Heavy metals (Pb, Cd, As, MeHg) as risk factors for cognitive dysfunction: A general review of metal mixture mechanism in Brain

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Abstract

Human exposure to toxic heavy metals is a global challenge. Concurrent exposure of heavy metals, such as lead (Pb), cadmium (Cd), arsenic (As) and methylmercury (MeHg) are particularly important due to their long lasting effects on the brain. The exact toxicological mechanisms invoked by exposure to mixtures of the metals Pb, Cd, As and MeHg are still unclear, however they share many common pathways for causing cognitive dysfunction. The combination of metals may produce additive/synergetic effects due to their common binding affinity with NMDA receptor (Pb, As, MeHg), Na^{+} - K^{+} ATP-ase pump (Cd, MeHg), biological Ca^{+2} (Pb, Cd, MeHg), Glu neurotransmitter (Pb, MeHg), which can lead to imbalance between the pro-oxidant elements (ROS) and the antioxidants (reducing elements). In this process, ROS dominates the antioxidants factors such as GPx, GS, GSH, MT-III, Catalase, SOD, BDNF, and CERB, and finally leads to cognitive dysfunction. The present review illustrates an account of the current knowledge about the individual metal induced cognitive dysfunction mechanisms and analyse common Mode of Actions (MOAs) of quaternary metal mixture (Pb, Cd, As, MeHg). This review aims to help advancement in mixture toxicology and development of next generation predictive model (such as PBPK/PD) combining both kinetic and dynamic interactions of metals.

Key words: Metal toxicity, Metal mixture, Cognitive dysfunction, Hippocampus, Mode of actions.

Abbreviations

ABCC = ATP-binding cassette, AchE = Acetylcholine esterase, As = Arsenic, BBB = Blood brain barrier, BDNF = Brain derived neuronal factor, CaM=Calmodulin, CAM-K= Calmodulin K, CAMKII = Calmodulin-dependent protein kinase-II, CAM-II=Calmodulin-II, Cd = Cadmium, CERB = c-AMP response element binding protein, CH_3 Hg = Methyl mercury, CNS = Central nervous system CP= Choroid plexus, CSF = Cerebrospinal fluid, DMT-I = Divalent metal ion transporter-I, ECHA = European Chemical Agency, GABA= ¥-amino butyric acid, Glu = Glutamate, GP_x = Glutathione peroxidase, GS = Glutathione synthase, GSH = glutathione, JNK3= c-Jun N-terminal kinase3, LTP = Long-term potentiation, MAPK = Mitogen-activated protein kinase, MDR = P-glyco protein, MT-III= Metallothionein, NMDA = N-methyl-D-aspartate, n-NOS= Neuronal nitric oxide synthase, Pb= Lead, PBPK/PD= Physiological based pharmacokinetic/pharmacodynamics model, PCB = Polychlorinated biphenyls, PKA= Protein kinase-A, PKC = Proteinkinase-C, PLC = Phospholipase-C, P^{38} MAPK= P^{38} mitogen-activated protein kinase, ROS= Reactive oxygen species, SOD = Superoxide dismutase, Tf = Transferrin.

1. Introduction

Heavy metals are naturally occurring elements with high atomic weight and are released by natural events and human activities. Their multiple industrial, domestic, agricultural, medical, and technological applications have led to their wide distribution in the environment, raising concerns over their potential effects on human health and the environment.

Human exposure to toxic metals has lately been decreasing in developed countries but in other parts of the world (mostly in developing countries), is increasing (Järup, 2003). Several population-based surveys indicate that metal exposure is still widespread (Nadal et al., 2004; Esteban-Vasallo et al., 2012; Alves et al., 2014; CDC, 2014; Mari et al., 2014;; Rovira et al., 2015; Vilavert et al., 2015). Major human exposure of metals results from anthropogenic activities such as mining and smelting operations, industrial production, domestic and agricultural use of metal-containing compounds (He et al., 2005). Metals are systemic toxicants that are known to induce multiple organ damage, even at lower levels of exposure (Hullmann et al., 2012). In general, lead (Pb), cadmium (Cd), methylmercury (MeHg) and arsenic (As) are some of the most toxic metals human are exposed to which target essential organs namely kidney, liver, and brain causing nephrotoxicity, hepatotoxicity, and neurotoxicity (WHO, 2007). According to world health organization (WHO) these four elements rank among the priority metals that are of great public health concerns (WHO, 2010).

The quaternary mixture of Pb, Cd, As and MeHg, a very frequently occurring metal mixture in the environment, have common exposure with common disease outcome such as cognitive dysfunction. The current literature of metal induced neuronal damage is primarily confined to single metal exposure (Zhu et al., 2014) and no published literature was found on quaternary mixture of Pb, Cd, As and MeHg evoked toxicity. Metal mixture exposure and their mode of action relation evaluating either independent or addition and synergistic effects are not well developed (Rodríguez et al. 1998; Sasso et al. 2010). However, the evidence for these kind of interactions continue to grow (Stackelberg, 2013). Predictive in-silico model developments are also constrained by lack of mixture experimental data. Growing evidence of neurotoxicity and their relation to common disease outcome demands further research in the area of cumulative risk assessment of these metals.

Most of these metals (with the exception of Cd) are known to increase susceptibility to cognitive dysfunction and neuro-degenerative outcomes (Clarkson, 1987). In case of Pb, experimental evidence have shown that, children who experienced Pb exposure were found their brain volume modified (Cecil et al., 2008). The Centre for Disease Control (CDC) limits the childhood Pb^{+2} intoxication to 10 µg/dl in blood (Landrigan, 2000). This level is thought to be the threshold for potential adverse effect of childhood cognitive deficits (Jusko et al., 2008). The exposure of Pb during early postnatal life produces a greater deficit in learning performance than in older animals (Kuhlmann et al., 1997). The Arsenic, also a potent neurotoxic, induces hippocampal-dependent behavioral deficits in rodent models (Martinez-Finley et al., 2011). Bellinger, (2013) proved that higher concentrations of As alters growth and development in children resulting in neurological deficits. Gong and O'Bryant, (2010)

found relationship between As exposure and Alzheimer's disease. Experimental animal models have disclosed potent alterations in hippocampal function with As exposure (Cronican et al., 2013). In vivo studies in rat showed that As exposure impacts on the synaptic activity of neurons localized in the hippocampus region of the brain (Krüger et al., 2006). In case of Hg, certain mercury compounds have well established link to neurotoxicity, affecting the normal development of the central nervous system (Crespo-López et al., 2009). However, exposure to inorganic mercury results in brain or nerve damage is not as certain, since it does not easily pass from the blood into the brain (Debes et al., 2006). Organic mercury (MeHg) may affect many different areas of the brain and their associated function. Exposure of MeHg to fetal brain is more susceptible to mercury-induced damage than the adult brain (Clarkson et al., 2003). In vitro studies in animals have indicated that MeHg can affect the biochemical processes believed to be involved in Alzheimer's disease (Leong et al., 2001), and axonal degeneration is unique to MeHg (Castoldi et al., 2003). In some recent studies, Cd has also been shown to produce free radicals in the brain (Czarnecki et al., 2012). In occupational and epidemiological studies with workers exposed to Cd evidence of memory loss was found (Hart et al., 1989). Chronic occupational exposure to Cd leads to slowing psychomotor functions of brain (Viaene et al., 2000). A in vivo study of Cd, reported the disruption of hippocampus region in brain resulting long term potentiation (LTP) function blocked (Luo et al., 2009).

