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Molecular Targets of Lead in Brain Neurotoxicity

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The detrimental effects of lead poisoning have been well known since ancient times, but some of the most severe consequences of exposure to this metal have only been described recently. Lead (Pb^{2+}) affects the higher functions of the central nervous system and undermines brain growth, preventing the correct development of cognitive and behavioral functions. As an established neurotoxin, Pb^{2+} crosses the blood-brain barrier rapidly and concentrates in the brain. The mechanisms of lead neurotoxicity are complex and still not fully understood, but recent findings recognized that both Ca^{2+}-dependent proteins and neurotransmitters receptors represent significant targets for Pb^{2+}. In particular, acute and chronic exposure to lead would predominantly affect two specific protein complexes: protein kinase C and the *N*-methyl-D-aspartate subtype of glutamate receptor. These protein complexes are deeply involved in learning and cognitive functions and are also thought to interact significantly with each other to mediate these functions. This review outlines the most recent hypotheses and evidences that link lead poisoning to impairment of these protein functions, as well as the *in vitro* experimental approaches that are most likely to provide information on basic mechanicistic processes.

Keywords: Metal uptake; Calcium channels; Glutamate-activated channels; NMDA receptor subunits; Synaptic plasticity; Protein kinase C

INTRODUCTION

Lead (Pb^{2+}) is widely recognized as a potent central neurotoxin that interferes with neuronal functions and causes a wide variety of long lasting adverse effects, especially in developing brains. Recent research has established a definite relationship between prenatal and postnatal low-level lead exposure and children's cognitive function disabilities (reviewed in Needleman, 1993; Markowitz, 2000). Epidemiological studies indicate a correlation between lead levels as low as 10 μg/dl in blood and bones and poor performance in attitude tests (IQ or psychometric tests) (Bellinger *et al.*, 1991; Stiles and Bellinger, 1993; Banks *et al.*, 1997). A similar correlation was found in behavioral studies carried out in experimental animals that had been exposed to lead during gestation, lactation and post-weaning (Cory-Slechta, 1997; Jett *et al.*, 1997; Kuhlmann *et al.*, 1997; Stewart *et al.*, 1998; Morgan *et al.*, 2001) and numerous reports showed how low-level lead exposure in the early postnatal period results in enduring neuroplastic deficits (Cory-Slechta, 1995; Bourjeily and Suszkiw, 1997; Murphy and Regan, 1999; Lasley and Gilbert, 2000). Although some observations indicated that lead-induced neurotoxicological deficits may be reversible (Finkelstein *et al.*, 1998; Nehru and Sidhu, 2001), chelation therapy was reported ineffective to cure neuropsycological impairments caused by blood lead levels between 20 and 45 μg in children (Rogan *et al.*, 2001) and residual cognitive shortfalls were reported in adults after 50 years from childhood lead poisoning (White *et al.*, 1993). In addition, cross-sectional studies suggested that the pathological processes associated with lead could be involved in the epigenesis of antisocial behavior (Dietrich *et al.*, 2001).

This hideous connection put lead among, and possibly on top of, the most dangerous neurotoxins that unsettle the higher function of the central nervous system (CNS).

Interest has recently been addressed mainly to those molecular targets that might be responsible for cognitive impairment. The effects of lead on the brain, causing cognitive deficit, are mediated by its interference with three major neurotransmission systems: the dopaminergic, cholinergic and glutamatergic systems (Cory-Slechta, 1995; Nehru and Sidhu, 2001). Reductions in dopaminergic activity have been observed in Pb^{2+}-exposed animals (Tavakoli-Nezhad *et al.*, 2001) and have been related to alterations in tyrosine hydroxylase activity (Jadhav and Ramesh, 1997). Early studies indicate that moderate lead exposure has long term repercussions on the cholinergic visual system of adult rats (Costa and Fox, 1983) and primates (Reuhl *et al.*, 1989), while more recent studies

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demonstrate developmental cholinotoxicity following low-level Pb^{2+} exposure, resulting in a persisting deficit in hippocampal cholinergic innervation (Bourjeily and Suszkiw, 1997). Expression of acetylcholinesterase and the acetylcholine receptor were also reduced in isolated tissue from neural retina of embryonic chick after *in vitro* Pb^{2+} treatment (Luo and Berman, 1997).

Although these effects bear striking importance, there are few doubts that the focal point of Pb^{2+}-mediated neurotoxicity is the glutamatergic synapse. Glutamate, the brain's essential excitatory neurotransmitter, mediates synaptic transmission by binding to different types of postsynaptic membrane receptors (Dingledine *et al.*, 1999). Micromolar concentrations of lead can block the ion flux through the membrane channel associated to a specific class of glutamate receptors, the *N*-methyl-D-aspartate (NMDA) type. Through the functioning of the associated ionic channel, the NMDA receptors play an important role in excitatory synaptic transmission and are key players in the processes of neural network creation and consequently in memory and learning functions. These very receptors appear to be one of lead's preferred targets in the neurons of the CNS. Moreover, Pb^{2+} also blocks voltage-dependent calcium (Ca^{2+}) channels (VDCC), which mediate neurotransmitter release, and interferes with the proteomic synaptic machinery (Husi *et al.*, 2000). The way in which all these actions result in an alteration of cognitive development is still partly unknown, but is starting to be unveiled thanks to a variety of experimental approaches, including *in vivo* treatment followed by *in vitro* measurements, and *in vitro* treatment and measurements.

This review will mainly address the correlation between alterations in CNS physiology and functions that are related to the NMDA receptors and to other synaptic proteins, specifically the protein kinase C (PKC) isozymes, studied in *in vitro* and *in vivo* animal models.

PATHWAYS OF LEAD BRAIN POISONING

Chemical Basis of Lead Toxicity

Lead is a xenobiotic metal and exerts its toxicity through mimicking physiological metals and competing for their binding sites, for which it frequently has a higher affinity. Most studies indicates that Pb^{2+} attacks primarily the Ca^{2+} and zinc (Zn^{2+}) binding sites. For many years, lead toxicity was linked to its ability to disrupt Ca^{2+}-dependent mechanisms (Bressler *et al.*, 1999). However, different from another xenobiotic metal, cadmium (Cd^{2+}), which exerts its toxicity through mimicking Ca^{2+}, the chemical basis for Pb^{2+} mimicking Ca^{2+} is not obvious. The ionic radii of Cd^{2+} and Ca^{2+} are fairly close (97 and 99 pm, respectively), while that of Pb^{2+} is 20% larger (120 pm, Weast, 1977). Also the electronic structure of the two elements are significantly different and Pb^{2+} has broader coordination chemistry than Ca^{2+}; the latter prefers oxygen ligands, whereas Pb^{2+} forms also complexes with other ligands, especially the sulphydryl group, and complex ions with OH^-, Cl^-, NO_3^- and $(CO_3)^{2-}$ (Clarkson, 1993). Despite these conflicting evidences, several experimental data demonstrat Ca^{2+}-mimicking actions of Pb^{2+}; this includes block of VDCC (Büsselberg *et al.*, 1993; 1994a,b; Bernal *et al.*, 1997), transport through pathways that are specific for Ca^{2+} (Simons and Pocock, 1987; Tomsig and Suszkiw, 1991; Kerper and Hinkle, 1997a,b; Mazzolini *et al.*, 2001) and binding to numerous Ca^{2+} binding proteins (Markovac and Goldstein, 1988; Goldstein, 1993; Murakami *et al.*, 1993; Long *et al.*, 1994; Tomsig and Suszkiw, 1995; Kern *et al.*, 2000). These Ca^{2+}-mimicking actions will be described in more detail in the following sections.

