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Contemporary Issues in Toxicology

New and evolving concepts in the neurotoxicology of lead ☆

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Abstract

Lead (Pb) is a xenobiotic metal with no known essential function in cellular growth, proliferation, or signaling. Decades of research characterizing the toxicology of Pb have shown it to be a potent neurotoxicant, especially during nervous system development. New concepts in the neurotoxicology of Pb include advances in understanding the mechanisms and cellular specificity of Pb. Experimental studies have shown that stress can significantly alter the effects of Pb, effects that could potentially be mediated through alterations in the interactions of glucocorticoids with the mesocorticolimbic dopamine system of the brain. Elevated stress, with corresponding elevated glucocorticoid levels, has been postulated to contribute to the increased levels of many diseases and dysfunctions in low socioeconomic status populations. Cellular models of learning and memory have been utilized to investigate the potential mechanisms of Pb-induced cognitive deficits. Examination of long-term potentiation in the rodent hippocampus has revealed Pb-induced increases in threshold, decreases in magnitude, and shorter retention times of synaptic plasticity. Structural plasticity in the form of adult neurogenesis in the hippocampus is also impacted by Pb exposure. The action of Pb on glutamate release, NMDA receptor function, or structural plasticity may underlie perturbations in synaptic plasticity and contribute to learning impairments. In addition to providing insight into potential mechanisms of Pb-induced cognitive deficits, cellular models offer an opportunity to investigate direct effects of Pb on isolated biological substrates. A target of interest is the 78-kDa molecular chaperone glucose-regulated protein (GRP78). GRP78 chaperones the secretion of the cytokine interleukin-6 (IL-6) by astrocytes. In vitro evidence shows that Pb strongly binds to GRP78, induces GRP78 aggregation, and blocks IL-6 secretion in astroglial cells. These findings provide evidence for a significant chaperone deficiency in Pb-exposed astrocytes in culture. In the long term, chaperone deficiency could underlie protein conformational diseases such as Alzheimer's Disease (AD). Lead exposure in early life has been implicated in subsequent progression of amyloidogenesis in rodents during old age. This exposure resulted in an increase in proteins associated with AD pathology viz., beta-amyloid precursor protein (β-APP), and beta-amyloid (Aβ). These four new lines of research comprise compelling evidence that exposures to Pb have adverse effects on the nervous system, that environmental factors increase nervous system susceptibility to Pb, and that exposures in early life may cause neurodegeneration in later life.

Keywords: Lead; Stress; Hypothalamic–pituitary–adrenal axis; Synaptic plasticity; Long-term potentiation; Chaperones; Conformational disease; Alzheimer's disease; Amyloid protein

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Contents

Introduction

Lead is a metal that has been used for over 8000 years for diverse applications in glass, pigments, makeup, water transport, wine, cooking, and more recently in antiknock fuel additives, electronic components, and batteries. It is a multimedia pollutant, meaning that human exposures occur via inhaled air, dust, food, and drinking water and, further, it has no known biological function. Lead is regulated under the Clean Air Act as a criteria air pollutant as well as under other federal and state laws. With the elimination of Pb in most fuels and paint and the decrease in the number of primary and secondary Pb smelters in the United States, Pb emissions have dropped from ~220,000 tons/year in 1970 to 3000 tons/year in 2004 (U.S. EPA, 2006). Currently, average U.S. ambient concentrations of Pb range from 0.10 to 0.22 μg/m^3, well below the current National Ambient Air Quality Standard of 1.5 μg/m^3. This decrease in environmental Pb exposure has brought about a concurrent drop in children's blood lead (PbB) levels from a geometric mean of 15 μg/dL in 1980 to ~1 to 2 μg/dL in 2004. Though this is considered a significant public health advance, a legacy of environmental Pb burden still exists (U.S. EPA, 2006). Additionally, there are still point sources of Pb, such as near incinerators, smelters, and foundries and in poor, inner-city areas where peeling Pb-based paint is a source of exposure to children. Thus, Pb is still a significant public health concern. Additionally, it has been shown that Pb is stored in bone and released over time, especially during times of bone demineralization such as pregnancy, lactation, and postmenopause. Thus, even though current environmental exposure is low, early childhood exposures to Pb create a body burden that moves from the bone to the blood, keeping other tissue levels elevated.

Lead has well-characterized effects on every organ system, including the cardiovascular (Vaziri, 2002), renal (Gonick, 2002), immune (Dietert and Piepenbrink, 2006), and reproductive (Bellinger, 2005) systems, as well as on bones and teeth (Hu et al., 1998). It has also been identified as a probable human carcinogen (Silbergeld, 2003). But the nervous system is especially sensitive to the effects of Pb. For more than 2000 years, its effects on cognitive function and behavior have been recognized. Major (1931) presented a history of lead poisoning that includes the observation by Dioscorides that "lead makes the mind give way." More recently, Bellinger and Bellinger (2006) presented an overview of lead neurotoxicity,

describing the realization throughout the 20th century that lower and lower PbB levels were recognized as causing deleterious effects on the nervous system, including decrements in IQ, decreased hearing and vision, and impaired peripheral nerve function. A science assessment completed by the Environmental Protection Agency (EPA) in 1977 (Air Quality Criteria for Lead; U.S. EPA, 1977) reviewed lead toxicology and epidemiology studies and concluded that (1) good evidence exists for the occurrence of encephalopathy at PbB levels of 80 to 100 μg/dL or higher, (2) PbB levels associated with neurobehavioral deficits in asymptomatic children appear to be in excess of 50 to 60 μg/dL, (3) the developing organism represents the population at greatest risk, and (4) exposures that result in PbBs levels ranging from 30 to 80 μg/dL disrupt cognitive function. A second assessment completed in 1986 (U.S. EPA, 1986) reported that (1) central and peripheral nerve dysfunction occurs at PbB levels of 40 to 60 μg/dL; (2) decrements in IQ occur in children with PbB levels of 30 to 50 μg/dL; (3) neurobehavioral effects in rats and monkeys occur with PbB levels of <20 μg/dL; (4) alterations in neurobehavioral function persist long after Pb exposure has stopped and Pb levels have returned to normal; and (5) Pb produces lasting changes in synaptogenesis, dendritic development, myelin and fiber tract formation, ionic mechanisms of neurotransmission, and energy metabolism. A third assessment has just been completed (U.S. EPA, 2006) with findings that include (1) developmental Pb exposures creating steady-state PbB concentrations of ~10 μg/dL result in behavioral impairments that persist into adulthood in rats and monkeys; (2) no evident threshold has yet been found for the effects of Pb on the nervous system; (3) in rats, Pb-related neurobehavioral deficits persist well into adulthood after prenatal, preweaning, and postweaning Pb exposure; (4) in monkeys, neurobehavioral deficits occur both with in utero-only exposure and with early postnatal-only exposure when peak PbB levels do not exceed 15 μg/dL and steady-state levels are ~11 μg/dL; (5) learning impairment occurs in animals at PbB levels as low as 10 μg/dL, with higher-level learning showing greater impairment than simple learning tasks; and (6) mechanisms associated with cognitive deficits include response perseveration, insensitivity to changes in reinforcement density or contingencies, deficits in attention, reduced ability to inhibit inappropriate responding, impulsivity, and distractibility.

Along with this information regarding the PbB levels at which deleterious effects occur comes important information advancing the understanding of the mechanisms of Pb's effects and cellular specificity. The four laboratories that have contributed to this review have followed four important new avenues of research to further elucidate the mechanisms by which Pb affects cognitive function and behavior. Experimental studies by Cory-Slechta and colleagues have demonstrated that environmental factors such as stress can interact with Pb exposure. Not only might such an interaction contribute to the central nervous system effects associated with Pb but this may also be a mechanism whereby Pb exposure can contribute to a host of diseases and disorders associated with dysfunction of the hypothalamic–pituitary–adrenal (HPA) axis. Elevated stress, with corresponding elevations in glucocorticoid levels, has been postulated to account for the increased incidence of various diseases and dysfunctions in low socioeconomic status (SES) populations. Additionally, they have identified important gender differences in these responses. Gilbert and colleagues used cellular models of learning and memory to investigate the potential mechanisms of Pb-induced cognitive deficits. They also investigated the impact of Pb on structural plasticity in the adult hippocampus. Tiffany-Castiglioni and colleagues demonstrated that Pb binds to GRP78 during the Pb accumulation process in astroglial cells. As GRP78 is also a stress protein and a chaperone for IL-6, this binding may contribute to increased susceptibility of the brain to stress. Zawia and colleagues have reported that Pb exposure in early life in both rodents and monkeys causes upregulation of APP mRNA expression and the levels of its amyloidogenic cleavage product Aβ. These studies show an association between developmental Pb-exposure and amyloidogenesis during old age. They propose epigenetics as one of the potential mechanisms that mediates such delayed consequences and hypothesize that early Pb exposure may inhibit the methylation of CpG dinucleotides in the APP promoter, causing increased responsiveness of the APP gene later in life.

Combined Pb exposure and stress: Consequences for the central nervous system (CNS) and the hypothalamic–pituitary–adrenal (HPA) axis

Pb and stress as co-occurring risk factors

A notable reduction in mean PbB levels in the United States accompanied the phase-out of lead from paint and gasoline. However, elevated Pb exposure, with its consequent effects, remains a significant public health problem for some segments of the U.S. population, specifically for low SES populations, and particularly for inner-city minority children who are medically underserved and live in old housing with Pb-based paint. For these children, elevated Pb exposure is largely a result of the residual contamination of dust and dirt from paint.