In mixtures, metals have competitive interactions with macromolecule/transporter because of their functional similarities. Normally metals are transported and eliminated through many common cellular mechanisms usually termed as a molecular mimicry (Bridges and Zalups 2005). In addition, toxic metals have significant interactions with essential metals (iron, manganese, calcium) which can influence the essential metal status in the human body (Goyer, 1997). The metal mixture toxic interactions could be either dose additive, interactive (synergistic or antagonistic) or independent of each other, which can generate high level biochemical changes in different regions of brain. Recently, Rai et al. (2010) found that subchronic exposure to the ternary mixture of Pb, Cd and As in wistar rat caused neuronal developmental disorder by synergistic action. This result gives a significant evidence of the metal mixture's neurotoxic activity and their potential interactions.

There is a consensus in the field of mixture toxicology that the classical chemical-by chemical approach to risk assessment might be too simplistic and it may be underestimating the risk of chemicals to human health and to the environment (El-Masri, 2007; Sharma, 2016). The scientific state of the art of mixtures toxicology is sufficiently advanced to make mixture risk assessment possible in a wide range of settings relevant to human toxicology. The issue of metal mixtures toxicology is timely and important, given the attention at EU level and form part of research objectives of recent EU projects. For example, cumulative lifetime exposure of various stressors including metals build up in the human exposome is being addressed in HEALS project (in relation to neurodevelopment risk) and general mixtures toxicity testing strategy in Euromix project. The aim of this study as summarized in Figure 1, was to review the current understanding of mode of action for major toxic metals (Pb, Cd, As and MeHg) in brain with particular emphasis on the metal-neurochemical

interactions. Additionally, this review elaborates metal mixtures mode of actions to summarize the prevailing proofs that support the hippocampus as the major target for metal mixture for inflicting the cognitive dysfunction/loss of memory in both children and adults. In the final part of the review an integrated framework is proposed for studying the metal mixture mode of toxicity in brain. The review is concluded with a future perspective on metal mixtures study and the development of next generation predictive model (such as PBPK/PD) combining both kinetic and dynamic interactions of heavy metals.

2. How metals enter the brain?

The microenvironment of the total brain is separated from the systemic circulation of remainder of the body by blood brain barrier (BBB) and choroid plexus (CP), that defend brain integrity from toxic chemicals/metals (Zheng et al., 2003). Metals are most frequently absorbed from the gastrointestinal tract, across the lungs, or through the skin and then enter the systemic circulation. Metal may enter into the brain from the blood by overcoming the BBB and by crossing the CP into the cerebrospinal fluid (CSF), and from CSF, it will reach to a specific part of the brain (Yokel, 2006). Ordinarily, BBB greatly limits the diffusion of non-lipophilic substances in and out of the brain (Bhowmik et al., 2015). Saunders et al. (2014) reported that protective efflux mechanisms like ATP-binding cassette (ABCC) and P-glyco protein (MDR) are there to forestall the entry of toxic chemicals in the brain. Despite these protective barriers, the weak integrity of blood-cerebrospinal fluid barrier allows these toxic chemicals to enter to the choroid fluxes regions of the brain (Wright and Baccarelli, 2007). Zheng et al., (2001) found that metals are accumulating in the BBB and CSF and then may reach the brain) Many recent studies have suggested that more investigation is required to know how metal exposure disrupts the BBB function during the early brain development of a foetus (Bridges and Zalups, 2005; Caserta et al., 2013).

The neurotoxic metals such as Pb, Cd, As and MeHg and their role in blood-brain barrier disruption for reaching the brain have been studied individually and reported in the literature. For crossing the BBB, toxic metals mimic the behaviour of essential nutrients for utilizing the physiological ionic transporters. Manton et al. (1984) reported 100-fold increase in the Pb levels in human choroid plexus compared to the amount present within brain cortex. These Pb accumulated in choroid plexus is most probably reaching the targeted region. The other neurotoxic metals such as Cd has also shown high permeability to cross the BBB in rats (Shukla and Chandra, 1987). In vivo study of Cd has been reported that Cd freely penetrates and accumulates in the developing and adult rats' brain (Méndez-Armenta and Ríos, 2007) and, therefore, the penetrated Cd powerfully binds with metallothionein (MT-III) (Uchida et al., 1991). MT-III is a sulphur containing macromolecule located in the cerebral cortical neurons (Xu et al., 2011). In case of As, exposure to pregnant rat has shown that fetal brain neurons initiate the apoptotic and necrosis process, which may be due to poor defense mechanism of the underdeveloped blood brain barrier during early brain development (Wright and Baccarelli, 2007). In case of mercurial compounds, they are found in two major forms specifically inorganic mercury (IHg) and organic mercury (MeHg). MeHg has shown higher neurotoxic behavior than IHg. The reason may be the limitation of brain transport mechanism or the chemical properties of the IHg. Whereas MeHg freely enters to brain by

binding with endothelial cysteine(AA) sulphhydral-groups (-SH) to form the neutral amino acid analog (i.e. methionine) that is mimicking the structure of methionine for entering the brain (Aschner and Aschner, 1990).

Exposure to metal mixtures takes place in different life stages (embryo, fetus, new born, child, adult, old age) which is termed as windows of exposure. The amount of internal dose of metal in a brain may have high inter-individual variability and highly dependence on the anatomical and physiological development in the brain barrier system (Yu et al., 2011). Some experimental studies have found proof of metals transfer in fetus stage, however, evidence is not clear for all four metals (Gundacker and Hengstschläger, 2012). Cd accumulates in the placenta during gestation but transfer of Cd to the fetus appears to be restricted (Lin et al., 2011). Early life exposure of arsenic causes deficits in intelligence and memory by influencing brain weight and neurotransmitter system (Tolins et al., 2014). Whereas, Pb does not accumulate within the placenta and therefore, the concentration of Pb in maternal blood is almost similar to that of fetal blood (Bhattacharyya, 1983). Choi et al. (1989) reported that MeHg initiates brain damage in prenatal stage.

Apart from BBB, Pb, Cd, As and MeHg metals additionally influence the essential metal transporters. Toxic metals have affinity for divalent metal ion transporter-I (DMT-I), and transferrin Tf-transporters that leads to toxic and essential metal interactions in BBB and brain tissues. In developing brain, essential metals (Fe, Cu, Mn) are transported from systemic circulation to brain by DMT-I, and Tf (Piloni et al., 2013). Rodriguez et al., (1998) reported that Mn and As had greater accumulation in rat brains relative to the single metal exposure. McCall et al. (1996) reported that Cd, Pb and As produce synergistic action for reducing the expression of glial fibrillary acidic protein (GFAP), which is crucial macromolecule in blood brain barrier. Rai et al., (2010) found that ternary mixture of As, Cd, and Pb has larger than the additive response on astrocyte toxicity by disrupting the BBB performance that culminate into neurological deficits in developing rats.

