediated synaptic activity plays an important role in early neuronal development and also in the later stages of synaptic maturation [47-50]. To further determine the role of NMDA receptors in HgCl\(_2\)-induced toxicity, we next explored whether HgCl\(_2\) acts to alter NMDA receptor-mediated synaptic activity such as synaptic currents or membrane discharges. We first tested whether HgCl\(_2\) affected spontaneous synaptic currents in pyramidal neurons. To test this, whole cell voltage-clamp experiments were performed on pyramidal neurons in culture for 7 to 15 days and spontaneous inward currents were recorded by holding the cells at \(-70\) mV. As shown in Figure 5A, spontaneous inward currents were recorded in pyramidal cortical neurons under control condition as shown in the insert (Ai). Upon local application of HgCl\(_2\) (25 \(\mu\)M), large amplitude inward currents were elicited. It is important to note that HgCl\(_2\)-elicited inward currents were often followed by high frequency, spontaneous postsynaptic events, which lasted for a long period (see insert Aii, indicated by an arrow). To test the involvement of NMDA receptors in HgCl\(_2\)-induced increase in synaptic currents, the inward currents were recorded from cells that were first exposed to MK 801 to block the NMDA-receptors, and then to both MK 801 and HgCl\(_2\). Figure 4B shows that spontaneous synaptic currents were again recorded under control conditions. Application of MK 801 eliminated events with bigger amplitudes indicating the involvement of NMDA-currents in these events. The remaining synaptic events in the presence of MK 801 are likely the non-NMDA currents. Interestingly, in the continued presence of MK 801, HgCl\(_2\) failed to further enhance synaptic currents that were observed in Figure 4A, while the frequency and amplitude of non-NMDA currents remained unchanged by HgCl\(_2\). These experiments suggest that HgCl\(_2\) mainly increased the NMDA-currents in pyramidal cortical neurons.
The above data suggest that HgCl$_2$-induced increase in synaptic inputs in pyramidal cortical neurons may in turn change the membrane excitability in these neurons. To test this possibility, we next monitored the membrane potential changes by performing whole-cell current-clamp experiments on pyramidal cortical neurons. Figure 5C shows a representative membrane potential trace recorded from a pyramidal neuron before and after the application of HgCl$_2$ (25 μM) at 25 μM. Application of HgCl$_2$ initially enhanced the spontaneous synaptic discharge and triggered firing of action potentials followed by sustained membrane depolarization to a potential of around −42 mV. Interestingly, subsequent application of MK 801 (10 μM) did abolish the firing of action potentials, but did not affect the sustained membrane depolarization indicating the involvement of secondary-mechanism in the sustained depolarizing components. It is interesting to note that pre-incubation of cells with MK 801 for 10 mins before and after HgCl$_2$ exposure. Pretreatment of cells with MK 801 greatly reduced HgCl$_2$-induced increase in action potential firing and sustained membrane depolarization. E. Statistical data shows that the increased amplitude of sustained membrane depolarization by HgCl$_2$ (25 μM) in MK 801-treated cells is significantly smaller than that in control cells.
shown in Figure 5E. These data reveal that the mean increase in membrane potentials of sustained components after 10 mins of HgCl₂ exposure in control neurons was 20.4 ± 2.8 mV (n = 7). The increased amplitude of membrane potential was significantly reduced to 6.86 ± 1.3 mV (n = 5) in the presence of MK 801. In summary, HgCl₂ increased synaptic current inputs and caused enhancement of membrane discharges in pyramidal cortical neurons, and this increase in synaptic activity could be substantially attenuated by NMDA receptor block.

**Inhibition of NMDA receptor activity prevents mercury-induced impairment of network integrity and disruption of cytoskeleton component in cortical neurons**

Perturbation of synaptic activity or Ca²⁺ homeostasis, especially excessive loading of cells with Ca²⁺, activates a series of downstream cascades. These steps include proteinases, reactive oxygen species, and mitochondrial failure, which are lethal to cell cytoskeleton components or DNA structures [51-53]. Based on our results showing that MK 801 could significantly inhibit HgCl₂-induced increases in synaptic activity and [Ca²⁺], we predicted that MK 801 may exert a protective action on cortical neurons during exposure to HgCl₂. To examine this, cells cultured for 10–12 days were exposed to the following four conditions: (1) control; (2) 25 μM of HgCl₂; (3) co-application of HgCl₂ at 25 μM and MK 801 at 5 or 10 μM, or following 30 mins of pretreatment with MK 801; (4) MK 801 alone for 16–24 hrs. Phase contrast pictures were taken the next day. We found that pretreatment or co-application of MK 801, at both 5 and 10 μM, prevented HgCl₂-induced disruption of neuronal network. Specifically, Figure 6 shows that control neurons exhibit healthy cell bodies with well-established networks (A). HgCl₂ alone severely disrupted neuronal network formation and triggered cell apoptosis (B). However, cells exposed to both MK 801 and HgCl₂ still exhibited healthy morphologies with extensive neurite outgrowth and integrated network (C). MK 801 alone did not cause damage to neurons and their networks (D).