Low SES itself is a significant risk factor for a broad range of diseases and disorders, even after access to medical care is considered. Gradients between SES status and disease have been reported for cardiovascular disease, diabetes, metabolic syndrome, arthritis, tuberculosis, chronic respiratory disease, gastrointestinal disease, and adverse birth outcomes (Adler et al., 1994). Relationships between SES and well being also occur in children, where consequences may be even more severe. Links between SES and intellectual/academic competence of children are also notable. Numerous studies report that poverty is associated with lower levels of school achievement and IQ later in childhood (Bradley and Corwyn, 2002). Moreover, low SES is associated with mental disorders such as schizophrenia, personality disorders, and depression.

The link between low SES and increased incidence of diseases and disorders has been hypothesized to result from the greater stress associated with low SES environments and an associated presumptive chronic elevation of stress hormones

(Lupien et al., 2001). This assertion is supported by an increasing number of studies. Low SES children from 6 to 10 years of age living in Montreal, for example, had higher morning salivary cortisol levels than children from more affluent families (Lupien et al., 2001). In another study, elevated salivary cortisol levels in children were associated with lower SES as well as with the mother's extent of depressive symptomatology, effects that emerged as early as 6 years of age (Lupien et al., 2000). In adults, job strain and the expression of anger were associated with elevation of free cortisol early in the working day (Steptoe et al., 2000). In another study, family SES was inversely related to initial cortisol levels in a population of young adult African-American males (Kapuku et al., 2002).

Thus, elevated Pb burden and heightened stress are co-occurring risk factors in low SES populations. It is notable that both are associated with similar adverse outcomes including hypertension in adults (Adler et al., 1994; Adler and Ostrove, 1999; Adler and Newman, 2002; Menke et al., 2006) and cognitive impairments in children (Bradley and Corwyn, 2002; Canfield et al., 2003, 2004). The obvious questions raised by the intersection of these risk factors include the extent to which Pb exposure and stress interact and whether Pb exposure itself contributes as a risk factor to the increased incidence of disease and dysfunctions associated with low SES.

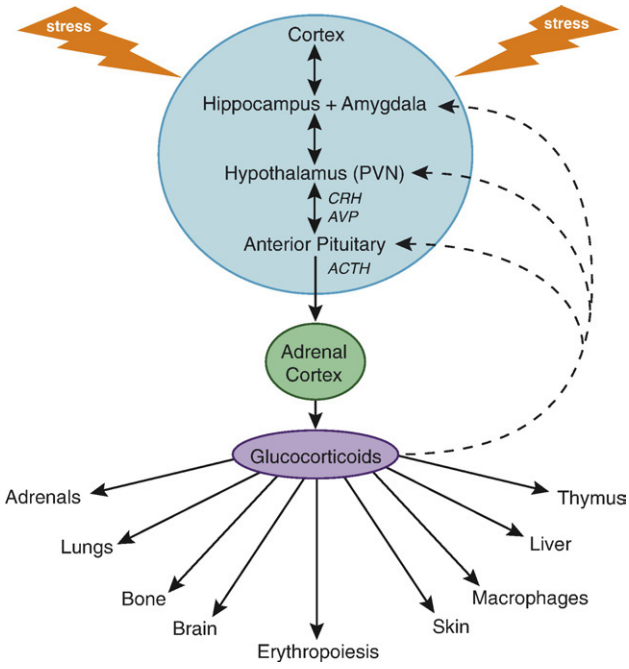

Fig. 1. Schematic of the impact of stress and its mediation via the hypothalamic–pituitary–adrenal (HPA) axis along with the organs and systems upon which the glucocorticoids produced, by the adrenal cortex act. Solid arrows show the sequence leading to production of glucocorticoids, whereas dashed lines show feedback loops regulating secretion of glucocorticoids.

HPA axis as the mediator of stress

Stressful stimuli result in the production of adrenal cortical glucocorticoids via the HPA axis, an effect considered an adaptive response to stress (Fig. 1). Physiological or psychological stressors cause release of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) from the periventricular nucleus (PVN) of the hypothalamus. This stimulates release by the adrenal pituitary of adrenocorticotropin (ACTH), which then acts on adrenal cortex receptors to elevate plasma glucocorticoids, particularly cortisol (corticosterone is the main glucocorticoid of the rat). Glucocorticoids act via two types of receptors. In the CNS, type I or mineralocorticoid (MR) receptors are located primarily in the septo-hippocampal system. Type II or glucocorticoid (GR) receptors are distributed throughout the brain and are preferentially activated by the higher levels of corticosterone associated with stress (Joels and de Kloet, 1994). Feedback loops to pituitary, hypothalamus, and hippocampus regulate glucocorticoid secretion.

Consequences of HPA axis dysfunction

Glucocorticoids are critically involved in virtually all organ systems of the body, as well as in associated physiological, cellular, and molecular networks and their associated activities (Fig. 1). As this signifies, the HPA axis and glucocorticoids are key participants in critical biological processes such as the organism's physiological and behavioral responses to stressors, organogenesis, control of energy homeostasis, sleep, and reproduction. In addition, the HPA axis and glucocorticoids have the ability to influence complex cognitive function through the hippocampus and its broader connections to the prefrontal cortex and the nucleus accumbens, comprising the mesocorticolimbic pathway.

In correspondence with this broad involvement of glucocorticoids in human physiological function, disturbances of the HPA axis contribute to a myriad of diseases and disorders. As has been noted (Chrousos and Kino, 2004), either too little or too much HPA axis and/or glucocorticoid activity can have far-reaching pathological consequences. Among diseases and disorders related to altered HPA axis function are cardiovascular disease, osteoporosis, asthma, arthritis, lupus, Crohn's disease, obesity (metabolic syndrome), diabetes, depression, anxiety, cognitive deficits, and insomnia.

Combined effects of Pb and stress and a multiple-hit hypothesis

The extensive demographic overlap of elevated Pb burden with low SES raises the obvious question of whether these risk factors interact. In addition to the demographic overlap of these risk factors, it is notable that both glucocorticoids and Pb act on mesocorticolimbic systems of the brain, systems critical to the mediation of complex cognitive function. If interactions occur, then experimental studies of Pb as a neurotoxicant in isolation may be misleading, both mechanistically and with respect to human health effect risks. Should Pb and stress interact, it might not be a coincidence that many of the functional deficits seen in low SES children are remarkably similar to those produced by Pb exposure in children and experimental models. Even more generally, stress is an inevitable experience in human life, and one not exclusive to low SES populations. Globally, therefore, the study of Pb+stress more accurately models the human

condition and corresponding results may have particular significance for understanding the true health risks posed by Pb, especially since the cycles of poverty and elevated Pb are so congruous. Indeed, a full understanding of the true risk posed by all environmental toxicants will ultimately require assessments of their interaction with other environmental and genetic risk factors.

Risk factors that target a common system of the brain but act via different mechanisms, such as Pb and stress and the mesocorticolimbic system, may be particularly problematic for the CNS (Cory-Slechta, 2005). The brain may be readily able to compensate for the effects of an individual chemical or risk factor acting on a particular target system of the brain. However, when multiple target or functional sites within that particular system are attacked by *different* mechanisms, the system may no longer be able to evoke homeostatic mechanisms, thereby leading to sustained or cumulative damage. Fig. 2 shows a hypothetical example of this multi-hit hypothesis of neurotoxicity, here featuring a dopamine terminal. Four concurrent insults are portrayed. While all four target the dopamine terminal, they do so by different mechanisms, i.e., at different sites of the system. Here, for example, insult A targets the vesicular monoamine transporter, insult B attacks the enzyme converting tyrosine to DOPA, insult C the metabolism of DOPAC to HVA, and insult D the dopamine transporter that takes dopamine back up from the synaptic cleft post-release. This multiplicity of insults occurring concurrently at different sites within the system may constrict the range and flexibility of compensatory mechanisms, thereby compromising the integrity of the system. As a consequence, multiple risk factors acting simultaneously could have effects that are more robust, more rapid in onset, or even differ in character from effects produced by a single risk factor.

Experimental models of Pb and stress

One issue requiring consideration in the experimental evaluation of Pb and stress interactions is the appropriate models to examine. Aspects of the current demographics of human Pb exposure are important to consider. Pb exposure, like poverty, now constitutes a cycle, with low SES mothers experiencing both high levels of stress and also having the highest Pb exposure levels. The Pb body burden accumulated over life, as well as the impacts of stress on the mother, including that experienced during gestation, are passed on to her children. Thereafter, these children, who are highly likely to remain in the cycle of poverty, will continue to be exposed to Pb over their lifetime as well and will also begin to experience similar environmental stresses associated with low SES conditions.