3. Heavy metals (Pb, Cd, As and MeHg) risk on brain: toxicological evidence

A common susceptibility factor for Pb, Cd, As and MeHg comprises the cognitive deficit but the risk level depends on exposure intensity and metal-biochemical interactions in the brain. Basha et al., (2005) observed that developmental exposure to Pb exhibits latent effects which are known to be epigenetic interaction of Pb with amyloid precursor protein gene that causes neuro degeneration in later age. Prenatal exposure to Pb influences the long-term potentiation (LTP) machinery in the developing brain by disrupting the N-methyl-D-aspartate (NMDA) receptor expression in hippocampus region that results in cognitive deficit in children (Sadiq et al., 2012). Cd produces neurological alterations including memory loss and mental illness (Wang and Du, 2013), that chiefly influences the discharging of repressing neurochemical glycine and gamma-amino butyric acid (GABA) into the extracellular space which upset the balance between excitation–inhibition in synaptic neurotransmission (Minami et al., 2001). Inorganic As exposure has shown effect on the cognitive function of brain (Tyler and Allan, 2014). Ex-vivo cell culture studies showed that As exposure increases the β- amyloid protein and induces hyperphosphorylation of tau protein which results in neurodegeneration (Giasson

et al., 2002). As also enhances the cellular apoptosis pathways such as caspase-3 and caspase-9 in all brain regions which raises oxidative stress and neuronal cell death (Kumar et al., 2013). Animal studies showed that the developmental exposure to MeHg have long-term consequences in the brain (Johansson et al., 2007), and also alter the homeostasis in brain (Atchison and Hare, 1994) resulting in oxidative stress (LeBel et al., 1990) and cognitive disability. Metal mixtures exposure could be a bigger risk for cognitive dysfunction, as well as behavior and impaired neurological (CNS) development than individual metal (Rai et al., 2010). In vivo studies showed that rats exposed to ternary mixture of As, Cd, and Pb bear the essential features of Alzheimer's-like pathology, and increased processing of hippocampal and cortical amyloid precursor protein (APP) gene causing cognitive dysfunction (Ashok et al., 2015). In another study, it has been found that interaction with mixture of essential metals like Zn, Cu and Fe may influence the amyloid-β (Aβ) aggregation (Atwood et al., 1998) and independently or in mixture Fe, Pb and Mn may raise the risk of Parkinson disease (Lai et al., 2002). Limited numbers of studies have found sufficient indication that quaternary metal mixture of Pb, Cd, As and Hg may have high capability to cause cognitive dysfunction, however the evidence of mixture and disease relation is not clear.

4. Critical Neurobehavioral and molecular changes induced by metals

The metals Pb, Cd, As and MeHg have toxic impact on the brain by different molecular mechanism in which metal ions interact with neurotransmitters, receptors and its' subunit, biological Calcium (Ca^{+2}), ion pumps, enzymes and amino acid functional groups. Each metal has unique nature of causing neuronal damage. In developing rats, experimental study (Rai et al., 2010) provides evidence that metal mixtures exposure during brain development has a greater impact on the neurological deficits compare to adult rats due to the lack of barrier system and poor development of defense mechanism (antioxidants). Among various regions in brain, metal mixtures majorly causes site specific damage to hippocampus (Angelica and Fong, 2014). The hippocampus is an important brain region for acquisition of memory (Snyder et al., 2005). In normal physiological situation NMDA receptor plays key role for the cognitive functions and neuronal synaptic plasticity (Lasley et al., 2001). Functional sensitivity of NMDA receptor depends on glutamate (Glu) release and Glu binding at postsynaptic region. The interaction of Glu-NMDA receptor enhances the influx of Ca^{+2} into post synaptic neuronal cytosol and activates the multiple Ca^{+2} dependent enzymes (kinases, calmodulin, phospholipases) for LTP function/memory (Li et al., 2007).

To understand the potential neurotoxicity of metal mixtures and their common cellular elements involved in different molecular mechanism, we have approached the rest of the study by reviewing individual metal's mode of action with hippocampus region of the brain as target site and finally summarising evidences of common molecular interactions. We hypothesize that summaries evidences from individual metal's study can give us potential direction for metal mixtures' molecular interaction for this particular region of brain, which can be tested in the future study.

4.1 Mode of neurotoxicity of Lead (Pb)

The brain damage induced by Pb depends on age (Landrigan, 2000) and level of exposure (Bradbury and Deane, 1993). Generally children absorbs significantly more Pb than adults (Goyer, 1996) due to an underdeveloped blood–brain barrier (Ruff et al., 1996). There is a very little proof that Pb will harm the functions of the blood-brain barrier at a lower dose of <80 µg/dl (Bradbury and Deane, 1993)**.** However, Slomianka et al., (1989) found hippocampus damage at blood Pb levels of 20µg/dl. Jett et al., (1997) reported that continuous low level exposure of Pb (250 ppm) caused hippocampus damage in adult rat (Jett et al., 1997). In brain, Pb mainly disrupts the hippocampus region by interacting with NMDA receptor. According to Guilarte & Miceli (1992), Pb and NMDA receptor interaction is voltage independent, and non-competitive (Guilarte and Miceli, 1992)

Pb interaction with NMDA receptor has two steps: synaptically and extra-synaptically. Pb primarily disrupts the Ca^{+2} ion signalling mechanism in neuronal synapsis by NMDA–Glu process. Pb modifies the NMDA-receptor subunit (NR2A,NR2B) expression, and forming complex, which leads to dysregulation of Ca^{+2}-sensitive signalling pathways in hippocampus (Toscano and Guilarte, 2005). Zhang et al., (2002) ascertained that chronic exposure to Pb decreases the NR2A content and increases NR2B content within the hippocampus. Hippocampus cell cultural study revealed that Pb could downregulates synaptic NR2A-NMDA receptor and concomitantly upregulates the NR2B-NMDA in the extra synaptic region (Neal et al., 2011). The NR2A, NR2B part is critical for NMDA receptor expression and neuronal activity (Kim et al., 2005).

In vivo rat studies showed that hippocampal expression of NR1/NR2A receptor assemblies could be altered because of Pb exposure which may be linked to persistent alterations of brain derived neuronal factor (BDNF), neuronal nitric oxide synthase (n-NOS), c-AMP response element binding protein (CERB) resulting in LTP function inhibition (Guilarte et al., 2001). The summarized molecular mechanism of Pb in hippocampal synaptic region is shown in Figure 2. Pb enters the hippocampus synaptic region and blocks the NMDA receptor function inducing the influx of NMDA ion channel dependent Ca^{+2} depletion. Therefore the effects of Ca^{+2} dependent processes like calmodulin-II (CAM-II), neuronal nitric oxide synthase (n-NOS) and cAMP response element-binding protein (CERB) are inhibition. The altered receptor performance could influence the neuronal plasticity, because of alterations linked with long-term potentiation (LTP) dysfunction (Baranowska-Bosiacka et al., 2012).

In the extra synaptically region, concentration at levels of picomolar is enough for substituting the micromolar concentrations of Ca^{+2}, which may activate the protein kinase-C (PKC) resulting in Pb induced neurotoxicity (Sanders et al., 2009). Wang et al. (2008) reported that due to the Pb exposure, PKC and Calmodulin (CAM) mRNA expression influenced the impairment of learning and memory. This could be another molecular mechanism of Pb evoked hippocampus impairment. The mode of actions within the extra-synaptically neurons is represented in Figure 3 which is antagonist to synaptical regions mechanism. In extra synaptical area, the exposure to Pb increases the Ca^{+2} ion concentration because of enhanced NMDA receptor NR2B expression (Jusko et al., 2008) and producing a

drastically increase level of intracellular endoplasmic reticulum calcium (Ca^{+2}), and phospholipase-C (PLC) (Yin et al., 1994). The enhanced PLC upregulates the protein kinase-A (PKA), the mitogen-activated protein kinase (MAPK), and the calcium/calmodulin-dependent protein kinase (CAMKII) functions leading to higher production of reactive oxygen species (ROS) and fall down of cellular protective elements such as CREB, BDNF, catalase and superoxide dismutase (SOD). The imbalance between defensive elements and ROS causes neuronal cell death (Yin et al., 1995).