Besides Ca^{2+} sites, Pb^{2+} interaction at Zn^{2+} binding sites is important. Zinc finger proteins are an important class of transcription factors that contain a Cys2-His2 Zn^{2+} binding domain. This site is highly reactive to metal ions and represents a significant target of toxic metals (Hartwig, 2001). Pb^{2+} has recently been shown to inhibit the DNA-binding mechanism of TFIIA, Sp1 and Egr-1 zinc finger proteins with significant specificity with respect to non finger proteins (Hanas *et al.*, 1999; Zawia *et al.*, 2000; Crumpton *et al.*, 2001). A simple competitive inhibition model could not be demonstrated, because excess exogenous Zn^{2+} did not reverse the inhibition (Hanas *et al.*, 1999) and it appears more appropriate to describe the action of lead as a structural alteration in the Zn^{2+} coordination site. Another putative target of lead neurotoxicity is the Zn^{2+} allosteric binding site on the NMDA receptor channel. Binding of Zn^{2+} to this site restrains receptor activity, so that free Zn^{2+} has been proposed to act as an "atypical neurotransmitter" (Baranano *et al.*, 2001). Pb^{2+} would interfere with the NMDA channel function through this or a closely related binding site (Guilarte *et al.*, 1995; Guilarte, 1997a,b; Lasley and Gilbert, 1999). This action of Pb^{2+} will be discussed in a later section.

A significant problem in modeling the effect of Pb^{2+}, particularly in *in vitro* systems, is to evaluate the actual Pb^{2+} concentration. Pb^{2+} easily forms complexes with simple anions, precipitates as $Pb(OH)_2$ and $Pb_3(PO_4)_2$, and is present as a contaminant in laboratory reagents. Although in principle Pb^{2+} complexes may also mediate toxicity, because they may be converted to free metal or have direct actions themselves (Matthews *et al.*, 1993), there is no evidence to support this possibility. The free

Pb^{2+} concentration may be controlled by carefully chosen buffers and measured by a Pb^{2+}-sensitive electrode (Kivalo *et al.*, 1976; Simons, 1985; 1993a).

Pathways of Pb^{2+} Entrance into the Brain

The ability of Pb^{2+} to gain access to the brain interstitial fluid is determined by the transport properties of the blood-brain barrier, a functional molecular barrier formed by brain capillary endothelial cells linked to one another by tight junctions. Pb^{2+} uptake through the blood-brain barrier proceeds at an appreciable rate, consistent with its action as a potent central neurotoxin (Smith *et al.*, 1997). Although severe lead poisoning may compromise the barrier's integrity, and significant abnormalities following Pb^{2+} administration have been reported (Struzynska *et al.*, 1997), there is little evidence that Pb^{2+} can damage the functions of the blood-brain barrier at a dose <80 μg/dl (Bradbury and Deane, 1993), i.e. at doses that mediate chronic or subacute poisoning. The mechanism by which Pb^{2+} crosses the blood-brain barrier endothelial tissue is not entirely known. Pb^{2+} was shown to traverse cellular membranes by VDCC (Simons and Pocock, 1987; Tomsig and Suszkiw, 1991) and store-activated Ca^{2+} channels (also called "Ca^{2+} release activated channels" or CRAC) (Kerper and Hinkle, 1997a,b) and possibly other passive transport systems. In erythrocytes, Pb^{2+} influx is mediated by the anion exchanger (Simons, 1986), while protein-independent lead permeation has been reported in myelin liposomes (Diaz and Monreal, 1995). Deane and Bradbury (1990) used both intravenous infusion and vascular perfusion of one cerebral hemisphere and found that Pb^{2+} transport across the blood-brain barrier was independent of Ca^{2+} and Mg^{2+} concentration and was not sensitive to Ca^{2+} channel blockers and anion exchanger inhibitors; the effect of K^+-induced depolarization and pH suggested that Pb^{2+} is transported mainly as low-molecular-weight complex, probably $PbOH^+$. Pb^{2+} uptake is mitigated by active back transport of lead into blood by the Ca-ATPase pump (Bradbury and Deane, 1993), a mechanism implicated also in Pb^{2+} extrusion from erythrocytes (Simons, 1993b). More recently, a role of the CRAC has been postulated also in this system (Kerper and Hinkle, 1997a,b).

Recent evidence suggests that the blood-brain barrier is an important filter of Pb^{2+} and its incomplete development makes young animals particularly susceptible to lead neurotoxicity. The amount of Pb^{2+} accumulated in the hippocampus reaches a peak in rats around postnatal day 15 (PN15), and then starts to decline, suggesting that after PN15 the blood brain barrier maturation can limit the amount of Pb^{2+} entering the brain (Guilarte and McGlothan, 1998; Zhang *et al.*, 2002).

Pb^{2+} is also sequestered at the level of the choroid plexus, a blood vessel structure that regulates part of the production and composition of cerebrospinal fluid (CSF) and manufactures and secretes proteins for the extracellular compartment in the CNS (Zheng, 2001). Apparently lead does not damage the choroid plexus structure directly, but it inhibits production and secretion of transthyretin, a major protein manufactured by it and responsible for the transport of thyroid hormones to the developing brain. This effect has been reported in experimental animals (Zheng *et al.*, 1996), *in vitro* (Zheng *et al.*, 1999) and in humans (Zheng *et al.*, 2001).

Lead Accumulation in Astroglia and Central Neurons

Once it has crossed the blood-brain barrier, Pb^{2+} specifically accumulates in brain tissue and its concentration does not fall rapidly even if the blood level of Pb^{2+} declines (Dyatlov *et al.*, 1998). Pb^{2+} influx and accumulation in astroglia and neurons has been mainly investigated in dissociated cultures. From studies in cultured astroglia, it was hypothesized that astrocytes may serve as a Pb^{2+} sink in the mature brain (Tiffany-Castiglion and Qian, 2001). These glia cells are interposed between neurons and the capillary endothelium that forms the blood-brain barrier, with their foot processes enfolding almost the entire circumference of the microvessels. Lead would penetrate the astrocyte membrane through L-type VDCC (Legare *et al.*, 1998) and would be sequestered in non mitochondrial sites, to protect not only the astrocyte's own respiratory apparatus, but also that of the more vulnerable neurons. This "lead sink hypothesis" is supported by measurements of lead accumulation in cultured astroglia, as compared to a neuroblastoma cell line (Lindahl *et al.*, 1999). However, similar studies performed *in vivo* were not conclusive, due to a number of technical problems (Tiffany-Castiglion and Qian, 2001).