To further evaluate the protective action of MK 801 on HgCl₂-induced neurotoxicity, we investigated whether MK 801 could also prevent HgCl₂-induced damage to cytoskeleton components. To test this, cortical neurons were exposed to four conditions overnight as listed above, and cells were then fixed and stained with antibodies against β-tubulin and F-actin the next day. Confocal images showed that the fluorescence intensity of β-tubulin but not F-actin was substantially reduced in cells treated with HgCl₂ alone (Figure 6F) as compared with control (Figure 6E). This result is consistent with what we observed previously (see Figure 3). Cells in dishes containing MK 801 at 5 μM and HgCl₂ at 25 μM exhibited intense fluorescence for both β-tubulin and F-actin (Figure 6G). MK 801 alone did not affect staining in both instances (Figure 6H). In fact, the fluorescence intensity of F-actin in cells exposed to HgCl₂ was even stronger in some cases as shown in Figure 5F & G.

In summary, our data indicate that an inhibition of NMDA-receptor activity indeed attenuates the degenerative effects of HgCl₂ on cultured cortical neurons. These data further support the notion that NMDA synaptic transmission or receptor function plays an essential role in initiating HgCl₂-induced toxicity in the CNS.

**Discussion**

This study is the first to investigate HgCl₂ neurotoxicity on rat cortical neurons from a variety of aspects – ranging from neuronal morphology to excitability. Specifically, our data demonstrate that HgCl₂ elicits detrimental effects on young, developing cortical neurons – ranging from reduced cell survival, impaired neurite outgrowth, compromised network assembly to the degeneration of mature networks. Importantly, our data caution against the possible detrimental effects of mercury at low nanomolar levels (sub-lethal doses). An apparent lack of short-term (3 days) or acute (< 24 hrs) effect of HgCl₂ at 25 and 100 nM, on cortical cell viability does not necessarily rule out cellular toxicity, as the detrimental changes may either be subtle or require longer exposures. In fact, HgCl₂ at 100 nM has been demonstrated to cause remarkable disruption of neurite membrane integrity, and induce robust retraction and collapse of growth cones in *Lymnaea Stagnalis* neurons [39]. Low nanomolar concentrations of mercury have also been shown to affect the production of pathological hall-marks of Alzheimers’ diseases in cultured neurons [40,54] as well as affect cardiac function [55,56]. Therefore, a detailed evaluation of the effect of HgCl₂ at these concentrations may still be required. Specially, the effect of HgCl₂ on the motile structure of growth cones, the length of neurite elongation, and cell viability would need to be monitored using proper neuronal model types in which these parameters can be easily measured as shown in our previous study [57].

Mercury neurotoxicity has been implicated to involve myriad mechanisms and cellular targets. These include perturbation of Ca²⁺ homeostasis, dysfunction of mitochondria, glutamatergic excitability, disruption of cytoskeleton structures, reactive oxygen species (ROS), and many others [22,28,58]. However, the steps that initiate, mercury-induced neuronal degeneration and the underlying mechanisms remain largely unknown. In this study, we first explored the potential involvement of Ca²⁺ in mercury toxicity because Ca²⁺ is an important integrator of neuronal viability and excitability. The proper regulation of intracellular Ca²⁺ level plays an important role in
regulation of growth cone motility, neurite initiation and outgrowth [59-61]. Intracellular Ca\textsuperscript{2+} levels either fall below or rise significantly above an optimal range have been shown to inhibit both of the growth cone motility and neurite outgrowth [59,62-64]. The data presented in this study show that HgCl\textsubscript{2} triggered a sustained rise in [Ca\textsuperscript{2+}]\textsubscript{i} in all cortical neurons examined and this effect on Ca\textsuperscript{2+} is not reversible (data not shown). The sustained elevation of [Ca\textsuperscript{2+}]\textsubscript{i} in cortical neurons by HgCl\textsubscript{2} may thus last for a long term and reach a level which would be detrimental for growth cone motility, neurite outgrowth and hence the network assembly during early neuronal development. Our data further demonstrated that HgCl\textsubscript{2} also induced degradation of mature neurite and network connectivity (Figure 3 & 6) suggesting that neuronal ultrastructure components such as cytoskeleton proteins may also be affected by HgCl\textsubscript{2}–induced disturbance in Ca\textsuperscript{2+} homeostasis. In support of this hypothesis, our study demonstrates that HgCl\textsubscript{2} indeed induced disassembly of cytoskeleton, primarily the β-tubulin proteins in cortical neurons. Our findings are consistent with previous studies showing that mercury induce disintegration of β -tubulin protein in several other species [39,65-67]. Neuronal cytoskeletal proteins have been shown to be extremely sensitive to intracellular Ca\textsuperscript{2+} levels and their assembly and disassembly can