Study 1: Maternal stress and maternal Pb exposure

An initial study by Cory-Slechta and colleagues focused on maternal contributions. It administered 0 or 150 ppm Pb acetate in drinking water from 2 months prior to breeding through gestation and lactation combined with (1) maternal stress alone, or with (2) maternal stress followed by adult offspring stress. Pb exposure was initiated 2 months prior to breeding to ensure that an elevated Pb body burden, as in the human environment, was sustained prior to pregnancy (Cory-Slechta et al., 2004) and was

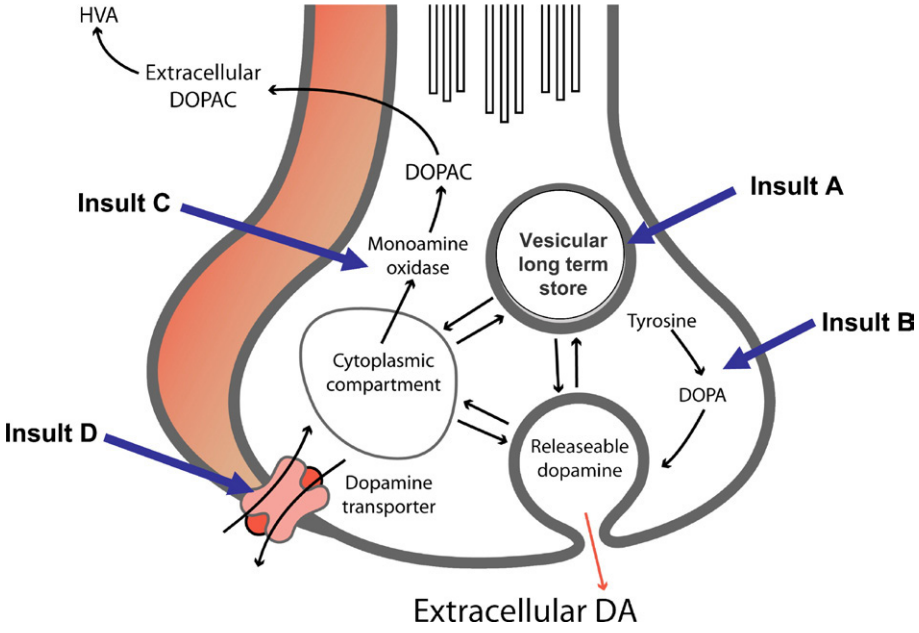

Fig. 2. Schematic depicting the multiple-hit hypothesis as applied to a dopamine terminal within the central nervous system. Four concurrent insults are depicted that occur at different target sites of the dopamine (DA) terminal: Insult A affecting the vesicular transporter, Insult B affecting the metabolism of tyrosine to DOPA, Insult C causing the breakdown of DOPAC and Insult D affecting the DA transporter. This multiple-hit hypothesis proposes that the brain may be readily able to compensate for the effects of an individual chemical acting on a particular target system of the brain. However, when multiple target or functional sites within that system are attacked by different mechanisms (i.e., multiple chemical exposures or chemical exposures combined with other risk factors), the system may no longer be able to homeostatically re-regulate itself, thereby leading to sustained or cumulative damage (Cory-Slechta, 2005).

associated with PbB levels of dams ranging from approximately 32 to 42 μg/dL. Dams were subjected to 45-min restraint stress 3 times daily on gestational days 16 and 17. Offspring were weaned at 21 days of age, and, using 1 male or female per litter to preclude litter-specific effects, behavioral (specifically Fixed Interval [FI] schedule-controlled responding) and neurochemical endpoints known to be altered by Pb exposure were subsequently evaluated. Offspring were also subjected to various stressors as adults, in accord with what would be anticipated to occur across the human life span. This design allowed a determination of the contribution of maternal Pb alone and of maternal stress as distinct from continuous stress that occurs across the lifetime and, also, provided the ability to assess potentially dormant effects associated with the maternal contributions (Virgolini et al., 2006). Corresponding group notations were 0NS, no Pb, no stress; 0S, maternal stress only; 150NS, maternal Pb exposure only; 150S, combined maternal Pb and stress.

Study 2: Postweaning Pb exposure and stress

A second study (Virgolini et al., 2005) examined the effects of Pb exposure beginning later in life (postweaning) in combination with repeated environmental stress. In this study, 21-day-old male rats were chronically exposed via drinking water to 0, 50, or 150 ppm Pb acetate associated with PbBs of <5, 8–27, and 15–43 μg/dL, respectively. Here too offspring were tested on the FI schedule and, at 3 different times during the course of FI testing, stressors were imposed prior to the FI session.

Some generalized consequences of Pb, stress, and the combination

It is clear from the studies carried out to date that the nature of the effects of combined Pb exposure and stress depend upon many experimental parameters. These include differential effects by developmental period of Pb exposure, the concentration of Pb utilized, the associated PbB concentration, and gender of the offspring, as examples. Further complexity includes the fact that for each gender, combined effects of Pb and stress (as well as each of these factors alone) can differ notably in different brain regions and by neurotransmitter under investigation and for the associated stress challenge response and behavioral outcomes.

Despite these expected complexities, a few interesting generalities of the effects of Pb and stress alone, as well as in combination with significant implications for risk assessment, have emerged from the studies in which full analysis of all outcome measures has been completed. Highlights of these findings, as well as their implications, are summarized here.

Permanent and dynamic effects of Pb on the HPA axis

It is clear from the two studies in which data analyses are fully completed that Pb exposure alters HPA axis function, as indicated by alterations in levels of corticosterone (Fig. 3), as well as in negative feedback as assessed via the dexamethasone suppression test (not shown). Fig. 3A shows changes in corticosterone levels in male offspring that had been maternally exposed to Pb, or to the combination of Pb and stress (Cory-Slechta et al., 2004). Basal corticosterone levels, measured in one group of male offspring at 9 months of age, were markedly enhanced by Pb exposure, with corresponding statistically significant increases in both the Pb alone (150NS) and Pb+stress (150S) groups, with levels about twice those of controls. These appear to be permanent alterations in HPA axis function, given that Pb exposure ended at 21 days of age in these offspring.

Measurements of other male littermates of these offspring, carried out at 14 months of age under basal conditions suggest, moreover, that the impact of Pb on HPA axis function is dynamic, rather than static. Corticosterone levels of controls (0NS) show little change between 9 and 14 months of age, whereas the marked increases associated with Pb exposure in males are no longer observed and, in fact, levels in Pb-treated groups, both 150NS and 150S, exhibit statistically significant reductions to values of 47% to 56% of control. The corresponding nature of the effects in the 150NS and 150S groups indicates that these are

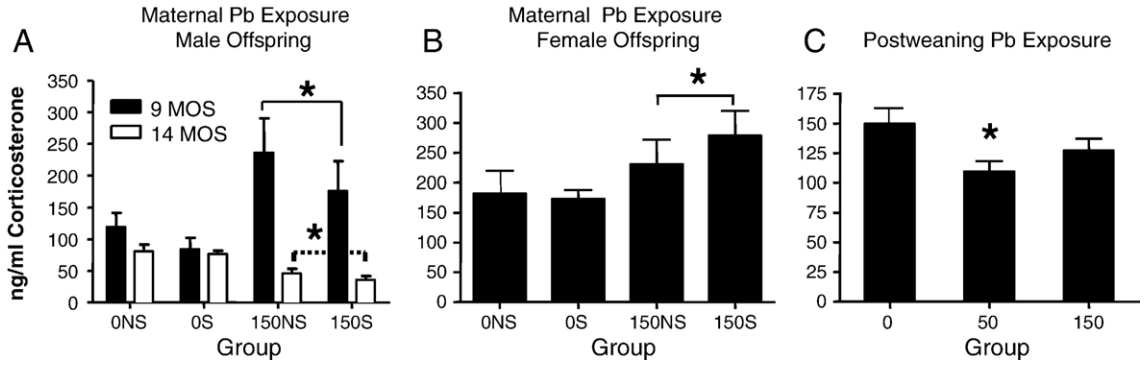

Fig. 3. Basal corticosterone levels. (A) Male offspring exposed via the dam to Pb, stress, or the combination measured at either 9 or 14 months of age (Cory-Slechta et al., 2004). (B) Female offspring exposed via the dam to Pb, stress, or the combination at approximately 9 months of age (Cory-Slechta et al., 2004). Group designations for panels A and B are 0NS, no Pb, no stress; 0S, maternal stress only; 150NS, maternal Pb exposure only; 150S, combined maternal Pb and stress. In panels A and B, brackets with asterisks indicate significant main effects of Pb in 2-factor ANOVAs examining Pb and stress No significant effects of stress or Pb×stress interactions were found. Panel C: male rats exposed to Pb at the designated concentrations (0, 50, or 150 ppm) from 21 days of age, as measured after approximately 5 months of exposure (Virgolini et al., 2005). A significant main effect of Pb in a one-factor ANOVA was confirmed; asterisk indicates that subsequent post-hoc tests confirmed that corticosterone levels of the 50 ppm group were significantly lower than those of the 0 ppm group.

Pb-mediated effects. Thus, significant Pb-induced alterations in corticosterone are observed out to at least 14 months of age, and the nature of the effect appears to differ across time.

Fig. 3B depicts basal corticosterone levels of female offspring following maternal Pb exposure with or without stress also measured at approximately 9 months of age (Cory-Slechta et al., 2004). As it shows, the increases in basal corticosterone levels associated with Pb exposure in males were also observed in female offspring. Statistically significant increases in females were on the order of 26% to 61% above corresponding control values. As with males, these effects were equivalent in the 150NS and the 150S groups, being driven, therefore, by the Pb exposure and apparently not modified by stress. These observations confirm permanent effects of Pb exposure on HPA axis function; no residual Pb burden would be expected in these offspring, as exposure ended at 21 days of age. They also indicate that the effects of Pb are dynamic across time, no doubt reflecting ongoing changes in the HPA axis system with time.