Pb^{2+} is rapidly taken up by neurons as well. The pathways of Pb^{2+} influx were studied in cerebellar granule cells from 8-day-old rats by the radiometric fluorescent indicator fura-2 (Mazzolini *et al.*, 2001), as previously described in chromaffin cells (Tomsig and Suszkiw, 1990, 1991). Binding of Pb^{2+} shifts the excitation peak of fura-2 to a shorter wavelength, a behavior similar to that of Ca^{2+} and Cd^{2+} (Usai *et al.*, 1999); as the affinity of Pb^{2+} for fura-2 is 5 orders of magnitude higher than for Ca^{2+} (Tomsig and Suszkiw, 1991), there is hardly any competition between the two ions for fura-2 binding. Pb^{2+} uptake can be further resolved by the membrane-permeant heavy metal chelator tetrakis(2-pyridylmethyl) ethylene-diamine (TPEN), whose affinity for Pb^{+2} is 10 orders

of magnitude higher than for Ca^{+2} or Mg^{+2} (Dyatlov *et al.*, 1998). Pb^{2+} permeates the neuronal membrane through at least three major pathways of permeation, an unspecific one, in the absence of any stimulus, VDCC and NMDA-activated channels (Mazzolini *et al.*, 2001). Different from chromaffin cells for which the VDCC involved were predominantly of the L-type (dihydropyridine sensitive) (Simons and Pocock, 1987; Tomsig and Suszkiw, 1991), in cerebellar granule neurons Pb^{2+} influx was neither antagonized by nimodipine, nor enhanced by BayK8644, but it was slackened by ω-agatoxin IVA (200nM), indicating an involvement of non-L type VDCC, which are the predominant type in these cells. In addition, Pb^{2+} caused a time-, dose- and stimulus-dependent saturation of the dye, whose intracellular concentration is ~10 μM, indicating that intracellular Pb^{2+} can readily reach concentration in the micromolar range in neurons.

PHYSIOPATHOLOGICAL MECHANISMS

Neurotransmitter Release

The ability of Pb^{2+} to use the same pathways of entry as Ca^{2+} has a double detrimental effect: first Pb^{2+} interferes with and damages Ca^{2+} transport systems, secondly Pb^{2+} takes advantage of these systems to penetrate into the cytoplasm and carry on its destructive mimicking action by occupying Ca^{2+} binding sites on numerous Ca^{2+}-dependent proteins that reside intracellularly. Pb^{2+} direct interference with synaptic function is an example of this twofold destructive action by both extra and intracellular mechanisms.

Chronic lead exposure in experimental animals causes a decrease in glutamate and GABA release stimulated by depolarization (Lasley and Gilbert, 1996, 2002). Patch-clamp experiments in a model preparation showed that Pb^{2+} exerts a double effect on neurotransmitter release. Pb^{2+} enhances background transmitter release (TTX-insensitive Braga *et al.*, 1999b) and impairs stimulated transmitter release (TTX-sensitive, Braga *et al.*, 1999a). The two sites of action are respectively located intracellularly and extracellularly. Pb^{2+} at a concentration as low as 100 nM facilitates the voltage-independent release of GABA and glutamate. The relatively long latency and the independence of Ca^{2+} suggested that the mechanism involved is mediated by interactions of Pb^{2+} with intracellular targets located within the presynaptic terminal (Braga *et al.*, 1999b). Pb^{2+} has similarly been shown to act as a high-affinity substitute for Ca^{2+} in triggering vesicular catecholamine release in chromaffin and PC12 cells (Tomsig and Suszkiw, 1990; Tomsig and Suszkiw, 1993; Westerink and Vijverberg, 2002). Because Pb^{2+} is effective at extracellular submicromolar doses, the intracellular mechanism must have a very high affinity for Pb^{2+}. Several synaptic proteins, which are involved in regulating transmitter release and bind Pb^{2+} with high affinity, are possible targets in this phase of poisoning (see also the following subsections). It is highly likely that Pb^{2+} mediates its disturbing effect by interfering with more than one protein.

The second effect of lead on transmitter release, i.e. inhibition of the stimulated Ca^{2+}-dependent component (Braga *et al.*, 1999a) appears to arise from presynaptic inhibition. This effect has a short latency, indicating that the site of lead action is extracellular, is dependent on extracellular Ca^{2+} and reversed by external Ca^{2+} chelators, and has an IC_{50} of between 50 and 100 nM. All these features, including the concentration range, have led to the hypothesis that inhibition of stimulated release might be mediated by a Pb^{2+} ability to block VDCC (Büsselberg *et al.*, 1993;, 1994a,b; Bernal *et al.*, 1997).

Inhibition of VDCC would also explain the decrease of stimulated release in chronically exposed animals (Lasley and Gilbert, 2002), although this observation still lacks direct proof. Because NMDA-stimulated release of glutamate was also reduced in cerebellar granule and glia cells of the offspring of lead-exposed mothers (Lim and Ho, 1998; Yi and Lim, 1998), an involvement of presynaptic NMDA channels cannot be completely ruled out. The effect on lead on the NMDA receptor complex will be discussed in greater detail in later sections.

Synaptic Proteins

Pb^{2+} and Ca^{2+} compete for the same binding site on proteins that utilize a helix-loop-helix motif known as EF hands. These proteins constitute a large family of cytoplasmic ion binding proteins. In particular, evidence has been provided that Pb^{2+} stimulates calmodulin-mediated phosphorylation and calmodulin activation of phosphodiesterase at subnanomolar doses (Goldstein, 1993; Kern *et al.*, 2000). This interaction would be sufficient to increase the availability of vesicles for release, thereby increasing the amplitude of spontaneously occurring synaptic currents.

Pb^{2+} in the nanomolar range can also alter the binding parameters of Ca^{2+} -binding synaptic vesicle protein synaptotagmin. At the presynaptic terminal, the Ca^{2+}-dependent interaction between this protein and phospholipids leads to fusion of the vesicles with the membrane. Nanomolar lead concentrations have been shown to induce interaction of synaptotagmin with phospholipid liposomes (Bouton *et al.*, 2001), suggesting that

Pb^{2+} may interfere with the synaptic machinery through this protein.