Figure 6 Inhibition of NMDA receptor by MK 801 prevents HgCl\textsubscript{2}-induced disruption of neuronal network and degradation of cytoskeleton components. To evaluate the protective effect of MK 801 on HgCl\textsubscript{2}-induced neurotoxicity, cells were grown for about 10 days and allowed to extend neurites and form network. Cultures were then maintained in the following conditions: control (A), 25 μM of HgCl\textsubscript{2} (B), 25 μM of HgCl\textsubscript{2} and 5–10 μM of MK 801 (C), and MK 801 alone (D). Phase contrast images were taken 16–24 hr later. Cells were subsequently fixed and stained with antibodies against β-tubulin and F-actin (E-H). Neurons exposed to HgCl\textsubscript{2} exhibited retraction of neurite processes (B) and lower fluorescence intensity of β-tubulin (F) as compared with control cultures (A & E). HgCl\textsubscript{2} in the presence of MK 801 failed to induce disturbance of network integrity and cytoskeleton staining (C & G). MK 801 alone did not affect neuronal network and fluorescence intensity of cytoskeleton staining (D & H). Scale bars, 25 μm.

Xu et al. Molecular Brain 2012, 5:30
http://www.molecularbrain.com/content/5/1/30
be influenced by Ca$^{2+}$ either directly or indirectly via regulation of cytoskeleton associated proteins such as tau, a tubulin binding protein [68,69]. For instance, studies have shown that experimentally-induced, sustained elevation of [Ca$^{2+}$], either by Ca$^{2+}$ ionophores, or depolarizing agents causes hyperphosphorylation of tau resulting in microtubule depolymerization and neuronal degeneration in cultured human cortical neurons [69]. As the microtubule cytoskeleton forms the basis for not only the structural integrity, but also for functional communications between neurons, the damage to the microtubules may result in abnormal physiological functions of the brain and hence aberrant animal behaviors.

Because Ca$^{2+}$ is also an important regulator of cell excitability and gene expression, its entry (via NMDA receptors)-mediated synaptic activity has been shown to play crucial roles in neuronal development, synaptic plasticity, cell survival, and synaptic circuitry refinement [47,48,50]. Disturbance of neuronal activity even within one element of the network has been found to perturb the development of the entire circuitry and its physiological functions [47,50]. Considering the predominant effects of HgCl$_2$ on NMDA receptor-mediated synaptic current inputs and membrane discharges in pyramidal cells, we postulated that Ca$^{2+}$ entry via NMDA receptors may alter the membrane excitability and cellular signaling in pyramidal neurons resulting in a deficit in overall network activity and/or Ca$^{2+}$ homeostasis. This is supported by our observations that HgCl$_2$-induced rise in Ca$^{2+}$ occurred almost simultaneously in a group of neurons within a network. The disturbance of Ca$^{2+}$ homeostatic or other cellular signaling pathways resulting from an over stimulation of NMDA receptors might ultimately lead to, cytoskeleton disruption and cell death which are hallmarks of HgCl$_2$-induced glutamatergic excitotoxicity. Future studies are however required to decipher the precise involvement of NMDA receptors by HgCl$_2$. In regard to the possibility of mercury directly modulating NMDA receptor function, the sulfhydryl group in cysteine residues of NMDA receptors has been found to be the potential targets for regulation of NMDA receptor function by redox or alkylating agents. Oxidizing agents are known to decrease while sulfhydryl reducing agents potentiate NMDA receptor activity [70,71]. For instance, Tang and Aizenman (1993) demonstrated that alkylation of the NMDA redox site by the sulfhydryl alkylating agent N-ethylmaleimide (MEM) enhanced NMDA receptor response in cortical neurons [72]. Based on the fact that HgCl$_2$ is a small molecular as MEM and has high affinity to sulfhydryl group, it is possible that HgCl$_2$ may potentiate NMDA receptor function by directly interacting with its cysteine residue sulfhydryl groups, rendering the NMDA receptor dysfunctional. These postulates however need to be determined experimentally.

Conclusions
Taken together, our data demonstrate that pre exposure of cells to MK 801 could remarkably inhibit HgCl$_2$-induced increase in neuronal excitability, Ca$^{2+}$ rise, and importantly prevent the damage to cytoskeleton components and network integrity. Our data suggest that over activation of NMDA receptors is likely the initial trigger for the downstream of cascades involved in HgCl$_2$-induced toxicity. Inhibition of NMDA receptor activity is a potential and effective way to block the initiation of injurious effect of mercury in central neurons.