A second experiment, as described above, utilized chronic postweaning Pb exposure to determine the impact of combined Pb and stress when exposures were initiated later in development (Virgolini et al., 2005). In that study, corticosterone levels of male rats were measured after approximately 5 months of exposure, a time point associated with stable PbB values. As shown in Fig. 3C, these later Pb exposures were likewise associated with alterations in corticosterone levels. In the case of postweaning exposure, however, Pb was associated with significant reductions in basal corticosterone levels. In addition, this effect was actually more pronounced at the lower Pb exposure concentration (50 ppm; associated with PbB values of 9 to 15 μg/dL) at a reduction of 27%, in contrast to a 15% reduction in the 150 ppm group that only attained marginal significance (PbB values of 23–27 μg/dL).

Studies currently underway will determine the extent to which continuous exposure over the lifetime impacts corticosterone levels. Moreover, it is likely that corticosterone levels will differ with respect to the behavioral history or lack thereof that these offspring experience over their lifetime. In addition, it is not yet clear how Pb exposure alters HPA axis function, but it seems likely that the mechanisms associated with the permanent effects seen with maternal exposure will differ from those associated with postweaning exposure. It is also notable that these outcomes confirm reports of Pb-induced changes in corticosterone in rat models from Vyskocil et al. (1990, 1991a,b,c), albeit they found effects only at far higher PbB concentrations than those at which effects have been observed to date in studies by Cory-Slechta and colleagues (Cory-Slechta et al., 2004; Virgolini et al., 2005, 2006).

Gender differences

A second observation that has emerged from the Cory-Slechta group's studies to date is that significant and often robust gender differences occur in response to Pb alone, to stress alone, and to combined Pb and stress. Fig. 4 depicts one example, showing long-term changes in levels of dopamine (DA) and in dopamine turnover (DA TO; defined as DOPAC/DA) in male vs. female offspring in each of three brain regions: frontal cortex, nucleus accumbens, and striatum, as measured at the termination

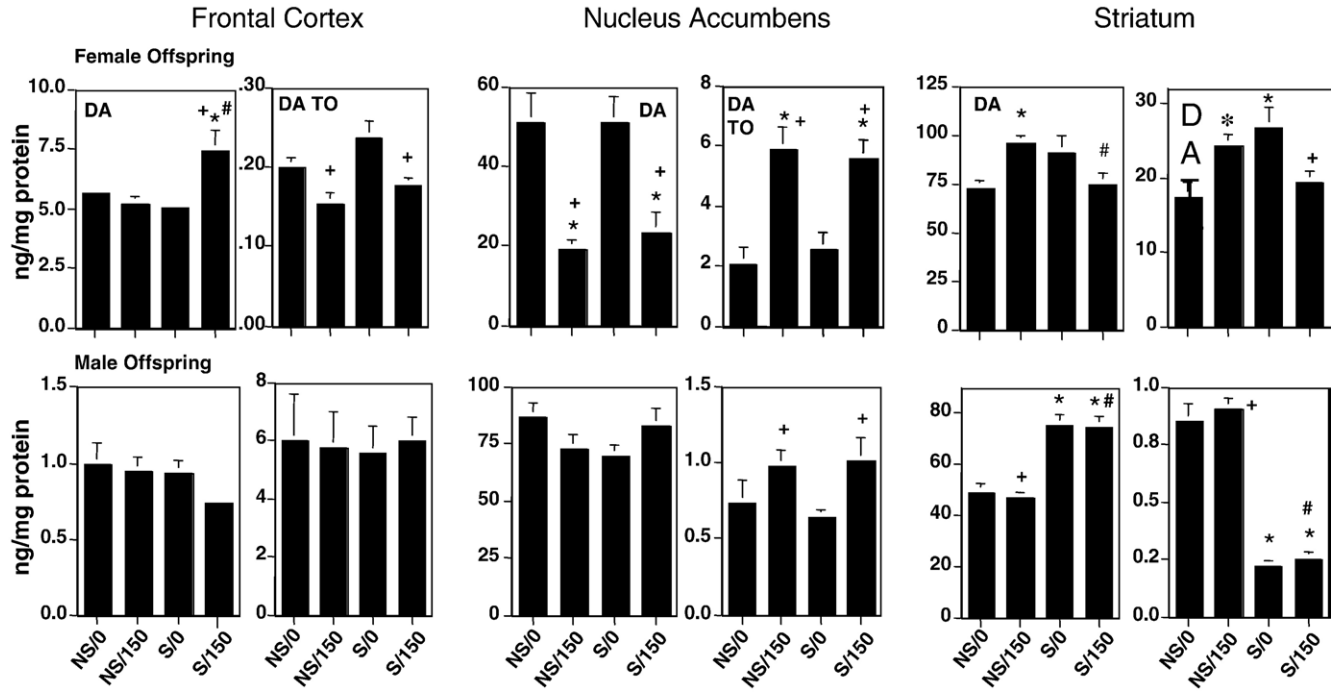

Fig. 4. Levels of dopamine (DA) or dopamine turnover (DA TO) measured in female (top row) or male offspring (bottom row) of dams that had been exposed from 2 months prior to breeding and through lactation to 150-ppm Pb exposure alone, maternal stress alone, or the combination (Cory-Slechta et al., 2004). Levels for frontal cortex (left), nucleus accumbens (middle), and striatum (right). *Statistically significant difference from NS/0 group; #significant difference from NS/150 group; +significant difference from S/0 group.

of the experiment when offspring were approximately 10 months of age following maternal exposures to Pb, stress, or the combination as described above (Cory-Slechta et al., 2004).

In the frontal cortex, a particular vulnerability of female offspring was found for dopaminergic systems as evidenced by potentiated effects, with marked increases in DA levels only in the combined Pb+stress group as compared to all other groups. DA turnover was lower in the NS/150 and S/150 groups than in the S/0 group, but these levels did not differ from control. In contrast, there were no significant changes in frontal cortex in male offspring in either levels of DA or DA turnover. In nucleus accumbens, Pb alone markedly reduced levels of DA in female offspring, while concurrently increasing DA turnover in both the NS/150 and S/150 groups, with effects that were of comparable magnitude. In contrast, for males, slight increases in DA turnover were noted in the NS/150 and S/150 groups as compared to the S/0 group, but not in comparison to controls. In the striatum, however, a very marked effect of stress was detected in male offspring, where notable increases in levels of DA and reductions in DA turnover were found in the S/0 and S/150 groups. Effects in females in striatum were mixed, with significant increases in DA observed only with Pb alone (NS/150 ppm), and increases in DA turnover found in response to Pb alone (NS/150) and stress alone (S/0), but not to the combination.

Implications for risk assessment

There are already significant implications of the outcomes of these studies.

- First, by permanently altering HPA axis function, Pb exposure could contribute to a myriad of diseases associated with alterations in glucocorticoids (Chrousos and Kino, 2004). Indeed, excess fetal glucocorticoid levels, such as appear to be associated with maternal Pb exposure, are well known to program pathologies in adult life that can include cardiovascular, neuroendocrine, and metabolic disorders (Seckl and Meaney, 2004). Should similar effects occur in humans, then elevated Pb burden in low SES populations could actually be a contributing factor to the increased risk of these diseases and disorders in low SES populations, not just serve as a co-occurring risk factor for them. As such, the possibility that elevated Pb exposure can similarly induce HPA axis dysfunction in humans clearly warrants evaluation.
- The permanent changes in HPA axis function produced by maternal Pb exposure, if they likewise occur in humans, suggest that screening for elevated Pb burden needs to be carried out in at-risk pregnant women rather than just in their in offspring, since postnatal screening would be too late to preclude these permanent effects on the HPA axis.
- Another hypothesis that emerges from these findings is that alterations in HPA axis function could be a mechanism by which Pb exposure adversely influences cognitive functions, based on known interactions of corticosterone with the mesocorticolimbic system, a system that has been shown to mediate specific behavioral effects associated with Pb exposure.
- Finally, risk assessments based on exposures to single agents in isolation from other co-occurring risk factors may not be sufficiently protective of human health.

Developmental Pb exposure and hippocampal function: Synaptic plasticity and transmitter release

Although it is well established that low-level chronic exposure to Pb results in long-lasting detrimental effects on intellectual function in children (Bellinger et al., 1991), it was proven challenging to progress from epidemiological observations to the identity of cellular mechanisms of action. At least part of the problem in linking cognitive dysfunction to deleterious actions of Pb on the developing CNS results from the disparate levels of experimental analysis at which these investigations are conducted. This section summarizes work performed in a rodent model on the physiological, neurochemical, and structural alterations in hippocampus induced by chronic developmental Pb exposure as they relate to cognitive function. As discussed in the previous section, cognitive function has been shown to be affected by Pb acting in brain regions including the hypothalamus, frontal cortex, and nucleus accumbens. The present section focuses on another important substrate for learning and memory, the hippocampus, where alterations in a cellular model of memory in the intact animal are compared with in vivo assessments of neurotransmitter release. These data demonstrate a consistency across these functional levels and suggest that Pb action on the properties of presynaptic transmitter release contribute to cognitive impairments associated with developmental Pb exposure.