Protein Kinase C

Pb^{2+} has been reported to interact, possibly through different binding sites, with PKC isozymes. At picomolar concentrations Pb^{2+} is an effective activator of PKC (Markovac and Goldstein, 1988; Long *et al.*, 1994), but it inhibits the enzyme in the micromolar range and through a mechanism not due to the competition with Ca^{2+} (Murakami *et al.*, 1993). These apparently conflicting observations led to a proposed model of Pb^{2+} as a partial agonist of PKC, capable of both activating and inhibiting the enzyme (Tomsig and Suszkiw, 1995) and this model was later tested and characterized using recombinant human isozymes (Sun *et al.*, 1999). More than 10 isoforms of the PKC family have been described. Of these, the 'conventional' forms (α, β, γ) are normally activated by modulators outside the cell (hormones, neurotransmitters, etc.) through an enzyme chain and in a Ca^{2+}-dependent manner; the direct intracellular activator is diacylglycerol, whose effect can be mimicked by phorbol esthers. The novel PKC isoforms, δ, θ, η and ε , are sensitive to diacylglycerol, but are Ca^{2+}-insensitive, while the atypical forms ζ, μ and ι are independent of both Ca^{2+} and diacylglycerol. The effect of Pb^{2+} is mediated through its interaction with the two coordination sites Ca1 and Ca2 within the C2 domain. Pb^{2+} partially activates the enzyme through pM-affinity interactions with the Ca2 site and inhibits divalent cation-dependent activity through nM-affinity interaction with the Ca2 site in the C2 domain; in addition, it inhibits constitutive kinase activity through μM-affinity interaction with the catalytic domain. As a result, Pb^{2+} has the capacity to both activate and inhibit the conventional PKCs, but it is solely inhibitory to the non-conventional and atypical PKC subspecies (Sun *et al.*, 1999). Pb^{2+}-induced PKC modulation has several implications in Pb^{2+} mediated neurotoxicity. PKC activation facilitates spontaneous transmitter release in several brain areas, and Pb^{2+} was likewise described to induce secretion of norepinephrine release from chromaffin cells (Tomsig and Suszkiw, 1993), possibly by a PKC-mediated mechanism (Tomsig and Suszkiw, 1995). However, although the interaction of Pb^{2+} with different proteins involved in exocytosis has been demonstrated, the mechanism through which Pb^{2+} exerts its action is still unresolved, because specific activators of PKC, but also of calmodulin and calcineurin, have failed to mimic the Pb^{2+} effect (Westerink and Vijverberg, 2002). A recent work (Nihei *et al.*, 2001) suggested that PKCγ gene and protein expression modulations by Pb^{2+} are correlated with Pb^{2+}-induced deficit in LTP maintenance (although not LTP induction) and spatial learning alterations; however the exposure-induced changes in spatial learning have not been reproduced in other laboratories so far. Finally, PKC modulation has been frequently correlated to altered Immediate Early Response Genes (IERG) and expression of other genes (reviewed in Bouton and Pevsner, 2000). In PC12 cells and human astrocytes, IERGs expression was induced by Pb^{2+} exposure through involvement of PKCδ and ε and not PKCα and β activation (Hossain *et al.*, 2000; Kim *et al.*, 2000; 2002).

ROLE OF THE NMDA RECEPTOR

The NMDA Receptor Channel

All functional NMDA receptors (NMDARs) are heteromeric complexes (tetramers or pentamers) and contain two different types of subunits, the essential subunit NR1 and at least one of the four different NR2 types (named A, B, C and D). The physiological and pharmacological properties of these receptors are mainly dependent on the NR2 subunit, although different NR1 splice variants may also influence channel performance (Cull-Candy *et al.*, 2001). The NR2 pattern of expression is tissue-dependent and differs remarkably during development (Wenzel *et al.*, 1997). In the immature brain, the NR2B subunit is widespread, but over the course of development this is supplemented with or replaced by NR2A or, in certain regions, NR2C subunit (Cull-Candy *et al.*, 2001). In the adult brain, the NR2B subunit is predominantly localized in the forebrain area (Monyer *et al.*, 1994) and in the superficial dorsal horn of the spinal cord (Boyce *et al.*, 1999). The NR2C subunit is expressed mainly in the mature cerebellum (Akazawa *et al.*, 1994; Farrant *et al.*, 1994; Guilarte and McGlothan, 1998).

The study of NMDAR ligands and modulators has progressed enormously with the use of heterologous model systems in which NMDAR complexes of known composition are selectively expressed. In these systems, it is possible to study the receptor complex in isolation or in a controlled protein environment and correlate results with those obtained from more complex neuronal model systems. The use of these model systems of different complexity provide a detailed description of the mechanicistic action of different substances, including neurotoxins such as lead, on this receptor.

The specific action of Pb^{2+} on the NMDAR function has been investigated in different systems and these studies are presented and summarized in Table I and will be discussed in the following sections.

TABLE 1 *In vivo* and *in vitro* studies of Pb^{2+} effects on NMDAR function

Experiment type	Tissue and experimental design	Effect	Citation
MK-801- binding sites *	*in vivo* chronic treatment	loss of binding sites in neonatal cerebral cortex, but not in adult	Guilarte and Miceli, 1992
	in vivo chronic treatment	decrease in frontal cortex, dentate gyrus, CA1 and striatum	Cory-Slechta *et al.*, 1997
	in vivo chronic treatment	increase in the hippocampus, occipital and temporal cortex at post-natal day 56 and 112.	Ma *et al.*, 1997
	in vivo subacute treatment, adult	decrease in hippocampus and cerebral cortex	Ma *et al.*, 1997
	in vivo chronic treatment	biphasic dose-effect relationship in hippocampus	Lasley *et al.*, 2001
NMDA-gated channels Patch-clamp native receptors	hippocampal neurons	inhibition with IC_{50} ~ 20 μM; no voltage depedence	Alkondon *et al.*, 1990
	hippocampal neurons	inhibition dependent on days *in vitro*	Ujihara and Albuquerque,
	acutely dissociated hippocampal neurons from 3-30 post-natal day rats	age-dependent inhibition	Ishihara *et al.*, 1995
	cerebellar granule cells	inhibition with IC_{50} ~ 4 μM; dependent on days *in vitro*	Gavazzo *et al.*, 2001
Recombinant receptors in *Xenopus oocytes*	mouse receptors	inhibition with IC_{50} ~ 40 μM all subunit combination	Yamada *et al.*, 1995
	mouse receptors	inhibition with IC_{50} ζ1ε1 ~ ζ1ε2 << ζ1ε1ε2 (from 0.9 to 6.6 μM)	Omelchenko *et al.*, 1996
	rat receptors	inhibition with IC_{50} NR1b-NR2A < NR1b-NR2C < Nr1b-NR2D < NR1b-NR2AC (from 1.5 to 8 μM)	Omelchenko *et al.*, 1997
	rat receptors	inhibition with IC_{50} NR1a-NR2B < NR1a-NR2A < Nr1a-NR2C (from 2.5 to 4.7 μM)	Gavazzo *et al.*, 2001
[^{3}H-] MK-801 binding†	cortical membranes	inhibition of binding in neonatal >> than adult	Guilarte and Miceli, 1992
	neuronal membranes PN14 and adult	binding competition with Zn^{2+} at post-natal day14	Guilarte *et al.*, 1995
	neuronal membranes PN 1-50	age-dependent two affinity binding sites	Guilarte, 1997
	neuronal membranes adults	inhibition with IC_{50} ~ 0.5 μM – no Zn competition	Lasley and Gilbert, 1999
NR subunit expression	*in vivo* exposure, *in situ* hybridization in hippocampus	mRNA NR1↑and NR2A ↓ -age-dependent effects	Guilarte and McGlothan, 1
	chronic *in vivo* exposure, Western blot in hippocampus	protein expression NR2A ↓	Nihei and Guilarte, 1999
	chronic *in vivo* exposure, *in situ* hybridization and immunoblotting	mRNA NR1 ↓ in hippocampus; NR1, NR2A ↓ in dentate gyrus; no change in NR2B	Nihei *et al.*, 2000
	chronic *in vivo* exposure, *in situ* hybridization	mRNA NR1-a ↑, NR1-b↑ ↑ NR1-1, NR1-4 ↑	Guilarte *et al.*, 2000
	RT-PCR and Western blotting in culture	in hippocampus NR2B ↑, in cortex NR1, NR2B ↓	Lau *et al.*, 2002
	in vivo exposure, *in situ* hybridization in hippocampus	NR1-2a, NR2D ↑, NR2A, NR3A ↓, no change in NR2B	Zhang *et al.*, 2002