4.2 Mode of neurotoxicity of Cadmium (Cd)

Cd causes a wide variety of toxic effects on the central nervous system (CNS). Kumar et al., (1996) reported that Cd affects the brain by disruption of specific membrane function, principally in the hippocampus region. Higher neurotoxicity is reported in newborn than adult, and this variation may be due to lack of blood brain barrier maturation in newborn (Wong and Klaassen, 1982). In new born, higher accumulation of Cd has been found in choroids plexus region of brain (Pal et al., 1993). In-vivo experimental studies of pups, it has been shown that the utero exposure to Cd could inhibit the acetylcholine esterase (AchE), Na^{+}/ K^{+} - ATPase pump which reduces the neuronal activity in pups (Chandra, 1991). Cd also mimics the ubiquitous intracellular ion Ca^{+2}, which acts as a signalling mediator in numerous cellular processes including cell proliferation, and differentiation (Xu et al., 2011). The Cd mode of action as represented in Figure 4, shows inhibition of all known pathways of cellular Ca^{+2} influx and acts as a competitive ion to Ca^{+2}. Consequently, it influences the membrane action potential and neurotransmitters release (Huguenard, 1996). Few studies also reported that Cd influences the Ca^{+2}-binding with molecules like calmodulin K (CAM-K) (Hayat et al., 2003).

Experimental dose-dependent studies disclosed that concentration of cytoplasmic and nuclear Ca^{+2} increased in neurons as result of higher Cd exposure (Orrenius and Nicotera, 1994). Changes in the homeostasis of cytosolic Ca^{+2} concentration affect the regulation of many cellular events (Alshuaib and Byerly, 1996). Principally it causes oxidative stress in the brain cells by induction of reactive oxygen species (ROS) and consequent reduction of intracellular glutathione (GSH), catalase and SOD activity (López et al., 2006). On the other hand, Cd exposure reduces the activity of MT-III and, BDNF, in the brain (Durczok et al., 2005) resulting in increased ROS levels. An experimental study in cerebral cortical neurons identified as targets of Cd-mediated induced neuronal cell apoptosis (Méndez-Armenta and Ríos, 2007).

4.3. Mode of neurotoxicity of Arsenic (As)

Low concentration exposure to As causes cognitive dysfunction (Naujokas et al., 2013), whereas growth delays and neuronal tube defects has been linked to high concentration exposure (Ahmed et al., 2011; Vahter, 2009). Studies in children have found evidence to poor performance scores after long-term exposure to As (Wasserman et al., 2004). In experimental animal models, it has been observed that As causes potent alteration in hippocampal region and dysfunctional cognitive behavior (Cronican et al., 2013). Another in

vivo study showed that As primarily impact the synaptic plasticity of neurons in the hippocampus region of the brain (Krüger et al., 2006). Another ex-vivo cell culture study provided evidence of As capacity to increases β-amyloid protein, which induces the hyperphosphorylation of tau protein resulting in neurodegeneration (Giasson et al., 2002). Cellular level experiments reported that As has property to alter the metabolism of assorted neurotransmitters like monoamines, acetylcholine, gamma amino butyric acid (GABA), and glutamate (Rodriguez et al., 2002). In a recent study, a big reduction in monoamines such as adrenaline, noradrenaline, dopamine, and serotonin was observed in corpus striatum, frontal cortex, and hippocampus areas of brain during chronic As exposure (Yadav et al., 2010). As suppresses the NMDA receptors in hippocampus, which play a pivotal role in synaptic plasticity, learning, and memory (Luo et al. 2009; Kruger et al. 2009).

As also affects the neurotransmitter metabolism by increasing AchE activity and glutamate decarboxylase (GAD) mRNA expression. The AchE has been suggested as potential biomarker of Arsenic neurotoxicity (Patlolla and Tchounwou, 2005). The toxic effects of As in the brain could be attributable not only to the change of neurotransmitter but also direct action on oxidative stress mode of action as represented in Figure 5. In vivo study in rat from prenatal to early life stage has found that As exposure produces imbalance in defensive antioxidative mechanism and neuro transmitter metabolism in the hippocampus region of brain (Xi et al., 2010), where As reduces GSH, glutathione peroxide (GPx) and glutathione synthase(GS) activity and elevates the lipid peroxidation at postnatal day 0, 28, and 42, respectively (Rigon et al., 2008). In As related oxidative stress, the released ROS and lipid peroxidation elements increase the activity of SOD and decrease the glutathione-related enzymes, which lead to change in the cellular redox status (Rao and Avani, 2004). Biochemically, As mediated toxicity involves the induction of apopotic factors in the cerebral neurons by activating p38 mitogen-activated protein kinase (P^{38} MAPK) and c-Jun N-terminal kinase 3 (JNK3) (Namgung and Xia, 2001), which enhances DNA damage and subsequently death of the brain cell (Felix et al., 2005) and resulting in impairments of neurobehavioral function (Prakash et al., 2015).

4.4 Mode of neurotoxicity of MeHg

Generally exposure to mercury can takes place as inorganic and organic mercury. The distribution, toxicity and metabolism of mercury is largely dependent on its chemical form (Aschner et al., 2007). Among all chemical forms, MeHg has the higher distribution rate than other forms of mercury due to its lipophilic nature and long half-life ($t_{1/2}$ =70 days) (Clarkson et al., 2003) and freely reaches the brain (Clarkson and Magos, 2006). In early life stage, fetal brain is more susceptible than the adult brain, because MeHg easily crosses the placental barrier thereby easily reaching the fetus (Björnberg et al., 2003). MeHg is also found in the mother milk, exposing newborn during the breastfeeding period (Grandjean et al., 1994). Johansson et al., (2007) ascertained that developmental exposure of MeHg has long term consequences in brain. Neurotoxicity expressions of MeHg are based on its interaction with cellular elements such as neurotransmitter, disruption of microtubules, and alteration of intracellular Ca^{+2} ion homeostasis (LeBel et al. 1990). Biochemically, MeHg has

high affinity to sulphur (-SH) containing molecules, mainly targeting cysteine and methionine-containing proteins (Suzuki et al., 1976).