The ability of Pb^{2+} to substitute for Ca^{2+} is one of the primary mechanisms proposed for Pb^{2+} action in the CNS. There is ample evidence from a variety of acute in vitro preparations that the calcium-mimetic properties of Pb^{2+} not only enhance spontaneous neurotransmitter release but also impede Ca^{2+} influx through voltage-sensitive Ca^{2+} channels to inhibit evoked neurotransmitter release (Minnema et al., 1988; Kober and Cooper, 1976; Atchison and Narahashi, 1984). Linking these well-documented cellular actions of Pb^{2+} to the syndrome of Pb toxicity expressed in children or animals has not been realized. To address this challenge, the following work focused on evaluating the effects of developmental Pb exposure on transmitter release and cognitive function in an intact rodent model. The model system reflects a significant increase in biological complexity for studies of transmitter release and a scaling back in complexity to assess the effects of Pb on cognitive function. Based on findings from cellular preparations, we examined the effects of Pb on transmitter release using in vivo microdialysis in developmentally Pb-exposed animals. In parallel studies, the complexity of behavioral impairments was reduced to the neural circuit level by evaluating cognitive function using a cellular model of learning, long-term potentiation (LTP). A number of laboratories have observed detrimental effects of Pb exposure on LTP in hippocampal slices from exposed animals (Altmann et al., 1993; Sui et al., 2000). In the work described herein, the intact preparation was employed in both neurochemical and electrophysiological approaches to facilitate the linkage between

transmitter release as measured by in vivo microdialysis and synaptic plasticity as measured by LTP following developmental Pb exposure.

Synaptic plasticity and neurotransmitter release – Developmental periods of vulnerability

In these studies, pregnant rats were placed on control or 0.2% Pb in the drinking water in late gestation. At weaning, offspring were maintained on the same solution of their dams or switched to the opposite water source, creating four independent exposure conditions: (1) control group with no Pb exposure, C; (2) perinatal Pb exposure terminating at weaning, W; (3) Pb exposure beginning at weaning and continuing to adulthood, WL; and (4) Pb exposure that encompassed the late prenatal period and was continued throughout life, L. As adults, animals were prepared with electrodes to activate cells across a monosynaptic circuit in the dentate gyrus subregion of the hippocampal formation. LTP is best characterized in the hippocampus, a structure known to be critical in learning and memory (see reviews by McNaughton, 1993; Massicotte and Baudry, 1991; Bliss and Collingridge, 1993). Stimulating perforant path afferents to dentate gyrus granule cells evokes release of glutamate from presynaptic terminals, driving a synaptic response. The coupling of released glutamate with postsynaptic receptors elicits a compound field potential that can be recorded from an electrode placed proximal to the dentate gyrus granule cell layer.

LTP in the dentate gyrus is induced by delivering trains of high-frequency stimulation to the perforant path to emulate patterns of cell firing that occur during a learning event. In this cellular model of learning and memory, the "learning" is indexed by a long-lasting increase in the amplitude of the evoked field potential. The difference in response amplitude before and after the delivery of train stimulation is a measure of the magnitude of evoked LTP. Deficits in both the synaptic (excitatory postsynaptic potential, EPSP) and the cellular (population spike) components of this field response were evident in all animals exposed to Pb from early life and persisted to adulthood despite termination of Pb exposure at weaning (Gilbert et al., 1996, 1999a). These findings are summarized in Figs. 5A and B which plot the differences in response amplitude before and 1 h after delivery of LTP-inducing trains and summed across a range of stimulus intensities. These data indicate that Pb exposure reduced LTP magnitude and thus impaired the efficacy of the cellular mechanisms that support learning in the hippocampus.

In a parallel series of experiments, microdialysis probes were implanted in the dorsal hippocampus of similarly exposed animals. Hippocampal neurons were depolarized to induce release of glutamate and GABA, the primary neurotransmitter substances in this region, by infusion of a high potassium solution through the dialysis probe. Samples of extracellular fluid were periodically collected before, during, and after stimulated transmitter release and subsequently analyzed for amino acid concentration. The method of stimulation and use of microdialysis essentially measures extracellular transmitter spillover from the synapse, and represents a combination of enhanced spontaneous release and diminished stimulated glutamate spillover. The synaptic, calcium-dependent component of evoked glutamate release was derived by subtracting calcium-independent release (measured in the absence of calcium in the perfusate) from total glutamate release (in the presence of calcium in the perfusate). On the background of a stable baseline of glutamate release, chronic Pb exposure reduced the magnitude of stimulation-induced glutamate release compared to responses in control animals. The pattern of Pb-induced reductions in glutamate release across differing windows and durations of exposure paralleled that seen in LTP studies (Lasley and Gilbert, 1996; Lasley et al., 1999). Findings as summarized in Fig. 5C demonstrate that chronic exposure beginning in utero (L) or in the early postweaning period and continuing throughout life (WL) altered presynaptic release of glutamate in the hippocampus. Transient exposure beginning in utero but terminating at weaning (W) was also effective in producing permanent impairment of release function. The latter findings are particularly important, as they indicate that the continued presence of Pb is no longer necessary to produce neurochemical deficits, but rather that limited exposure during critical periods of brain development is sufficient to irreversibly alter transmitter release function. The pattern of effects is strikingly similar to that obtained with electrophysiological assessments of synaptic function in the LTP model. Comparing the pattern of effects across developmental periods of exposure as depicted in the top panel of Fig. 5 (A–C) suggests that impairments in LTP may result, in part, from Pb-induced disruptions of presynaptic transmitter release.

Synaptic plasticity and neurotransmitter release – Dose–response analysis

The properties of LTP and glutamate release as a function of Pb exposure level were investigated in animals continuously exposed to Pb from birth. Exposure levels above and below those used in developmental exposure studies were utilized ranging from 0.1% to 1.0% Pb acetate. Increasing levels of Pb in the drinking water produced, not surprisingly, monotonic increments in blood (~25 to 118 μg/dL) and brain Pb (~220 to 1800 ng/g, wet weight) (Gilbert et al., 1999b). Contrary to expectation, synaptic release of glutamate was affected by dose in a biphasic manner. Lower levels of Pb resulted in diminished release, consistent with our earlier findings, yet this effect was reversed at higher levels of exposure (Lasley and Gilbert, 2002; Fig. 5F). Biphasic dose–response profiles clearly indicate the presence of more than one mechanism of Pb action. The decline in release at lower exposure levels is consistent with the blocking effects of Pb^{2+} on voltage-sensitive calcium channels, elegantly demonstrated using in vitro preparations (Kober and Cooper, 1976; Evans et al., 1991). A plausible basis for the reversal at higher exposure levels of the Pb-induced diminution of the K^+-stimulated transmitter response can be proposed. In the exposure groups in which the Pb effect is partially or fully reversed (0.5–1.0% Pb), the Ca^{2+}-independent component of release is elevated (Lasley and Gilbert, 2002), suggesting a compensation for the deleterious effects of exposure on K^+-stimulated glutamate release evident at lower exposure levels. However, the Ca^{2+}-independent release is less sensitive to the effects of