* as a measure of NMDA receptor density
† as a measure of NMDA receptor status

Block of the NMDA Channel in Native and Recombinant Receptors

Acute exposure to Pb^{2+} in the micromolar range causes a reversible inhibition of the current activated by glutamate through the NMDAR channel in cultured and acutely dissociated neurons (Alkondon *et al.*, 1990; Ujihara and Albuquerque, 1992; Büsselberg *et al.*, 1994a,b; Ishihara *et al.*, 1995). Pb^{2+} was also shown to inhibit [H^3]MK-801 binding to rat neuronal membrane *in vitro* (Alkondon *et al.*, 1990; Guilarte and Miceli, 1992), a biochemical indicator of NMDAR activation. Because MK-801 is a non-competitive channel blocker, it binds inside the NMDAR pore and displays higher affinity for open channels than for closed (non conducting) ones. Pb^{2+} does not compete with MK-801 either, but it binds a specific (still undefined) site on the channel and prevents both channel opening and MK-801 binding inside the pore. Several features of NMDA channel inhibition were described in early studies based on whole-cell clamp and single channel recordings and have later been confirmed. Some of these features are summarized in fig. 1, which show experiments carried out in the author's laboratory. (see fig.1). First, the inhibition is specific to NMDA channels, which are significantly more sensitive to Pb^{2+} inhibition than other glutamate channels (Alkondon *et al.*, 1990; Ishihara *et al.*, 1995; fig. 1A). Secondly the channel block is independent of voltage (Alkondon *et al.*, 1990; Ujihara and Albuquerque, 1992; Ishihara *et al.*, 1995; fig. 1B) and therefore the interaction site is likely to be located away from the electric field, or outside the conducting pore; accordingly, single channel conductance and mean open time were not altered by Pb^{2+}, which, however, caused a reduction in opening frequency, as expected from an agent which is not an open channel blocker, but binds to the channel in both the open and close states with comparable affinity. Thirdly, the effect is noncompetitive since increasing the glutamate or glycine concentration could not overcome either the block of the current (see fig. 1C) or [^{3}H]MK-801 binding to rat neuronal membrane *in vitro* (Alkondon *et al.*, 1990; Guilarte and Miceli, 1992).

As mentioned before , another metal ion, Zn^{2+}, mediates a voltage-independent allosteric modulation in certain NMDA channels, notably those containing the NR2A subunit (Paoletti *et al.*, 1997). The Zn^{2+} binding domain has been clearly identified and described at the *N*-terminal of the NR2A subunit, close to the agonist binding domain (Paoletti *et al.*, 2000). Because of significant analogies obtained by the [^{3}H]MK-801 binding assay (Guilarte *et al.*, 1995; Schulte *et al.*, 1995), it was suggested that Pb^{2+} would interact with the NMDA channel at the Zn^{2+} allosteric binding site. Pb^{2+} significantly altered the inhibition of [^{3}H]MK-801 binding by Zn^{2+} (Guilarte *et al.*, 1995), and, similar to Zn^{2+}, the inhibitory effect of Pb^{2+}, displayed two components, a component of higher affinity, with IC_{50} ranging from 0.3 to 4.7 μM depending on the age of the rat, and a low affinity component (IC_{50}~70 μM; Guilarte *et al.*, 1995; Guilarte, 1997a,b). The two components were correlated to different populations of NMDA receptors that are differently expressed during development (Guilarte *et al.*, 2000). Finally, a hypothesis was formulated that Pb^{2+} and Zn^{2+} bind to a site that allosterically modulates glycine binding. In fact, while Ca^{2+} and Mg^{2+} increase the affinity of NR for glycine (Gu and Huang, 1994; Paoletti *et al.*, 1995; Wang and MacDonald, 1995; Hashemzadeh-Gargari and Guilarte, 1999), both Zn^{2+} and Pb^{2+} antagonize this glycine-dependent potentiation (Hashemzadeh-Gargari and Guilarte, 1999). This possibility is interesting, but not conclusive. Because of significant analogies and findings obtained by the [^{3}H]MK801 binding assay (Guilarte *et al.*, 1995; Schulte *et al.*, 1995), it was suggested that Pb^{2+} would interact with the NMDA channel at the Zn^{2+} allosteric binding site and would interfere with the NMDA channel function through competition with Zn^{2+}. A later study, conducted with the same binding assay, indicated that, although the two metals similarly affect the NMDA receptor channel, they act via independent allosteric binding sites (Lasley and Gilbert, 1999). This work, in which concentrations of metal ions were set by means of a chelating agent, also established the IC_{50} for Pb^{2+}-mediated NMDA channel blockade in the low μM range. However, different from previous work, the [^{3}H]MK-801 binding assay was conducted under equilibrium conditions (see Yeh *et al.*, 1990) and only one allosteric binding site was observed for Zn^{2+} inhibition. For these discrepancies and because of lack of both conclusive electrophysiological data and structural evidences, competition between Zn^{2+} and Pb^{2+}, as well as the location of the Pb^{2+} binding site, is still undefined. Based on the lack of voltage-dependence, it is conceivable that Pb^{2+} would interact with the NMDAR channel through a binding site located outside the conducting pore, possibly in the same area as that of Zn^{2+}, i.e. close to the agonist binding domain, but this has never been demonstrated. Moreover, unlike Zn^{2+}, which inhibits NR2A-containing receptor channels with an affinity that is several orders of magnitude higher than that for other NMDAR channels, Pb^{2+} does discriminate between NR2A- and NR2B NR2B-containing receptors, but the difference in affinity is much smaller (see below and fig.1 D).