Methods
Cortical neuron cultures
All animal procedures were approved by the University of Calgary institutional animal use and care committee. Conditions were met with the standards established by the Canadian Council on Animal Care. The culture of cortical neuron was made using Sprague–Dawley rat pups on the day they were born. Dissociated cortical neurons were plated onto cover slips coated with poly-D-lysine (30 μg/ml, Sigma P6407) and laminin (2 μg/ml, Sigma L2020). Cortical neurons were cultured in neurobasal medium (Invitrogen, no. 21103–049) supplemented with 2% B27 (Invitrogen, no. 17504–044), L-Glutamine (200 mM) (Invitrogen, no. 25030–081), 4% FBS (Invitrogen, no. 12483–020), and penicillin-streptomycin (Invitrogen, no. 15140–122). Approximately 80% of the culture media was replaced every 3–4 days. Cultures were maintained at 37°C in an incubator circulated with air and 5% carbon dioxide. For counting studies, cortical neurons were plated onto cover slips with grids (Bellco Glass, no. 1916–91818).

To study the effect of Mercury (II) Chloride (HgCl$_2$) on neuronal process initiation, neurite outgrowth and network formation, freshly dissociated cortical neurons were cultured either in the absence (control) or presence of different concentrations of HgCl$_2$ (25 nM, 100 nM and 25 μM) for 3 days. Neurite outgrowth, cell viability and network formation were evaluated on day 3. To examine the acute effect (up to 24 hrs) of HgCl$_2$ on newly established neuronal processes and network, cells were first cultured for 4 days to allow for the establishment of neurite outgrowth and network. Neurons were then exposed to HgCl$_2$ at different concentrations overnight, and the effects of HgCl$_2$ were examined the next day. To study the chronic effect (7 days exposure) of HgCl$_2$ on well-established mature network, 10 day-old cells cultured on cover slips with grid were exposed to different HgCl$_2$ concentrations and the effect was evaluated on day 17.

Live/dead cell viability assay for cortical neurons
To determine and quantify the impact of HgCl$_2$ on cell viability, cortical cultures that were maintained in
control or drug-treated conditions were subsequently loaded with the LIVEDEAD® ViabilityCytotoxicity Kit (Molecular probes, L-3224) for 30 mins at room temperature (21-22°C). This two-color assay was developed based on the fact that intracellular esterase activity and an intact plasma membrane are unique characteristics of live cells. The LIVEDEAD® Viability-Cytotoxicity Kit discriminates live from dead cells by simultaneously staining with green-fluorescent calcein-AM to indicate intracellular esterase activity and red-fluorescent ethidium homodimer-1 to indicate loss of plasma membrane integrity. Preparations were visualized using confocal microscopy (LSM 510 Meta, Zeiss, Germany) under a 10x or 20x objective at 488 nm excitation (green) and 548 nm (red) wavelength and images were collected using a band pass filter (560–615 nm). The number of cells labeled with both colors was subsequently counted using imageJ software.

Whole-cell patch-clamp recordings in cortical neurons
Whole-cell recordings of synaptic inward currents and membrane potentials were performed on pyramidal cortical neurons after 7 to 15 days in culture at room temperature (21-22°C) using a Multiclamp 700B amplifier (Axon Instruments; Sunnyvale, CA, USA) connected to an analog-to-digital interface Digidata 1322 (Axon Instruments). Signals were acquired and stored through pClamp 9.2 software (Axon Instruments). Synaptic currents were recorded under voltage-clamp mode with the holding potential of ~70 mV throughout. Membrane potentials of pyramidal cells were recorded under current-clamp mode. The external solution contained (in mM) NaCl, 135; CaCl_2, 3; KCl, 5; MgCl_2, 2; HEPES, 10; D-Glucose, 10; pH adjusted to 7.3 with NaOH. The internal pipette solution was composed of (in mM) K+ Gluconate, 130; NaCl, 7; MgCl_2, 0.3; HEPES, 10; EGTA, 0.1, ATP-Mg, 3; GTP-Na, 0.6; pH adjusted to 7.3 with KOH. The osmolarity for internal solution was approximately 300 mOsm (295–305) and for external solution approximately 330 mOsm (320–340). Borosilicate patch pipettes (A-M Systems, Inc; Sequim, WA, USA) were pulled using a horizontal micropipette puller (Model P-97, Sutter instrument Co., USA) and had a tip resistance ranging from 3–5 MΩ after filling with internal solutions. Only cells with series resistances less than ~15 MΩ and leaks less than ~100 pA were selected for the analysis. Data were analyzed using Clampfit 9.0 software (Axon Instruments), and traces were plotted using OriginPro 8.0 SRO (OriginLab Corporation; Northampton, MA, USA).