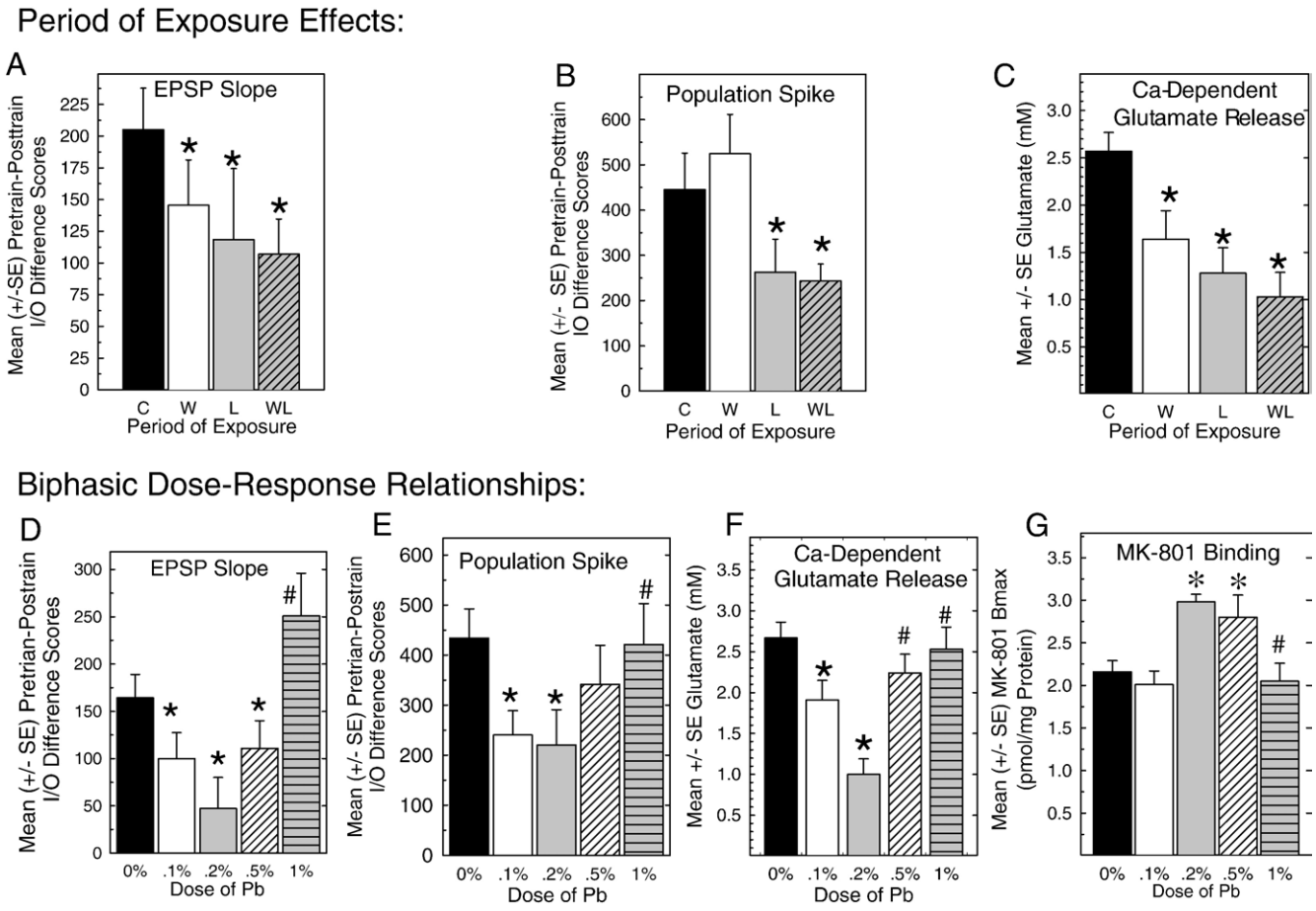

Fig. 5. Top panel: Parallel changes were observed in long-term potentiation (LTP) of the excitatory postsynaptic potential (EPSP, A) and population spike (B) measures of synaptic response in the dentate gyrus and calcium-dependent glutamate release as measured by in vivo microdialysis in the hippocampus as a function of developmental period of Pb exposure (C). Bottom panel: Biphasic dose–response relationship (D–G). Graded levels of exposure to Pb produced a similar U-shaped pattern in LTP (D, E), transmitter release (F), and NMDA receptor binding (G). Postsynaptic NMDA glutamate receptor binding as depicted in (G) was increased at low Pb exposures, consistent with a reduced sensitivity to MK-801 (see text). Collectively, these findings argue for a presynaptic locus of Pb action to reduce transmitter release and impair synaptic plasticity, postsynaptic increases in NMDA receptor number being secondary to Pb-induced reductions transmitter release. The consistency of effects of Pb based on developmental timing and exposure level in transmitter release and synaptic plasticity suggests that transmitter release deficits may underlie deficits in synaptic function that lead to learning impairments. *Significantly different from control values, $P<0.05$; #significantly different from 0.2% values indicative of reversal of effect with increasing dose and constituting a U-shaped dose–response relationship.

exposure than the Ca^{2+}-dependent component, which exhibits a reduction in response at the lowest exposure level utilized. A differential sensitivity of these two mechanisms has also been noted with acute application of Pb^{2+} to cultured hippocampal neurons (Braga et al., 1999a,b). Thus, the U-shaped dose–response function can be conceptualized as the summation of two monotonic dose–effect relationships displaced by differential sensitivity, one decreasing and one increasing as a function of exposure level.

In a similar fashion, the dose–response relationships described for glutamate release were reflected by results of LTP experiments. Reduced LTP magnitudes at lower exposure levels were reversed at higher doses in both components of the compound field potential in response to LTP-inducing trains of electrical stimulation (Gilbert et al., 1999b; Figs. 5D, E). Thus, the effects of Pb exposure on LTP and glutamate release as a function of either exposure period or exposure level are remarkably similar. Consequently, the Pb-induced decrease in glutamate release is proposed as a significant contributing factor to LTP/learning impairments associated with developmental Pb exposure, and it supports a presynaptic locus of action of this neurotoxicant. It is notable that in humans, PbB levels equivalent to those attained at 1.0% Pb in the animal studies produce severe cognitive impairment and gross encephalopathy. In rodents, these doses were not associated with overt signs of toxicity, indicative of clear species differences in sensitivity to high concentrations of Pb. These observations also underscore the complexity of learning phenomena and neural circuitries that subserve them, some of the inconsistencies of isolated model systems, and the multiplicative ways in which Pb interacts with nervous system function.

Does Pb target the postsynaptic component?

LTP requires presynaptic glutamate release and subsequent activation of the postsynaptic *N*-methyl-D-aspartate (NMDA)

subtype of glutamate receptor (Collingridge and Bliss, 1987; Massicotte and Baudry, 1991; McNaughton, 1993). LTP in area CA1 of the hippocampus and in the dentate gyrus can be readily blocked by NMDA receptor antagonists (Gilbert and Mack, 1990; Robinson and Reed, 1992; Morris et al., 1986). Acute exposure to Pb^{2+} reduces NMDA-mediated currents in cells in culture and access to the NMDA receptor channel in brain tissue homogenates (Alkondon et al., 1990; Ujihara and Albuquerque, 1992; Guilarte and Miceli, 1992; Büsselberg et al., 1994; Lasley and Gilbert, 1999). Glutamate receptor subunit expression changes have also been reported in developmentally Pb-exposed animals (Nihei et al., 2000; Zhang et al., 2005). To examine the potential role of the postsynaptic NMDA receptor alterations, LTP was evaluated in control and Pb-exposed animals in the presence and absence of an NMDA receptor antagonist. If NMDA receptor function is compromised in developmentally Pb-exposed animals, it is reasonable to expect that these animals will display an increased sensitivity to the LTP-blocking properties of NMDA antagonists. This hypothesis was tested by challenging control and Pb-exposed animals with a modest dose of the NMDA antagonist, MK-801. Animals were prepared with chronic indwelling electrodes and allowed to recover from surgery for several weeks. A counterbalanced design was utilized such that each animal received LTP stimulation under saline control and MK-801 conditions. A dose of MK-801 was identified from a series of pilot studies in naïve animals that would reduce but not completely block LTP in controls (0.05 mg/kg, i.p.). Our previous work indicated that the threshold for LTP was increased by developmental Pb exposure, but this deficit could be overcome by stimulation of control and Pb-exposed animals at higher intensities (Gilbert et al., 1996). Therefore to facilitate data interpretation, LTP was induced by administering high-frequency trains at 1500 μA that are sufficient to alleviate Pb LTP induction impairments and produce comparable levels of LTP in control and Pb animals under saline conditions. This permitted an equivalent basis for comparing the effects of NMDA antagonism of evoked LTP in the two populations of animals. Following decay to baseline of any induced LTP from the first treatment (saline or MK-801), the same animals from each exposure condition were challenged under the opposite drug condition. As expected, LTP was significantly impaired in control animals administered MK-801. Contrary to expectations of an enhanced sensitivity to the LTP-blocking properties of NMDA antagonists, however, LTP was relatively spared in Pb-exposed animals administered MK-801 (Gilbert and Lasley, 2007).

NMDA currents are not induced by single pulse stimulation. However, when short bursts of single pulses are delivered at high frequencies that are effective in inducing LTP, the area of the response evoked by the burst is substantially increased and represents a more direct indicator of NMDA receptor activation. The increase in area of the train-evoked response is NMDA-mediated and is reduced in animals following administration of MK-801 (Racine et al., 1991). In support of findings with MK-801 on LTP magnitude described above, the NMDA-sensitive component of the train response was reduced to a lesser degree in Pb-exposed animals relative to controls. So contrary to enhanced sensitivity to MK-801, Pb-exposed animals experienced a degree of protection. One possible explanation is that LTP was produced in Pb-exposed animals through mechanisms distinct from NMDA receptors and thus were not impacted by the antagonism of this receptor system. However, decreased sensitivity to the blocking properties of MK-801 was also associated with an increase in MK-801 binding in the hippocampus of chronically Pb-exposed animals (Guilarte et al., 1993; Lasley et al., 2001). The dose–response relationship is biphasic and reminiscent of observations with glutamate release and LTP (Fig. 5G). Based on findings in binding studies with acute administration of Pb^{2+} in vitro (Guilarte and Miceli, 1992; Lasley and Gilbert, 1999) and on subunit receptor changes reported as a function of chronic developmental Pb exposure (Nihei et al., 2000; Guilarte et al., 2000), the present results with MK-801 challenge experiments were unexpected. The data are consistent, however, with behavioral reports of subsensitivity to MK-801 in repeated acquisition (Cohn and Cory-Slechta, 1993) and drug discrimination (Cory-Slechta, 1995, 1997) paradigms. In the absence of any data to suggest altered drug uptake, metabolism or elimination of MK-801 in Pb-exposed animals, the most parsimonious explanation of a decreased sensitivity to MK-801 results from an upregulation of postsynaptic NMDA receptors in response to diminished presynaptic glutamate release (Lasley and Gilbert, 2000). The consistency in the biphasic dose–response relationships across LTP, microdialysis, and binding studies depicted in the bottom panel of Fig. 5 (D–G), further supports this contention.

Long-term retention of LTP

It has long been postulated that neuronal mechanisms of memory encompass distinct short-term, intermediate, and long-term phases reflecting corresponding cellular properties at the synaptic, synaptosomal and nuclear level. Different phases of LTP paralleling these memory phases have also been described, each based on a distinct mechanistic substrate (Matthies et al., 1990; McNaughton, 1993; Reymann and Frey, 2007; Riedel et al., 1996). The initial early phase with a time course of ~1 h is NMDA-dependent. With sufficient glutamate release to induce postsynaptic depolarization, a relief of a voltage-dependent block of the NMDA receptor channel is achieved. A series of postsynaptic signalling and phosphorylation events then follows – synaptic tagging, receptor shuttling to the postsynaptic density and insertion into the membrane, and maintained increases in transmitter release at the presynaptic site are all initiated by coincident glutamate release and NMDA receptor activation (Reymann and Frey, 2007). These events are reflected as persistent increases in synaptic response amplitudes and represent the "intermediate phase" of LTP, the "consolidation phase" of memory. Late phase LTP follows thereafter with a time line defined in slice experiments of ~6 to 8 h, but can last several days to weeks in chronic in vivo preparations. Late phase LTP entails dendritic growth, increases in the number of spines, and modification of the physical structure of the synapse and may embody the "retention" of the memory (McNaughton, 1993).