The effect of Pb^{2+} on the NMDA channel is age- (Ishihara *et al.*, 1995) and subunit-dependent (Yamada *et al.*, 1995; Omelchenko *et al.*, 1996; 1997; Gavazzo *et al.*,

2001). Ishihara *et al.* (1995) described Pb^{2+} effect and NMDA current development in neurons from 3-30 day-old rats. A fast decaying component of the current was more prominent in neurons from young animals and diminished with age; this component bound glycine with lower affinity and was blocked by Pb^{2+} in an age-independent manner (Kew et al., 1998). Conversely a slowly decaying component was inhibited by Pb^{2+} to a larger extent in neurons from younger rats than those from older ones. Although these authors did not attempt to correlate the age dependency with the appearance of different NMDA subunits, their results can now be interpreted in the light of established properties of NR2 subunits: the fast decaying component is related to the expression of the NR2A subunit, which has been reported to display more prominent inactivation (Krupp *et al.*, 1996) and lower glycine sensitivity, while the slowly decaying current may be due to the presence of either NR2B in early development or NR2C later. These two subunits would confer a differing Pb^{2+} sensitivity to the NMDA channel. In recombinant receptors, Pb^{2+} potency of inhibition was found to be NR1a-NR2B > Nr1a-NR2A > Nr1a-NR2C (Gavazzo *et al.*, 2001), a sequence not in contrast with the above described pattern. These differences support the hypothesis of enhanced Pb^{2+} sensitivity in immature neurons, but are not sufficiently wide to be used as a distinguishing feature for a given subunit, as can be done for Zn^{2+}.

The inhibitory action of lead on the NMDA receptor was proposed to induce changes in NMDA receptor subunit expression (Nihei and Guilarte, 1999; Nihei *et al.*, 2000; see also later subsection) in animals exposed during development and whose Pb^{2+} levels are consistent with those in Pb^{2+}-exposed children. This would imply direct involvement of NMDA inhibition in Pb^{2+} poisoning in chronic intoxication, but this point is controversial. Other authors state that the inhibitory effect of Pb^{2+} on the NMDA channel does not occur at environmentally significant exposure levels (Lasley and Gilbert, 2000) and therefore the effect on receptor conducting properties is more likely to play a role in acute intoxication, which is less dependent on neuronal development.

Effect of Lead on the Expression of the NMDA Receptor Subunits

Chronic exposure has been shown to cause age-dependent changes in NMDAR gene expression *in vivo* (Guilarte and McGlothan, 1998; Guilarte *et al.*, 2000; Nihei *et al.*, 2000; Zhang *et al.*, 2002) and in cultured cells *in vitro* (Lau *et al.*, 2002).

Both the density and the NMDAR status were studied *ex vivo* by comparing binding of [^{3}H]MK-801 after subacute (Ma *et al.*, 1997a,b) or chronic treatment. (Guilarte and Miceli, 1992; Guilarte *et al.*, 1993; Schulte *et al.*, 1995; Cory-Slechta, 1997; Ma *et al.*, 1997a,b; Lasley *et al.*, 2001). Although these studies involved a similar autoradiographic approach, they provided rather scattered results, probably because variable paradigms of treatment were used. MK-801 binding sites are heterogeneously distributed throughout the brain (Ma *et al.*, 1997a,b) and chronic lead exposure causes significant and sustained modifications of binding. The results of these works are not always consistent and the discrepancies can be only partially attributed to differences in the experimental protocol. An increase in receptor density was found in the brain of adult animals after long-term Pb^{2+} administration during gestation, lactation and post-weaning (Guilarte *et al.*, 1993; Schulte *et al.*, 1995; Ma *et al.*, 1997a,b), but other authors, which employed a similar autoradiographic technique as Ma *et al.* (1997a), reported a significant decrease (30%) in MK-801 binding in all brainfour different brain regions of rats treated during weaning and exibiting similar mean blood Pb^{2+} values (16-74 μg/100ml, Cory-Slechta *et al.*, 1997). Finally, a biphasic dose-effect relationship for hippocampal binding was determined for rats treated with Pb^{2+} from early gestation until adult age (Lasley *et al.*, 2001). The increase in receptor density seen at higher Pb^{2+} doses correlates with the decrease in glutamate release and can be due to compensatory up-regulation. Further work is required to identify the mechanisms regulating NMDA receptor density that are sensitive to Pb^{2+} exposure.

Current studies have relied on *in situ* hybridization histochemistry, reverse transcriptase-polymerase chain reaction (RT-PCR), as well as measurements of protein levels by immunoblotting techniques and provided rather more precise information on selective Pb^{2+} modulation of the expression of different NMDAR subunits. In rats, NR1 and NR2A mRNA levels are known to increase during the first postnatal weeks, while the NR2B level decreases. Although the protocol of *in vivo* treatment may vary from one study to another, most authors conducted investigations of Pb^{2+} effects in chronically exposed animal, which received Pb^{2+} in their diet from as early as gestation until weaning and sometimes beyond it. With such treatments, a significant decrease in the expression of the NR2A subunit and subsequent protein expression have been reported by different authors in animals aged 14-28 PN days (Guilarte and McGlothan, 1998; Nihei and Guilarte, 1999; Zhang *et al.*, 2002). The expression of the NR2B subunit appeared to be largely Pb^{2+} insensitive *in vivo* (Nihei and Guilarte, 1999; Nihei *et al.*, 2000; Zhang *et al.*, 2002) and dependent on the neuron type in culture (Lau *et al.*, 2002).