Ca²⁺ Imaging
Fura-2 acetoxymethyl ester (AM) ratiometric Ca²⁺ imaging experiments were performed to study the action of HgCl₂ and/or N-methyl-D-Aspartate (NMDA) receptor activity on intracellular Ca²⁺ level in cortical neurons. Specifically, neurons that were cultured for 5 to 10 days were loaded with the membrane permeable and ratiometric Ca²⁺ sensor Fura-2 AM (Molecular Probes, Carlsbad, CA) at 5 μM for 30 mins at 37°C. Cells were subsequently washed three times with normal saline solution containing 0 Mg²⁺ and 3 μM glycine to activate NMDA receptor at resting level. The composition of normal saline included (in mM): NaCl, 135; CaCl_2, 3; KCl, 5; HEPES, 10; D-Glucose, 10; pH adjusted to 7.3 with NaOH. Culture dishes were then mounted on an inverted microscope and neurons were exposed to excitation wavelengths of 340 and 380 nm delivered using a LAMBDA DG4 high-speed wavelength switcher (Sutter Instrument, Novato, CA) through a 40x oil objective. The emitted fluorescence signal was collected at 510 nm by a Regina Exi camera. Images were acquired with the Northern Eclipse software ionwave program (Empix Imaging, Canada). The change in intracellular Ca²⁺ level was reflected by the ratio of fluorescence intensity obtained at 340 nm and 380 nm.

Immunochemistry and confocal microscopy
Cultured cells were fixed for 1 h with pre-warmed 4% paraformaldehyde and subsequently washed four times with 1x PBS. Cells were permeabilized for 1 h with incubation media (IM) (0.5% Triton in 1x PBS with 10% goat serum) and then incubated overnight with monoclonal anti-β-tubulin antibody produced in mouse (1:500) (Sigma, T0198). Cells were rinsed two times with 1x PBS the next day. Cells were then incubated with AlexaFluor 488 goat anti-mouse IgG secondary antibody (1:100) (Invitrogen, A-11001) for 1 h at room temperature (21-22°C) under dark conditions. Cultures were subsequently rinsed two times with 1x PBS and incubated for 30 minutes with rhodamine phalloidin (1:20) (Invitrogen, no. R415). Following two time wash with 1 × PBS and one quick rinse with double distilled H₂O cover slips were mounted using MOWIOL mounting media with 4’6-diamidino-2-phenylindole dihydrochloride (Sigma-Aldrich). Samples were viewed using confocal microscopy (LSM 510 Meta, Zeiss, Germany) under a 20x dry or 40x water objective at 488 nm excitation (green, β-tubulin) and 548 nm (red, F-actin) wavelength. Images were collected using a band pass filter (560–615 nm). To assess the level of β-tubulin and F-actin staining, image acquisition parameters for control and drug-treated neurons were kept the same.

Chemicals
All chemicals including Mercury (II) chloride (HgCl₂, M1136) and MK 801 (M107) were purchased from Sigma-Aldrich (Oakville, Ontario, Canada). Fura-2...
acetoxyethyl ester (AM) was purchased from (Molecu- lar Probes, Carlsbad, CA). HgCl \textsubscript{2} was dissolved in deio- nized water (for electrophysiological and Ca\textsuperscript{2+} imaging experiments), or in culture medium (for toxic study) to a stock concentration of 10 mM or 10 μM. The applied solutions (0.025-25 μM) were diluted with normal exter- nal solution or culture medium.

**Statistical analysis**

Data were analyzed statistically using Student’s t test or one-way analysis of variance (ANOVA) as appropriate. Post hoc analysis was conducted using Tukey’s test. Values were considered statistically significant at the level of P < 0.05. The data are presented as mean ± S. E.M. Each experiment was replicated a minimum of four times; the actual number of replicates for each experiment is listed in the corresponding figure legend or in the text.

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

FX, SF, SS, FXZ and LC conducted experiments and data analyses. Specifically, FX and FXZ conducted the calcium imaging and electrophysiological experiments. FX, NIS and GWZ drafted and edited the manuscript. All authors read and approved the final manuscript.

**Acknowledgements**

This work was supported by Natural Sciences and Engineering Research Council (NSERC) grant to NIS. GWZ is supported by a grant from the Canadian Institutes of Health Research (CIHR). He is a Canada research Chair Council (NSERC) grant to NIS. GWZ is supported by a grant from the

**References**

1. Clarkson TW, Magos L: The toxicology of mercury and its chemical compounds. Crit Rev Toxicol 2006, 36:659–662.

2. Clarkson TW: The Three Modern Faces of Mercury. Environ Health Perspect 2002, 110(Suppl 1):11–23.

3. Choi BH: Kim R C and Peckham N H: Hydrocephalus Following Prenatal Methylmercury Poisoning. Acta Neuropathol 1988, 75:325–330.

4. Clarkson TW, Magos L, Myers G J: The Toxicology of Mercury – Current Exposures and Clinical Manifestations. N Engl J Med 2003, 349:1731–1737.

5. Meyer-Baron M, Scharper M, Seiber A: A Meta-Analysis for Neurobehavioural Results Due to Occupational Mercury Exposure. Arch Toxicol 2002, 76:127–136.