The impact of Pb exposure on the persistence of memory functions as reflected in very long lasting LTP was examined in control and Pb-exposed animals equipped with chronic indwelling electrodes. Optimal LTP parameters were identified that produced robust LTP in control and chronically exposed Pb rats (0.2% Pb from birth). The same suprathreshold stimulus parameters were delivered to both groups of animals to overcome the initial LTP deficits associated with developmental Pb exposure and to produce comparable, saturating levels of LTP in all subjects. This magnitude of potentiation recorded 1 h after train delivery was defined as maximal (LTP_{max}) and was similar in both groups. Stimulus–response curves collected at 24 and 48 h, and weekly thereafter for the next 4 weeks, displayed a gradual decline in potentiation back toward the baseline pretrain levels in both exposure groups (Gilbert and Mack, 1998). The rate of decline represents the decay of LTP, the "forgetting function". Difference scores between stimulus–response functions collected at 1 h (LTP_{max}) and several time points thereafter were calculated to simplify evaluation of decay functions. The relative decline from LTP_{max} was similar in control and Pb animals over the first 2 days post-LTP induction. By 1 week, however, Pb animals had larger difference scores, indicating greater declines from maximal levels of LTP. Repeated monitoring of stimulus–response relationships over time permitted the calculation of decay time constants (τ=tau, time to decay by 63%) for pooled data for each exposure group. In control animals, τ was 17.4 days, a value comparable with previous determinations (Jeffery et al., 1990). In the Pb-exposed group, the time constant of decay τ was reduced to 13.4 days. Consistent with these calculations, statistical analysis of individual animal data revealed that 45% of Pb animals had decayed by one time constant within 7 days of train delivery relative to only 12% of controls within the same time frame. These data indicate that in addition to elevations in threshold for LTP induction, a decrease in the maintenance of synaptic plasticity was evident as a result of developmental Pb exposure even under conditions where strong LTP was achieved (Gilbert and Mack, 1998).

Structural plasticity – Adult neurogenesis in the hippocampus

In addition to alterations in synapse structure and number that support long-lasting LTP, the hippocampus also exhibits another form of structural plasticity known as neurogenesis. Neurogenesis refers to the capacity to generate new neurons in the adult nervous system and represents another means whereby the brain can change its functional circuitry. Although the functional significance of neurogenesis is a topic of intense research and debate, this type of plasticity has been implicated in learning and memory and affective disorders (e.g., Kempermann and Kronenberg, 2003). Neurogenesis is also triggered in response to injury or pathological stimulation and has been proposed to play a role in regeneration and repair in the adult nervous system (Lledo et al., 2006).

A number of factors inherent to the organism, its experience, and the environment in which it resides have been shown to modulate hippocampal neurogenesis (see reviews by Lledo et al., 2006; Ming and Song, 2005). The potential for developmental Pb exposure to interfere with this type of structural plasticity in the hippocampus was investigated in the following way. Control and 0.2% Pb-exposed animals, one group continuously exposed from birth and another group exposed only during lactation, were administered a series of injections of bromodeoxyuracil (BrdU) to label newly born cells. BrdU is a thymidine analog that becomes incorporated into the DNA of dividing cells. Immunohistochemistry of BrdU provides a means of identifying cells actively dividing at the time of its administration. A protocol for BrdU administration used in studies of environmental enrichment and activity-induced changes in neurogenesis was adopted. Half of the animals were euthanized 24 h after the last BrdU dose to estimate "cell proliferation." The remaining animals were euthanized 28 days later to assess "survival" of the cells born over the 12-day BrdU injection period. No differences in the number of BrdU-positive profiles were seen among control and Pb-exposed animals at the early sacrifice time, indicating that, with this BrdU dosing protocol, cell proliferation was not impacted by Pb exposure. At 28 days, however, the number of BrdU-positive profiles was substantially diminished in animals continuously exposed to Pb (Gilbert et al., 2005). A recent report has confirmed these observations of developmental Pb exposure on hippocampal neurogenesis (Verina et al., 2007). Using a different BrdU dosing protocol in which higher doses of BrdU were repeatedly administered over a single day, reductions in cell proliferation have also been reported (Jaako-Movitts et al., 2005; Schneider et al., 2005).

These data indicate that chronic exposure to Pb can modify neurogenesis in the adult hippocampus. Further, investigation of this process may have implications for the actions of Pb on neuroplasticity and learning, CNS responses to stress, regeneration and repair in the aging nervous system, and affective and neurodegenerative diseases (Fig. 6 and other papers presented in this symposium). Functionally, many features of adult neurogenesis appear to recapitulate the sequence of events that occurs during neuronal development (Lledo et al., 2006). In this manner, the adult hippocampal neurogenesis may provide a model system to study the effects of potential developmental neurotoxicants in a less complex setting.

What does the future hold?

Metal chelation therapy to reduce circulating levels of Pb has proven ineffective in treating low level environmental exposures to Pb and has failed to reverse the associated learning deficits (e.g., Rogan et al., 2001; Stangle et al., 2004). Therefore, it is proposed that treatment strategies directed to the neuronal actions of Pb may prove more effective in reversing or alleviating the impact of Pb on brain function. Given the findings described herein, such strategies might include manipulations that augment synaptic and structural plasticity in areas of the brain critical for cognition (Kramar et al., 2004; Rex et al., 2006). Recent discoveries of Pb exposure interactions with the process of neurogenesis may serve to further elucidate the role of Pb in IQ deficits in children. These observations also suggest that Pb effects on neurogenesis may be exacerbated by stress as

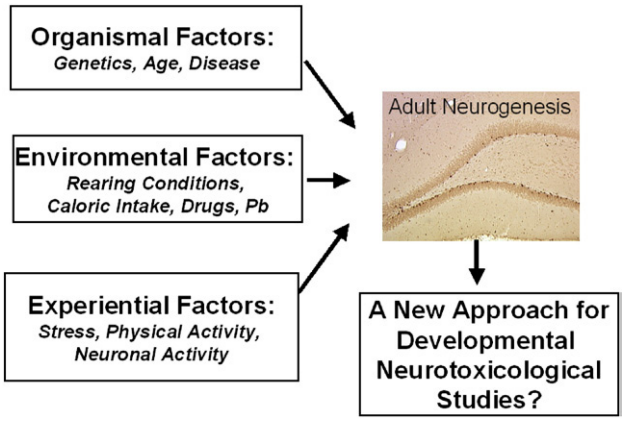

Fig. 6. Since its rediscovery in the last decade, the observation that the adult nervous system is capable of generating new neurons that become functionally incorporated into working neural networks has spawned a rich field of scientific inquiry as to the mechanisms and potential benefits to the organism. Neurogenesis has been implicated in response to cell injury and repair mechanisms, neurodegenerative diseases, and affective disorders. Factors that have been demonstrated to influence the neurogenesis process in the adult include those inherent to the organism, the environment in which it resides, and the interactions of the organism within that environment. Recent data indicate that chronic Pb exposure can alter the animal's potential for neurogenesis. The extent to which the impact of Pb on neurogenesis contributes to cognitive impairments associated with Pb exposure has yet to be determined. It is also possible that the action of Pb on neurogenesis could potentially contribute to perturbations in the animal's adaptation to stress, cognitive decline with age, and depression, aspects of which are discussed by other contributors to this chapter. Furthermore, to the extent to which this process recapitulates the processes of neuronal development in early brain ontogeny, neurogenesis in the adult hippocampus may offer a simplified model system in which to evaluate the potential for xenobiotics to produce developmental neurotoxicity.

outlined in the previous section, may underlie perturbations in emotion and affect, and may contribute to neurodegeneration and age-related declines in cognition (see below). Given the parallels between developmental processes that are recapitulated in adult neurogenesis, examination of this phenomenon may provide a simplified framework to evaluate the potential of Pb and other neurotoxicants to disrupt brain development.

In vitro responses of neural cells to Pb: Focus on chaperones

Cellular targets of Pb in vitro

The two previous sections described the effects of Pb on various brain regions, demonstrating how Pb's effects on the CNS are both temporally and spatially specific. This section describes work in in vitro models, specifically mammalian cell and tissue cultures, which offer an opportunity to investigate direct cellular and molecular effects of Pb on isolated biological substrates. Issues pertaining to the use of in vitro systems in neurotoxicity testing have been extensively reviewed and critically examined in recent years. Comparisons of in vitro and in vivo data for neurotoxicants such as Pb and organophosphorus insecticides support the validity of results obtained from in vitro systems and indicate areas where their greatest value may be found (Tiffany-Castiglioni, 2004; Tiffany-Castiglioni et al., 2006a,b). These comparisons demonstrate that neurons and glia are affected at toxicologically relevant concentrations in culture, that direct mechanisms of toxicity may be unmasked in culture, and that cell type is an important variable in attempting to define pathways of toxicity. Conversely, cell and tissue cultures have specific limitations for neurotoxicity studies, notably that they can model young but not aging cells and that modeling sporadic, long-term, and incremental exposures to toxicants is difficult in vitro (Harry et al., 1998; Pentreath, 1999; Tiffany-Castiglioni, 2004; Tiffany-Castiglioni et al., 2006a,b). Exposures to mixtures and multiple toxic elements in culture have not been studied extensively and this area must be developed in order to provide useful information for risk assessment (Tiffany-Castiglioni et al., 2006a,b).