In Figure 6, the MeHg mode of action represented, in neuronal synapsis MeHg initiated the inhibiting astrocytic glutamate (Glu) uptake process and then stimulating its efflux from cytosol vesicles, resulting in higher Glu concentration in the extracellular fluid (Brookes and Kristt, 1989; Dave et al., 1994). Brookes et al., (1992) reported that Hg^{+2} ion markedly inhibits the clearance of extracellular Glu in astrocyte cultures and spinal cord cultures and does not impair the sensitivity of neurons to the excitotoxic action of Glu. The enhanced Glu targets Glu based NMDA receptor in brain (Choi, 1992) resulting in receptor hyperactivation which raises the Na^{+} and Ca^{+2} influx in neuronal cells (Lafon-Cazal et al., 1993). The elevated Ca^{+2} acts as a second messenger causing alterations of protein phosphorylation (Sarafian, 1993). On the other hand, MeHg directly disrupts the mitochondrial activity by generating the uncontrolled release of Ca^{+2} from the mitochondria and inhibiting the mitochondrial enzymes function and phosphorylation (Atchison and Hare, 1994). Farina et al. (2011a) reported that MeHg inhibits the mitochondrial electron transport chain (ETC) in brain cultured cell line. In another interesting study found that MeHg exhibits a direct inhibitory effect on the activity of GP_x in mouse CNS resulting in increased lipid peroxidation and decreased Glu uptake into cerebrocortical slices (Farina et al., 2005). Further, it has been reported that production of NO (nitric oxide) following microglial activation causes a decline in cellular GSH levels (Moss and Bates, 2001). MeHg has high affinity to bind GSH (Franco et al., 2007) resulting in weakening of antioxidant level (Johansson et al., 2007). In the mitochondria, MeHg raises the inner membrane action potential which up-regulate the hydroxyl, superoxide, peroxide (OH, O_2^{-}, H_2O_2) and simultaneously down regulate defensive enzymes such as SOD, catalase, GP_x, GR functions, resulting in oxidative stress (Mori et al., 2007). Experimental evidence found that 48 hours after a single injection of MeHg (1 mg/kg, intraperitoneal) in mice and 1 week after a single injection of MeHg (5 mg/kg, intraperitoneal) in mice, the rate of formation of ROS in both rat and mouse cerebellum increased significantly (Ali et al., 1992).

5. Analyzing the metal mixture (Pb, Cd, MeHg & As) mode of toxicity in brain (Hippocampus)

In previous experimental studies (Jiri Patocka, 2014; Wang and Fowler, 2008; Wright and Baccarelli, 2007; Zheng et al., 2003), individual metal neurotoxicity was observed. Some studies carried out with binary metal mixtures showed that metal mixture has more strength to produce common outcome such as cognitive dysfunction. For instance, some studies concluded that the binary mixture of Pb and Cd has greater than additive effect on divalent metal transporter (DMT1) protein synthesis in the developing rat brain, which results in enhanced metal transport rate and finally higher cognitive dysfunction. Similarly, experiment on pregnant rat showed that combined exposure of Cd and Pb have additive effect on decreasing Na^{+} /K^{+} -ATP ase function, in which Cd activity is potentiated by Pb for causing failure of the Na^{+}/K^{+}-ATPase pump. The Na^{+}/K^{+}-ATPase pump inhibition generates depletion of intracellular K^{+}, accumulation of intracellular Na^{+} and increases in intracellular free Ca^{+2} resulting in intensified cognitive dysfunction (Antonio et al., 2003). In another

binary mixture study of Pb and As in brain, Mejia et al., (1997) ascertained that the binary mixture affects the hippocampus by drastically enhanced mode of action of Pb in presence of As. Rai et al., (2010) observed that the ternary mixture of Pb, As and Cd triggers the release of intracellular Ca^{+2}, generates the ROS, stimulates the extra cellular signal-regulated pathway (ERK), c-Jun N-terminal kinases (JNK) pathway, and mitogen-activated protein kinase3 (MAPK3), resulting in neuronal oxidative stress. However, it failed to prove the clear epidemiological relationship between ternary mixture (Pb, As, and Cd) exposure and cognitive dysfunction.

The above evidence strengthens effects of Pb, Cd, As, and MeHg mixture, which produces common adverse outcome termed as cognitive dysfunction or loss of memory by influencing the different interactions and common interactions in hippocampus. The exact toxicological mechanisms invoked by exposure to mixtures of the metals Pb, Cd, As and MeHg are still unclear. It is also found that each metal share common cellular elements such as receptors, enzymes, neurotransmitters for causing cognitive dysfunction. Therefore, it can be safely assumed that the modes of actions of individual metal toxicity can be used as a baseline for understanding potential mechanisms associated with mixture (Pb, Cd, As, and MeHg). Further, we hypothesize that concurrent exposure to Pb, Cd, As, and MeHg may have greater-than additive/synergistic toxic responses to cause cognitive dysfunction. Metal mixture containing Pb, Cd, As and MeHg, have high permeability to blood-brain barrier. However, these metals have specific mode of action. For example Pb interacts with NMDA receptor by two different mechanisms. Guilarte et al., (2000) reported that hippocampal expression of NR1/NR2A receptor assemblies may alter the physiological properties of NMDA receptor during brain development, resulting in deficits in LTP. Cd mainly inhibit the Na^{+} /K^{+} -ATP ase pump and increases the intracellular Ca^{+2} ion, produces neuronal cell stress (Méndez-Armenta and Ríos, 2007). Studies in the rat showed that As impacts the synaptic activity of neurons localized in the hippocampus by inhibiting the NMDA function similar to Pb and upregulating the AchE function. MeHg inhibits the GAD, Glu transporter affecting the Glu uptake and NMDA over expression (Farina et al., 2011). MeHg also disrupts the microtubules in the brain due to high affinity to binding the sulphur containing amino acids -SH (Farina et al., 2011).

Using this hypothesis, in the following Figure 7, we present common mode of actions of Pb, Cd, As and MeHg in the hippocampus region of brain. Figure 7 emphasizes the common links between Pb, Cd, As and MeHg to cause the cognitive dysfunction. Pb^{+2} have been shown to interfere with the Glu transmission and may disrupt the NMDA expression in synaptic and extra synaptic region. Cd^{+2} has shown to interfere with Na^{+}/K^{+} -ATP ase and biological Ca^{+2}. As has the property to bind with AchE, and GAD. MeHg has high affinity to Na^{+} / K^{+} -ATPase enzyme, extra synaptic NMDA receptor, Glu and biological Ca^{+2}. The functional interaction of the four metals seems to cause common adverse outcome by influencing the anti-oxidant elements status.

The dynamic interaction of metals (Pb, Cd, As, and MeHg) with neurochemicals may alter the essential neuronal cell integrity by down-regulation of elements such as n-NOS, MT-III, catalase, SOD, BDNF, GP_x, GSH and GS. Therefore, common susceptibility factor of these

four metals is the imbalance between the defensive elements and reactive oxygen species which is called as oxidative stress. The oxidative stress leads to programmed neuronal cell death by cellular signalling pathways which results in cognitive dysfunction (Ceccatelli et al., 2010; Gavazzo et al., 2008; Stackelberg, 2013; Wang and Du, 2013; Wright and Baccarelli, 2007).

6. Conclusion and Future Perspective

We have reviewed the neurotoxicity of Pb, Cd, As and MeHg extensively and found that prolonged exposure of these environmental toxicants have extreme susceptibility to the brain. During this review, we explored each of these metals' modes of action on brain hippocampus region based upon disease outcome evidence. With the available information, we found clear correlation between metals' exposure and cognitive adverse effects. We have also found many pieces of evidences from in vivo interaction studies of metal and biochemical molecules in neurons, which points to the consistent similarity with down-regulation of homeostasis of neuron functionality.