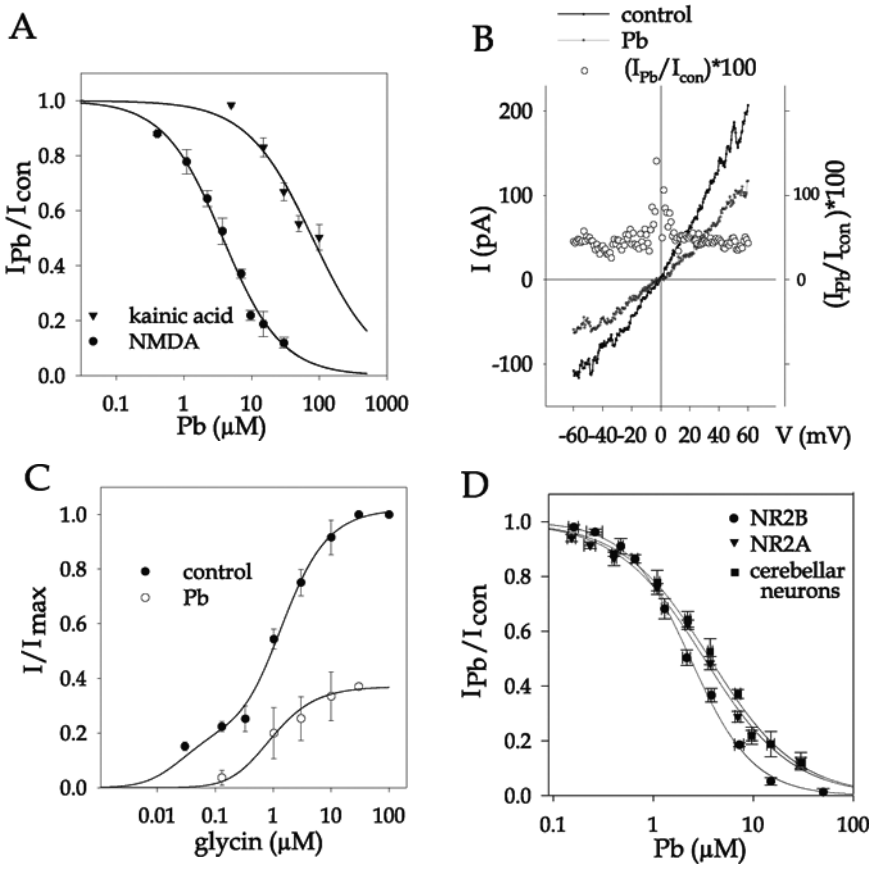

FIGURE 1 Specificity of block by Pb^{2+} on NMDA channel. Data were obtained from rat cerebellar granule cells in dissociated cultures (A through D) and from *Xenopus laevis* oocytes expressing recombinant NR1a-NR2A or NR1a-NR2B receptors (D), in the author's laboratory, as described in Gavazzo *et al.* (2001). (A) NMDA channels are significantly more sensitive to Pb^{2+} inhibition than other glutamate channels (Alkondon *et al.*, 1990; Ishihara *et al.*, 1995). The current was elicited by either 50 μM NMDA in the presence of 30μM glycine (●) or 100μM kainic acid (▼) and different doses of Pb^{2+}. Experimental points were fitted to the equation:
$I/I_{control} \rightarrow 1/[1+([Pb^{2+}]/IC_{50})^{n}]$ where $I/I_{control}$ is the average value of the fraction of current resistant to Pb^{2+} inhibition, $[Pb^{2+}]$ is the free Pb^{2+} concentration and IC_{50} is the free Pb^{2+} dose which provokes a 50% block of the NMDA current. The best fit gave IC_{50} = 3.8 μM and a Hill coefficient n_H = 1 for the NMDA current and IC_{50} = 80 μM and n_H = 0.9 for the kainate current. (B) The channel block is independent of voltage (Alkondon *et al.*, 1990; Ujihara and Albuquerque, 1992; Ishihara *et al.*, 1995). Granule cells were stimulated by voltage ramps from -60 to +60 mV in the presence of NMDA+glycine (control) and of the same agonists plus 10μM Pb^{2+}. The percentage of residual current was calculated as $[I_{Pb}/I_{control}]$*100 and was not dependent on voltage. (C) Pb^{2+} block is not competitive at the glycine site. In control (●), currents were elicited by 50μM NMDA in the presence of increasing concentrations of glycine, and experimental points were best fitted to a 2 X 2 equivalent binding site model (Kew *et al.*, 1998; Gavazzo *et al.*, 2001) with the equation
$I([A]) = I_{maxH}/(1 + mK_{DH}/[A])^2 + I_{maxL}/(1+mK_{DL}/[A])^2$ + where I_{maxH} and I_{maxL} are the current amplitudes of the high and low affinity components, [A] is the concentration of glycine and mK_{DH} and mK_{DL} are the microscopic dissociation constants for the high- and low-affinity components of the curve. The best fit yielded mK_D . 15 and 800 nM respectively. The same experiments were performed in the presence of 10μM Pb^{2+} (❍) and points were fitted by a single binding site model (equation 2 with I_{maxH} = 0), because the high affinity component was too small to be resolved (I_{maxH} = 0). However, there was no significant correlation between the amount of current blocked by Pb^{2+} and the amplitude of the current in low (300 nM) glycine and the effect of Pb^{2+} was independent of glycine concentration. (D) Pb^{2+} block depends on the subunit composition. In voltage-clamped *Xenopus laevis* oocytes expressing recombinant NR1a-NR2A or NR1a-NR2B receptors, Pb^{2+} inhibited glutamate-activated peak currents with IC_{50} (mean ± sem) equal to 2.4 ± 0.1 (●, NR2B, 9 oocytes) and 3.2 ± 0.2 (▼, NR2A, 6 oocytes). These values were significantly different from each other with $p < 0.01$. The Pb^{2+} sensitivity in native channels from cerebellar granule cells (■, see also part A) is closer to that of NR2A-containing receptors. In these experiments, free ionic Pb^{2+} concentrations were measured by a Pb^{2+}-sensitive electrode and are expressed as mean ± sem in 5 to 40 measurements for each dose.

The NR2A subunit confers to the NMDA channel several peculiar features, including fast desensitization and low glycine sensitivity. Possibly linked with these features is the critical role of NR2A subunit in development (Losi *et al.*, 2002). Moreover, the regulation of NR2A mRNA was shown to be strongly dependent on synaptic activity (Hoffmann *et al.*, 2000), while NR2B mRNA expression was not influenced by activity blockade. It is thus possible to speculate that block of NMDA channel conduction by Pb^{2+} may affect NR2A expression and subsequent synaptic development.

MOLECULAR CORRELATES OF COGNITIVE IMPAIRMENT

Models of Synaptic Plasticity

Although compelling evidence exists that the NMDAR subunit proteins are key targets for Pb^{2+}-induced neurotoxicity (Nihei and Guilarte, 2001), how all these effects are related and whether they bear any link to the Pb^{2+}-caused impairment of higher mental functions is still a matter of debate.

The best available electrophysiological model of learning and memory is long term potentiation (LTP), a long-lasting increase in synaptic efficacy in response to high-frequency stimulation of afferent fibers, which can last from minutes to days. LTP is a complex phenomenon, comprising different components, but its most studied form, the LTP at the CA1 synapses in the hippocampus, is largely dependent on NMDAR activity. The important role of this form of synaptic plasticity in learning and memory formation has been widely debated, but has been reinforced by recent findings, which have also showed how it plays an essential role in shaping synaptic connections (Toni *et al.*, 1999).

Pb^{2+} treatment was reported to impair LTP induced by high frequency stimulation following acute exposure *in vitro* (Hori *et al.*, 1993; Carpenter *et al.*, 1994) as well as in *ex vivo* approaches, using tissue taken from chronically exposed animals (Gutowski *et al.*, 1998; Cai *et al.*, 2001). In *in vivo* models Pb^{2+} caused a decrease of LTP amplitude (Lasley *et al.*, 1993; Gilbert *et al.*, 1996; 1999; Ruan *et al.*, 1998) and an elevation of LTP-threshold (Gilbert *et al.*, 1996; 1999). The mechanism of LTP blockade by Pb^{2+} was not clear in early studies because the concentration of lead that totally blocked LTP had no effect on NMDA current (Hori *et al.*, 1993). However, more recent studies revealed a significantly lower value of IC_{50} for Pb^{2+} block of NMDA channel conduction (Lasley and Gilbert, 1999; Gavazzo *et al.*, 2001). In addition there are evidences that NMDA-dependent forms of synaptic plasticity are more susceptible to chronic lead exposure than NMDA-independent processes, because the NMDA-independent mossy fiber potentiation in the CA3 region of the hippocampus was not influenced by lead treatment (Gutowski *et al.*, 1998). Then the deficit in LTP in lead-exposed animals seems to have a postsynaptic locus and may involve NMDA channel inhibition.