6. Mutter J, Naumann J, Schneider R, Wielch H, Halley B: Mercury and Autism: Accelerating Evidence? Neurotoxicol Teratol 2005, 26:439–446.

7. Ferraro L, Tomatis MC, Tangarini S, Mazza R, Colucci A, Canata MR, Gaetani S, Cuomo V, Antontelli T: Developmental exposure to Methylmercury elicits early cell death in the cerebral cortex and long-term memory deficits in the rat. Int J Dev Neurosci 2009, 27:165–174.

8. Eto K, Yasutake A, Kikuma T, Kurogi Y, Akima M, Shimozeki T, Tokunaga H, Kaneko Y: Methylmercury poisoning in common mammals: a study of selective vulnerability within the cerebral cortex. Toxicol Pathol, 2001, 29(5):565–573.

9. Fonfria E, Vilaro MT, Babot Z, Rodriguez-Farre E, Sunol C: Effects of Inorganic Mercury and Methylmercury on the Ionic Currents of Cultured Rat Hippocampal Neurons. Cell Mol Neurobiol 1997, 17:273–288.

10. Farina M, Rocha JBT, Aschner M: Mechanisms of Methylmercury-Induced Neurotoxicity: Evidence From Experimental Studies. Life Sci 2011, 89:555–563.

11. Pekel M, Platt B, Busselberg D: Mercury (Hg2+) Decreases Voltage-Gated Calcium-Channel Currents in Rat Drg and Aplysia Neurons. Brain Research 1993, 632:121–126.

12. Peng SQ, Haja R, Atchison WD: Effects of Methylmercury on Human Neuronal L-Type Calcium Channels Transiently Expressed in Human Embryonic Kidney Cells (HEK-293). J Pharmacol Exp Ther 2002, 302:424–432.

13. Siros JE, Atchison WD: Effects of Mercurials on Ligand- and Voltage-Gated Ion Channels: A Review. Neuro toxicol 1996, 17:63–84.

14. Castoldi AF, Cocconi T, Cecellati S, Manzo L: Neurotoxicity and Molecular Effects of Methylmercury. Brain Res Bull 2001, 55:197–203.

15. Yuan Y, Atchison WD: Methylmercury Differentially Affects GABA\textsubscript{A} Receptor-Mediated Spontaneous IPSCs in Purkinje and Granule Cells of Rat Cerebellar Slices. Journal of Physiology-London 2003, 550:191–204.

16. Aschner M, Aschner JL: Mechanisms of Methylmercury Induced DNA Damage in Rat Frontal Cortex. Toxicology 2005, 207:223–229.

17. Ishig U, Datar S, Couch C, Shapiro IM, Shenker BJ: Methyl Mercuric Compounds Inhibit Human Monocyte Function by Inducing Apoptosis: Evidence for Formation of Reactive Oxygen Species, Development of Mitochondrial Membrane Permeability Transition and Loss of Reductive Reserve. Toxicology 1997, 124:211–224.

18. Linske TL, Atchison WD: Acute Exposure to Methylmercury Opens the Mitochondrial Permeability Transition Pore in Rat Cerebellar Granule Cells. Toxicol Appl Pharmacol 2002, 178:52–61.

19. Atchison WD, Harm MF: Mechanisms of Methylmercury-Induced Neurotoxicity. FASEB J 1994, 8:622–629.

20. Aschner M, Yao CP, Allen JW, Tan KH: Methylmercury Alters Glutamate Transport in Astrocytes. Neurochem Int 2000, 37:199–206.

21. Allen JW, Mutsuk LA, Aschner M: Methylmercury, Inhibits Glutamine Synthetase Activity in Primary Cultures of Cortal Astrocytes. Brain Research 2001, 891:148–157.

22. Brookes N: Invitro Evidence for the Role of Glutamate in the Cns Toxicity of Mercury. Toxicology 1992, 76:245–256.

23. Basu N, Scheuhamer AM, Rouvinen-Watt K, Grochowina N, Evans RD, O’Brian M, Chan HML: Decreased N-Methyl-D-Aspartic Acid (NMDA) Receptor Levels Are Associated With Mercury Exposure in Wild and Captive Mink. Neuro toxicology 2007, 28:587–593.

24. Ndeouette LT, Chan HM: Methylmercury Increases N-Methyl-D-Aspartate Receptors on Human SH-SYSY Neuroblastoma Cells Leading to Neurotoxicity. Toxicology 2008, 249:251–255.

25. Miyamoto K, Nakashita H, Moriguchi S, Fukuyama N, Eto K, Wakamiya J, Murao K, Arimura K, Osame M: Involvement of Enhanced Sensitivity of N-Methyl-D-Aspartate Receptors in Vulnerability of Developing Cortical Neurons to Methylmercury Neurotoxicity. Brain Research 2001, 901:252–258.