In the individual metal mode of action studies, Pb, As and MeHg have been found as potent neurotoxicants. However, some recent studies have proved neurotoxic character of Cd which acts by mimicking the biological cellular Ca^{+2} and potentially inhibiting the Na^{+}/K^{+} -ATPase ion transporter in neuronal cell membrane. This behavior of Cd may influence the protective elements such as BDNF, MT-III, and deprivation of protective elements leading to neuronal cell apoptosis. Evidence of Pb interference with neuronal functions by modulating the NMDA receptor subunit expression was found which results in alteration of neuronal plasticity by down-regulation of n-NOS and upregulation of PLC causing neuronal cell apoptosis. As produces cognitive deficit even at low doses by modulating the NMDA receptor and significantly deactivates the defensive enzymes (anti-oxidants) such as GP_x, GS and GSH leading to programmed cell death. In case of mercury compounds, many chemical forms are present, but MeHg has high capacity to target hippocampus region by two possible mechanisms. First one is interacting with Glu, GAD, NMDA receptor, another is intracellular disruption, due to high affinity to sulphur containing (-SH) enzymes and microtubules, resulting in ROS upregulation which leads to cell death.

The exact toxicological mechanisms invoked by exposure to mixtures of the metals Pb, Cd, As and MeHg are still unclear. Chemically heavy metals are polar in nature and this may be the reason for associations with common neuronal elements. However, until now, no study was found to prove mechanistic view of metal mixture external exposure and organ/brain internal exposure relation for finding the common disease end point. There is a need of more focused investigation to know the common mechanism of metal mixture causing neuronal damage.

Recommendations for study design and evaluation of combined effects of metal mixtures (binary, ternary, quaternary) are unknown due to lack of information and experimental studies on mixtures were often not well designed. It was observed that one of the disadvantages of experimental studies is the expensive large combinatorial in vivo studies

involving animals. Furthermore regulatory frameworks such as REACH in the EU are becoming more and more critical regarding the use of animal testing. Therefore, better predictive tools are needed to use limited in vivo study and enable in vitro data on toxicological effects to be interpreted for wider mixtures study and comprehensive risk assessment of mixtures.

It is practically impossible to test all these possible metals mixtures (binary, ternary, quaternary) experimentally, especially in vivo. Therefore, rational and alternative strategies are needed to assess the metal mixtures toxicity in brain (hippocampus). Ideally these tools should be more robust in providing the necessary neurotoxic information of defined metal mixtures (El-Masri, 2007). The concentration addition (CA) approach could be helpful for finding the combination of mixtures (binary, ternary and quaternary) risk assessment in target based toxicity (Cedergreen, 2014). In this review, we explored the biological target site of metal mixtures and found brain hippocampus region as common site of action. Similarly, Adverse Outcome Pathway (AOPs) might provide insight into the relevance of combinational effects when assessing the toxicity of mixtures (Caldwell et al., 2014), whereas, in vitro mechanistic assays can be used to elucidate the mixtures mechanism of action in broader context (Ankley et al., 2010). Omics based on in vitro methodology increasingly is applied to gain insight in the mechanism of action of mixtures at the transcription level, the protein level, and at the metabolome level (Borgert, 2007). Omics study of metal mixture may help in generating sufficient information to understand possible interactions of metals with different molecular components. Finally using friendly in silico Physiological Based Pharmacokinetic/Pharmacodynamic (PBPK/PD) models may be helpful to incorporate known mechanisms and biological processes into use-friendly software for studying different exposure scenarios and their possible risk, which can be finally used for regulatory purposes. PBPK/PD are built using the body as a set of interconnected compartments of differential mathematical equations describing the absorption, distribution, metabolism, elimination (ADME) of a specific chemical and/or its metabolite, and then they connect the internal dose to the dose response of the adverse dynamic effect for a chemical (WHO, 2010). Generally speaking, PBPK/PD models can provide a tool to estimate the internal concentration of a chemical and also useful for establishing tolerance levels for mixtures of neurotoxicity (Tan et al., 2011). In the past few decades, numerous individual PBPK or metal kinetic models have been developed for Pb, Cd, As, MeHg (O'Flaherty 1991; Yu 1999; Carrier et al. 2001). One of the early pioneer of metal research, O'Flaherty had stated in his work of 1998 that further work from mechanistic point of view and experimental data are needed to support further development and refinement of these models (O'Flaherty, 1998). Generally these individual kinetic models have different set of modelling assumptions and lack interaction mechanism even at kinetic stage (such as metabolism). Integration of these individual models is difficult for mixtures toxicity estimation. However, we greatly appreciate the individual metal neurotoxicity studies, which is a key line for modelling design and development. These studies help in understanding the metal mixtures (binary, ternary and quaternary) and disease relation by application of predictive mixture modelling framework such as PBPK/PD. Recently some attempt has been made to integrate these models (Sasso et al., 2010). However, it has been mostly loose integration without harmonization of modelling

assumption. So far, comprehensive mixture modelling efforts have not been pursued in the field of toxic metals, despite ample evidence of interactions of these toxic metals and with other essential elements. Advance predictive model like PBPK/PD may be very useful since they provide a highly refined tool, in which it should be possible to reduce uncertainty for higher tier risk assessments of single and multiple chemicals. The availability of commonly accepted user-friendly physiological based modelling platform is crucial for toxicological study (Ashauer et al., 2011). The above all alternative novel methods may contribute to the 3R (Replacement, Reduction and Refinement) principal of ECHA (Joseph et al., 2015) for more human relevant approach to the risk assessment of metal mixtures.

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Figure Captions

Figure 1. Conceptual diagram of metal mixture exposure- toxicology-disease outcome scenario (brain) (Ankley et al. 2010). Potential interactions of metal mixture for crossing the blood brain-barrier (BBB) gives internal exposure, critical for estimating effective concentration of individual metal in the mixture responsible for potential risk of cognitive dysfunction.

Figure 2. Schematic representation of Pb^{+2} ion entry into hippocampal synaptic region by competitive mechanism with glutamate (Glu), and high affinity to binding with NMDA receptor forming Pb-NMDA complex influences the expression of NR2A subunit resulting in low release of Ca^{+2} ion and inhibited LTP function.

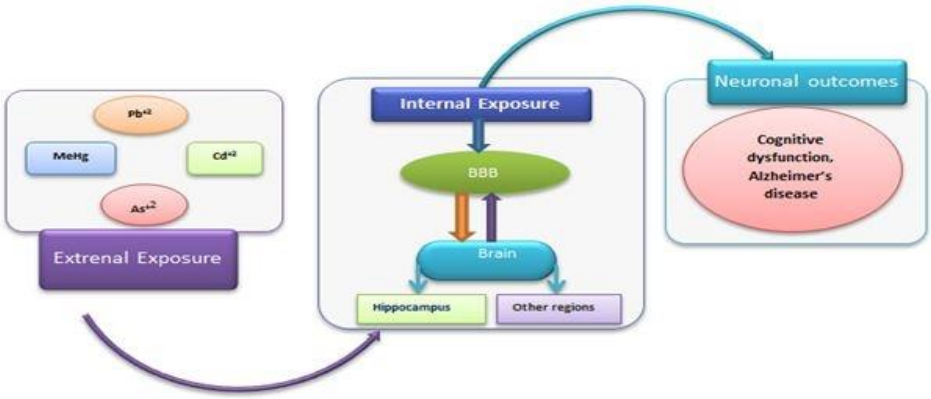
Figure 3. In extra synaptical region Pb^{+2} directly binds with NMDA receptor's NR2B subunit, causing positive enhancement of NR2B expression which results into enhanced Ca_i^{+2} ion flow, finally leads to imbalance between apoptosis factors and antioxidants elements.