Chronic low-level Pb^{2+} exposure also affects an additional mechanism of synaptic plasticity, the long-term depression or LTD. This is an activity-dependent decrease in synaptic efficacy, which occurs in CA1 neurons as well as in other parts of the brain. The effects were measured *in vitro* in hippocampal slices from previously exposed rats (Zhao *et al.*, 1999; Sui *et al.*, 2000a,b). Two components of LTD, i.e. the NMDA-dependent and VDCC-dependent component, were identified and both were affected by previous Pb^{2+} exposure in both area CA1 and dentate gyrus (Sui *et al.*, 2000b), consistent with the specific action of Pb^{2+} on the NMDA receptor complex and VDCC.

These results show how effectively Pb^{2+} can reduce the range of synaptic plasticity. These effects, together with the described disturbances in neurotransmitter release, led to describing the action of Pb^{2+} at the synaptic level as a "decrease in the signal to noise ratio" (Johnston and Goldstein, 1998), which during the critical period of postnatal development may permanently disable the refinement of synaptic connections.

In this respect, a positive correlation has recently been demonstrated between spatial learning and *in vivo* LTP, while changes in NMDAR subunit expression have been identified in animals that showed Pb^{2+}-induced cognitive deficits (Nihei *et al.*, 2000). If confirmed by other studies, this correlation is highly significant because, due to high gastrointestinal absorption and permeability of the undeveloped blood-brain barrier, the same exposure results in a higher brain Pb^{2+} concentration at an age when functional correlates in NMDAR function, such as the magnitude of LTP induction, are maximal (Zhang *et al.*, 2002).

Proteomic Synaptic Machinery

Possible molecular targets for Pb^{2+} in the brain are numerous, as described in a great variety of *in vivo* studies (Nihei and Guilarte, 2001) and this suggests that the neurotoxic action of Pb^{2+} is not confined to a single specific protein, but involves several proteins and their proteomic unit. A proteomic structure has been identified in the glutamatergic synapse and has been named after its electron microscopy appearance "postsynaptic density" (PSD). This structure contains postsynaptic receptors

and their cytoplasmic interacting proteins, such as the protein kinases that regulate receptor function. Mutual interactions among these proteins have been described in rat brain (Suen *et al.*, 1998; Linden *et al.*, 2001). In recent works the NMDAR is regarded as part of a large multiprotein signaling machine, or NMDAR multiprotein complex (NRC, Husi *et al.*, 2000), whose role in the process of information storage and learning is a matter of intense investigation. The signaling machinery has been described as composed of different kinds of proteins, including neurotransmitter receptors, enzymes and cytoskeletal proteins and other kind of proteins (Grant and O'Dell, 2001). Therefore, the interactions of Pb^{2+} with NMDAR, PKC isoforms and other synaptic proteins cannot be regarded as separate phenomena, but are part of a complex neurotoxic attack to the synaptic plasticity machinery. This would result in long-lasting impairment, also in conditions and levels of exposure at which direct toxicity is not apparent.

CONCLUSIONS

Lead neurotoxicity research has progressed steadily in recent years and the interest in its mechanism of action is multifaceted. The obvious medical and social concern for the environmental hazard that lead still represents has fostered studies on how this toxic metal is absorbed, as well as on the conditions of exclusion, tolerance or resistance in cells that accumulate Pb^{2+}. Then, understanding the action mechanism of Pb^{2+} would help to gain an insight into the higher mental functions that it undermines. Moreover Pb^{2+} represents a model neurotoxic agent that is suitable for use as a control substance to design, calibrate and evaluate *in vitro* tests, in toxicokinetics and organ-specific toxicity studies (Clemedson *et al.*, 2002; Holme and Dybing, 2002).

Critical molecular targets for Pb^{2+}-induced injury appear to be present during neuritogenesis and/or synaptogenesis and in this respect the potential of *in vitro* models for studies of synaptic plasticity, neurite extension and synapses formation has opened a new area for neurotoxicological research. The obvious advantages are that *in vitro* cells are directly exposed to the substances under examination at precisely controlled concentrations and it is possible to manipulate the cellular environment. It is also possible to compare different cellular types from different tissues or from the same tissue, but from different species, including the human species. However, *in vitro* systems lack the complexity of intact brain tissue with a number of host factors that could modify the interaction of Pb^{2+} with molecular targets. *In vitro* models, especially those derived from chronically exposed animals, have proven valuable to the aim of identifying mechanisms of Pb^{2+} action, but the correspondence between Pb^{2+}-induced changes following acute exposure *in vitro* and chronic exposure *in vivo* is not always particularly good. Moreover *in vitro* studies are in general considered of little use in quantitative hazard characterization, or in the process of defining the risk-specific doses. In this respect it is important to determine the actual Pb^{2+} concentrations in the brain. Although blood levels provide an index of recent exposure, they do not reflect (Guilarte and McGlothan, 1998; Gutowski *et al.*, 1998; Cai *et al.*, 2001) and are not simply related with the concentration in the brain. Pb^{2+} concentration in the brain appears to be dependent on the animal age (Sui *et al.*, 2000; Zhang *et al.*, 2002), but independent of the brain region, with no selective accumulation in any brain area (Widzowski and Cory-Slechta, 1994, but see Nihei *et al.*, 2000).

The selective vulnerability of neonatal and young animal to Pb^{2+} poisoning is linked to a number of concurrent factors. These include: (i) higher gastrointestinal absorption of Pb^{2+} and higher permeability of the undeveloped blood brain barrier to Pb^{2+}; (ii) enhanced sensitivity to synaptical dysfunctions in the critical early period of shaping synaptic connections; (iii) selective impairment in the expression of a specific NMDAR subunit, the NR2A; (iv) interference with the expression and function of several proteins present in the PSD in glutamatergic synapses, including NMDAR subunits and PKC isoforms; (v) compromised neurotransmitter release, with concurrent enhancement of background noise and decrease in controlled release; (vi) reduction in the range of synaptic plasticity, which results in (vii) alteration in synaptic formation and refinement.

As these factors contribute to open the developing brain to the assault of Pb^{2+} poisoning, it is not surprising that no safety threshold can be defined (Finkelstein *et al.*, 1998) and any exposure to Pb^{2+} is to be considered potentially harmful for the young CNS.

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