26. Aschner M, Aschner JL: Mercury Toxicity - Mechanisms of Blood-Brain-Barrier Transport. Neuroscience and Bio behavioral Reviews 1999, 14:69–76.

27. Katsolos AF, Cocconi T, Cecellati S, Manzo L: Neurotoxicity and Molecular Effects of Methylmercury. Brain Res Bull 2001, 55:197–203.

28. Szics A, Angiello C, Salanki J, Carpenter DD: Effects of Inorganic Mercury and Methylmercury on the Ionic Currents of Cultured Rat Hippocampal Neurons. Cell Mol Neurobiol 1997, 17:273–288.

29. Friberg L, Mottet NK: Accumulation of Methylmercury and Inorganic Mercury in the Brain. Biological Trace Element Research 1989, 21:201–206.
34. Gopal KV: Neurotoxic Effects of Mercury on Auditory Cortex Networks Growing on Microelectrode Arrays: a Preliminary Analysis. Neurotoxicology and Teratology 2003, 25:365–369.

35. Hock C, Drach G, Golombowski S, Muller-Spahn F, Willershausen-Zonnchen B, Schwarz P, Hock U, Growdon JH, Nitsch RW: Increased Bleed Microtubule Levels in Patients With Alzheimer’s Disease. Journal of Neural Transmission 1998, 105:59–68.

36. Mutter J, Naumann J, Schneider R, Walach H: Mercury and Alzheimer’s Disease. Fortschritte der Neurologie Psychiatrie 2007, 75:528–540.

37. Mutter J, Naumann J, Schneider R, Walach H, Haley B: Mercury and Autism: Accelerating Evidence? Neuroendocrinol Lett 2005, 26:439–446.

38. Sieger FAS, Silva GAD, Ardila GP, Garcia RG: Mercury Chronic Toxicity Might Be Associated to Some Cases of Hydrocephalus in Adult Humans? Medical Hypotheses 2012, 79:13–16.

39. Leong CCW, Syed NI, Lorscheider FL: Retrograde Degeneration of Neurite Membrane Structural Integrity of Neurite Growth Cones Following in Vitro Exposure to Mercury. Neuro report 2001, 12:733–737.

40. Olivieri G, Brack C, Muller-Spahn F, Stahelin HB, Herrmann M, Renard P, Brockhaus M, Hock C: Mercury Induces Cell Cytotoxicity and Oxidative Stress and Increases Beta-Amyloid Secretion and Tau Phosphorylation in SHSY5Y Neuroblastoma Cells. J Neurochem 2000, 74:231–236.

41. Poulain FE, Sobel A: The Microtubule Network and Neuronal Morphogenesis: Dynamic and Coordinated Orchestration Through Multiple Players. Mol Cell Neurosci 2010, 43:15–32.

42. Feng J: Microtubule: A Common Target for Parkin and Parkinson’s Disease Toxins. Neuroscientist 2006, 12:469–476.

43. Johansson C, Castoldi AF, Onishchenko N, Manzo L, Vahter M, Ceccatelli S: Neuro behavioural and Molecular Changes Induced by Methylmercury Exposure During Development. Neurotox Res 2007, 11:241–260.

44. Ankarcrona M, Dybub JI, Orenius S, Nicotera P: Calcineurin and Mitochondrial Function in Glutamate-Induced Neuronal Cell Death. FEBS Lett 1996, 394:321–324.

45. Chung RS, McCormack GH, King AE, West AK, Vickers K: Glutamate Induces Rapid Loss of Axonal Neurofilament Proteins From Cortical Neurons in Vitro. Exp Neuro 2005, 193:481–488.

46. Stemmer JE, De Zeeuw CI, Weber JT: Don’t Get Too Excited: Mechanisms of Glutamate-Mediated Purkinje Cell Death. Creating Coordination in the Cerebellum 2005, 148:367–390.

47. Blankenship AG, Feller MB: Mechanisms Underlying Spontaneous Patterned Activity in Developing Neural Circuits. Nat Rev Neurosci 2010, 11:18–29.

48. Rao A, Craig AM: Activity Regulates the Synaptic Localization of the NMDA Receptor in Hippocampal Neurons. Neuron 1997, 19:801–812.

49. Shu YS, Hasenstaub A, McCormack DA: Turning on and Off Recurrent Balanced Cortical Activity. Nature 2003, 423:298–303.

50. Thivierge JP: How Does Non-Random Spontaneous Activity Contribute to Brain Development? Neural Networks 2009, 22:901–912.

51. Piovavaro NB, Nguyen HV, Winters CA, Brantner CA, Smith CL, Andrews SB: Excitotoxic Calcium Overload in a Subpopulation of Mitochondria Triggers Delayed Death in Hippocampal Neurons. J Neurosci 2004, 24:5611–5622.

52. Orenius S, Zhitkovich B, Nicotera P: Regulation of Cell Death: The Calcium-Apoptosis Link. Nat Rev Mol Cell Biol 2003, 4:522–565.