Figure 4. Cd^{+2} ion enters neuron cytosol by mimicking the voltage gated Ca^{+2} channel, causing downregulation of BDNF, $GP_{x,}$ Catalase, SOD, AchE, MT-III, CAM-K, and upregulation of intra cellular $Ca^{+2}{}_i$ producing free radicals which causes neuronal cell death.

Figure 5. Schematic representation of arsenic metal neurotoxicity, - sign indicates inhibition of cellular elements and + sign shows rising apoptotic factors.

Figure 6. The mode of action of methyl mercury (MeHg) by two pathways: I) Neurons physiological functions and II) Reductive defensive mechanism (– sign indicates inhibition)

Figure 7. Common mode of actions of metal mixtures in brain. Interactions with receptor (NMDA), enzyme (Na^+ - K^+ ATP ase, AchE), ion (Ca^{+2}), alter the n-NOS, MT-III, Catalase, SOD , BDNF, CERB, GP_x , GSH, GS levels, causing potential risk of cognitive dysfunction.



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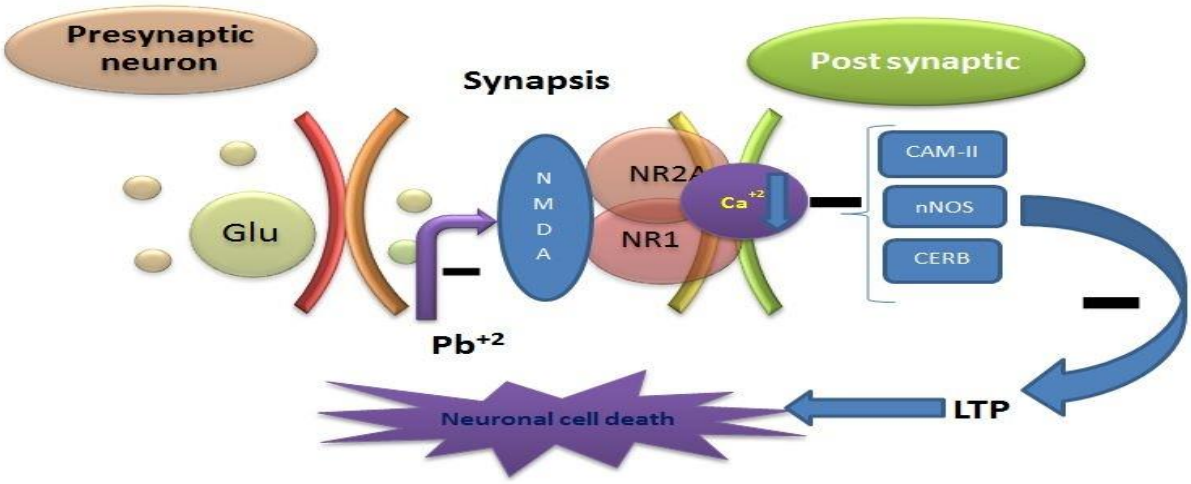

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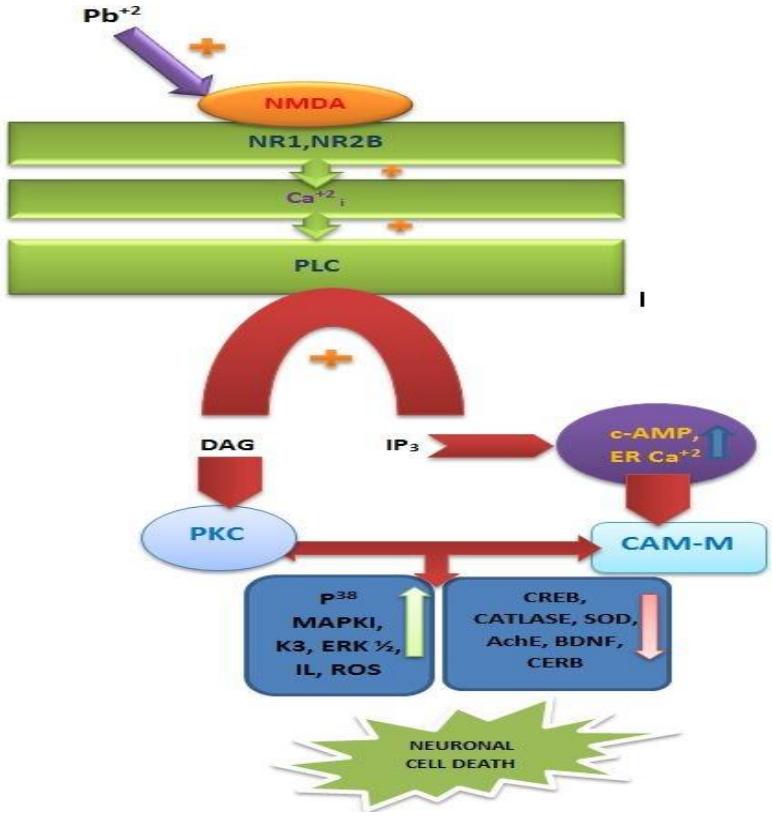

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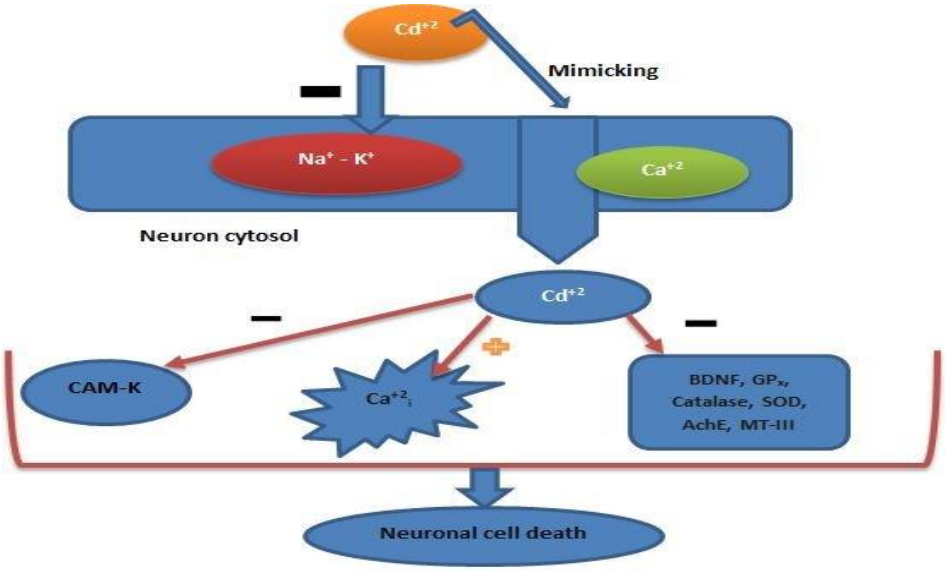