53. Mattson MP, Duan WZ: “Apoptotic” Biochemical Cascades in Spastic Compartmental: Roles in Adaptive Plasticity and Neurodegenerative Disorders. J Neurosci Res 1999, 58:152–166.

54. Pendergast JC, Haley BE: Inhibition of Brain Tubulin-Guanoine 5’-Triphosphate Interactions by Mercury: Similarity to Observations in Alzheimer’s Disease. Metal Ions in Biological Systems 1997, 34:461–478.

55. de Assis GPS, Silva CEC, Stefanon I, Vassallo DV: Effects of Small Concentrations of Mercury on the Contractile Activity of the Rat Ventricular Myocardium. Comparative Biochemistry and Physiology C-Toxicology & Pharmacology 2003, 134:375–383.

56. Wiegand GA, Stefanon I, Padilha AS, Pecanha FM, Vassallo DV, Oliveira EM: Low Nanomolar Concentration of Mercury Chloride Increases Vascular Reactivity to Phenylphrine and Local Angiotensin Production in Rats. Comparative Biochemistry and Physiology C-Toxicology & Pharmacology 2008, 147:252–260.

57. Xu F, Luk C, Richard MP, Zaidi W, Farkas S, Getz A, Lee A, van MJ, Syed N: Antidepressant Fluoxetine Suppresses Neuronal Growth From Both Vertebrate and Invertebrate Neurons and Perturbs Synapse Formation Between Lymnaea Neurons. Eur J Neurosci 2018, 31:994–1005.

58. Farina M, Rocha J, Aschner M: Mechanisms of Methylmercury-Induced Neurotoxicity: Evidence From Experimental Studies. Life Sci 2011, 89:555–563.

59. Kater SB, Mills LR: Regulation of Growth Cone Behavior by Calcium. J Neurosci 1991, 11:891–899.

60. Kolek T: Nerve Growth Factor-Induced Neurite Outgrowth of Rat Pheochromocytoma PC-12 Cells - Dependence on Extracellular Mg2+ and Ca2+. Brain Research 1983, 280:293–303.

61. Schubert D, Lacroberre M, Whitlock C, Stallcup W: Alterations in the surface properties of cells responsive to nerve growth factor. Nature 1978, 273:718–721.

62. Almohanna FA, Cave J, Bolsover SR: A Narrow Window of Intracellular Calcium-Concentration Is Optimal for Neurite Outgrowth in Rat Sensory Neurons. Developmental Brain Research 1992, 70:287–290.

63. Mattson MP: Calcium As Sculptor and Destroyer of Neural Circuitry. Exp Gerontol 1992, 27:29–49.

64. Mattson MP, Kater SB: Calcium Regulation of Neurite Elongation and Growth Cone Motility. J Neurosci 1987, 7:4034–4043.

65. Miura K, Inokawa M, Imura N: Effects of Methylmercury and Some Metal-Ions on Microtubule Networks in Mouse Gioma-Cells and Invitro Tubulin Polymerization. Toxicol Appl Pharmacol 1984, 73:218–231.

66. Stoiber T, Degen GH, Bolt HM, Unger E: Interaction of Mercury(II) With the Microtubule Cytoskeleton in IHR-32 Neuroblastoma Cells. Toxicol Lett 2004, 151:99–104.

67. Cadrin M, Wastenez GJ, Jonesvilleneuve EM, Brown DL, Reuhl KR: Effects of Methylmercury on Retinoic Acid-Induced Neuroectodermal Derivatives of Embryonal Carcinoma-Cells. Cell Biology and Toxicology 1988, 4:61–80.

68. Weisbeek RC: Microtubule Formation In-Vitro in Solutions Containing LowCalcium Concentrations. Science 1972, 177:1104.

69. Mattson MP, Engle MG, Rychlik B: Effects of Elevated Intracellular Calcium Levels on the Cytoskeleton and Tau in Cultured Human Cortical-Neurons. Mol Chem Neuropathol 1991, 15:117–142.

70. Azizeman E, Lipton SA, Loring RH: Selective Modulation of Nmda Responses by Reduction and Oxidation. Neuron 1989, 2:1257–1263.

71. Lazarewicz JW, Wroblewski JT, Palmer ME, Costa E: Reduction of Disulfide Bonds Activates Nmda-Sensitive Glutamate Receptors in Primary Cultures of Cerebellar Granule Cells. Neurosci Res Comm 1989, 4:91–97.

72. Tang LH, Azizeman E: The Modulation of N-Methyl-D-Aspartate Receptors by Redox and Alkylation Reagents in Rat Cortical-Neurons In vitro. Journal of Physiology-London 1993, 465:303–323.

Cite this article as: Xu et al: Mercury-induced toxicity of rat cortical neurons is mediated through N-methyl-D-aspartate receptors. Molecular Brain 2012 5:30.