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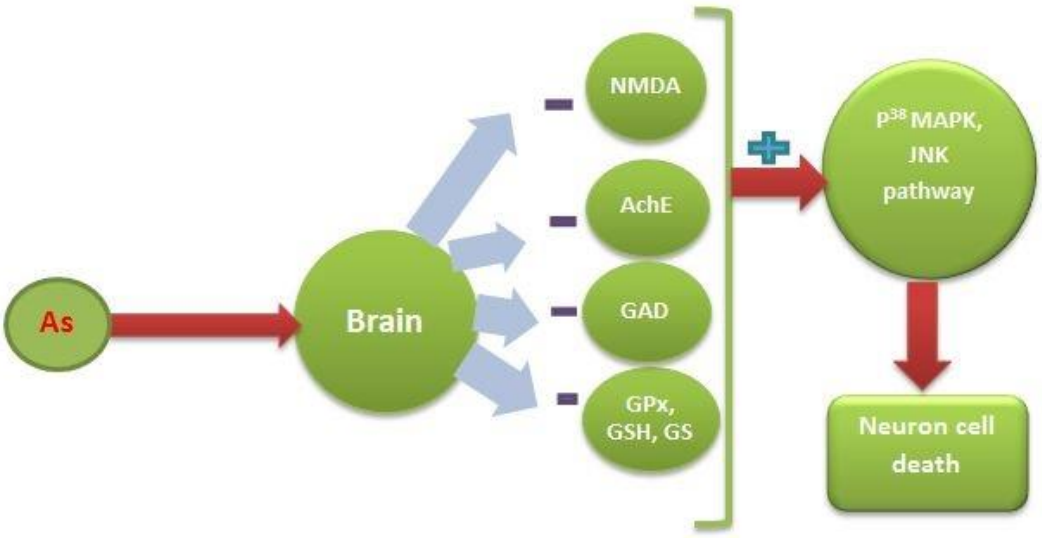

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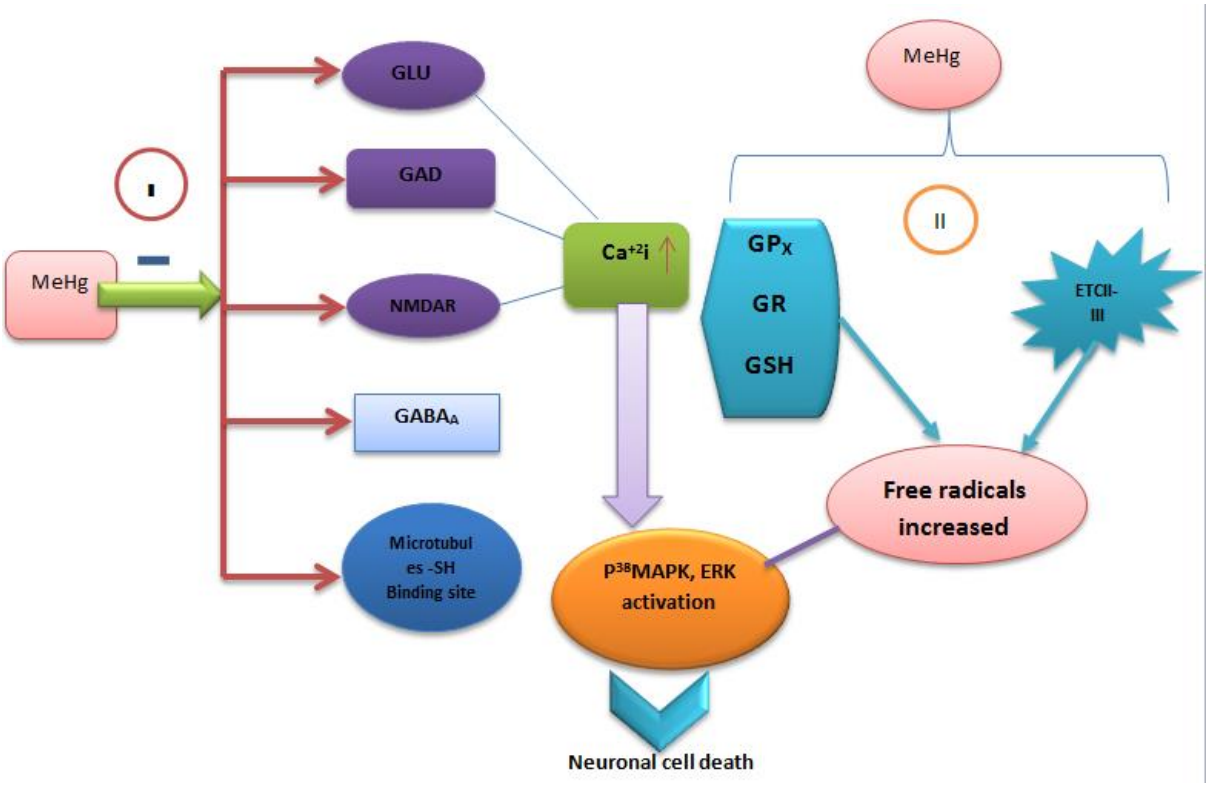

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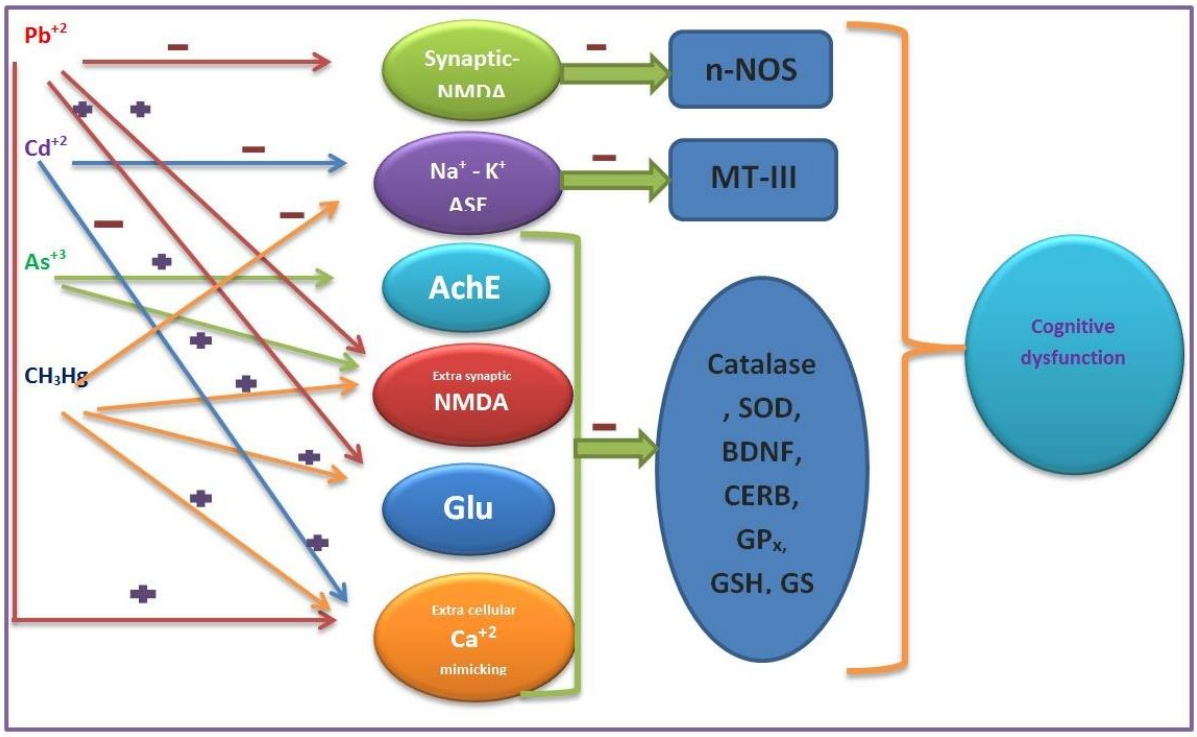

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