Inorganic Pb has been studied more extensively in vitro than most other neurotoxicants, because unlike other neurotoxicants, Pb acts directly on neural cells without prior systemic metabolism or biological activation (Lasley and Gilbert, 2004; Tiffany-Castiglioni and Qian, 2004, 2005). Neuronal effects induced by Pb at 200 mg/L in the dam's drinking water and at 0.1 μM in neuronal cultures include alterations in morphology, neurite growth, ion channels, and both pre- and postsynaptic neuronal function (Morgan et al., 2000; Afano and Petit, 1982; McCauley et al., 1982; Cookman et al., 1987; Reuhl, 1991; Kern and Audesirk, 1995; Patrick and Anderson, 1995; Ishihara et al., 1995; Cline et al., 1996; Gilbert et al., 1996; Omelchenko et al., 1996, 1997; Wilson et al., 2000). Pb-induced effects on cultured astroglia or model cell lines at micromolar concentrations include intracellular accumulation of Pb (Tiffany-Castiglioni et al., 1987), altered glutamate metabolism (Engle and Volpe, 1990; Sierra and Tiffany-Castiglioni, 1991; Fitsanakis and Aschner, 2005), altered homeostasis of calcium and copper ions (Tiffany-Castiglioni et al., 1987; Rowles et al., 1989; Dave et al., 1993; Qian et al., 1995, 1999; Legare et al., 1998), oxidative or mitochondrial stress (Legare et al., 1993; Qian et al., 2001; Qian and Tiffany-Castiglioni, 2003), and reduced basal respiratory rate (Holtzman et al., 1984, 1987). Effects on Schwann cells include partial inhibition of myelination (Windebank, 1986) and ultrastructural abnormalities (Tang et al., 1996). Oligodendrocyte progenitor cells show delayed differentiation as a result of exposure to Pb (Deng et al., 2001). The breadth of cellular effects indicates that Pb interacts with diverse proteins to impair cell function. As many aspects of Pb neurotoxicity in vitro have been reviewed recently (Lasley and Gilbert, 2004; Tiffany-Castiglioni and Qian, 2004, 2005), this discussion will focus on a new aspect of Pb neurotoxicity recently uncovered in vitro: the effects of Pb on chaperone proteins.

Chaperone deficiency hypothesis in disease

Molecular chaperones are highly conserved proteins that assist nascent proteins in folding into their correct conformations. Chaperones are also linkage molecules for communication between signaling pathways of the cell. Because chaperones are induced by a number of environmental stressors, they are also known as heat shock proteins and stress proteins (Little et al., 1994). Chaperones thus have a critical role in cell survival and recovery after stress. The Tiffany-Castiglioni laboratory is

testing the hypothesis that certain environmental neurotoxicants such as Pb produce a deficiency in chaperone function that compromises protein secretion, exacerbates protein aggregation, and increases sensitivity to oxidative stress. In the long term, chaperone deficiency could underlie age-related neurodegenerative diseases that exhibit protein aggregation such as in Alzheimer's disease (AD), Parkinson's disease (PD), and prion-related diseases.

In eukaryotic cells, secretory and membrane proteins are synthesized on membrane-bound ribosomes and extruded into the endoplasmic reticulum (ER) for processing before they are transferred to their destination. Nascent proteins begin to fold co-translationally and then undergo post-translational modification necessary for optimal function, including completion of folding, assembly of subunits, and oligomerization. Each co-translational and post-translational step requires a sequential interaction with a distinct chaperone protein. The processing of proteins in the ER is under a tight quality control. However, with environmental stress or with certain genetic mutations, some proteins escape the quality control process, become unfolded or misfolded, and accumulate in the ER (Wickner et al., 1999). The accumulation of unfolded or misfolded proteins results in ER stress and then activation of the unfolded protein response (UPR), the three hallmarks of which are upregulation of ER molecular chaperone gene expression, transient suppression of the rate of protein synthesis, and activation of unfolded protein ubiquitination so that they are tagged for degradation. Degradation occurs through lysis by the proteosome (Gorell et al., 1997, 1999; Kaufman, 1999; Mori, 2000). These cellular responses minimize the accumulation and aggregation of defective proteins by increasing the capacity of the ER machinery for folding and degradation. Prolonged ER stress leads to the induction of specific cell death pathways (Rao and Bredesen, 2004; Zhang and Kaufman, 2004).

Glucose-regulated protein of 78 kDa (GRP78), also known as immunoglobulin heavy chain-binding protein (BiP) and heat shock 70 kDa protein HSPA5, is a key chaperone in the UPR. GRP78 has been termed the "master regulator" chaperone because quality control pathways triggered by misfolded proteins converge on this molecule (Rao and Bredesen, 2004). GRP78 negatively regulates the UPR by binding to three ER stress transducer proteins or sensors to maintain them in an inactive conformation: inositol requirement 1 (IRE1), phosphorylated extracellular signal-regulated kinase (PERK), and activating transcription factor 6 (ATF6). IRE1 and PERK are prevented from homodimerizing and autophosphorylating to their active states when they are bound by their luminal domains to GRP78. ATF6 undergoes proteolytic activation in the Golgi when it is released from GRP78. When misfolded proteins accumulate in the ER, they bind to a peptide-binding domain of GRP78 and disrupt its interaction with these three stress sensors, releasing them from negative inhibition. The three sensors coordinately regulate the UPR through various signaling pathways. Thus, for example, activated IRE1 acts as a signal transducer from the ER to the nucleus and indirectly upregulates GRP78 gene expression through TRE kinase/ribonuclease (Bertolotti et al., 2000; Liu et al., 2000; Okamura et al., 2000; Kimata et al., 2003). Fig. 7 shows the IRE1–ATF6–XBP-1 pathway-dependent upregulation of the GRP78 gene expression.

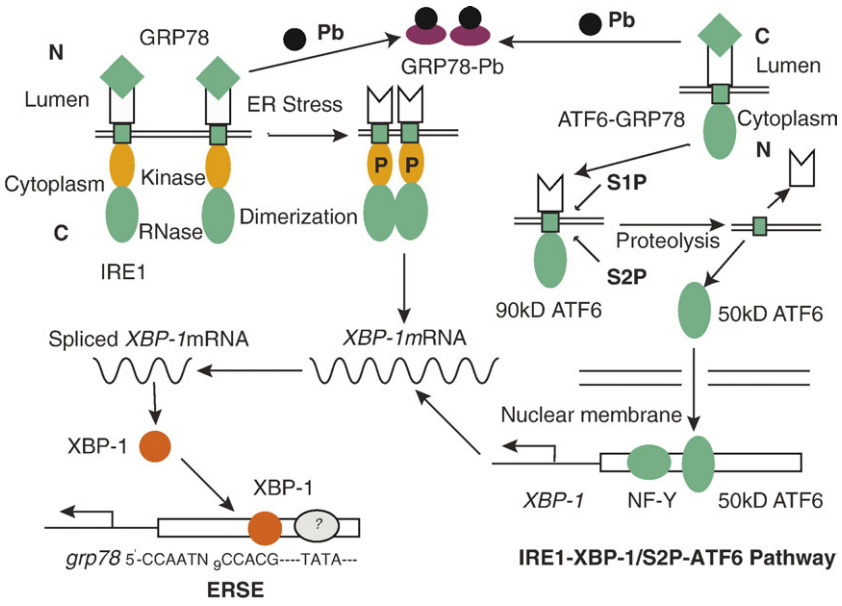

Fig. 7. IRE1–ATF6–XBP-1 pathway for GRP78 upregulation. Under normal conditions, GRP78 binds to inositol requiring protein 1 (IRE1) and activating transcription factor 6 (ATF6) in the endoplasmic reticulum (ER) lumen. When unfolded or misfolded proteins accumulate in the ER, GRP78 is recruited to bind to unfolded proteins, which results in GRP78 dissociation from IRE1 and ATF6, two membrane-bound signal transducers. The dissociation of GRP78 from IRE1 activates IRE1 autophosphorylation and dimerization, resulting in the activation of IRE1 RNA nuclease that splices X-box binding protein 1 (XBP-1) mRNA. XBP-1 translated from spliced XBP-1 mRNA binds to GRP78 promoter and contributes to activate GRP78 transcription. At the same time, after dissociation from GRP78, a 90-kDa ATF6 is proteolysed by S2P, an intramembrane protease, and a 50-kDa fragment is released for binding to the XBP-1 promoter to activate XBP-1 gene expression with nuclear factor Y (NF-Y).

Chaperone deficiencies are proposed to play a major role in cell and tissue aging and the development of neurodegenerative diseases (Rao and Bredesen, 2004). This subject is pertinent to Pb exposure because epidemiologic studies suggest a link to PD, as will be discussed in the next section (Duckett et al., 1977; Kuhn et al., 1998; Gorell et al., 1997, 1999). Many neurodegenerative diseases exhibit the intracellular accumulation of proteins in neurons and glia of the brain, owing to the misfolding of proteins, thus implicating chaperone deficiencies as a potential mechanism of disease. In age-related neurodegenerative diseases, specific proteins escape post-translational quality control and undergo a conformational rearrangement that endows them with a tendency to misfold or unfold, aggregate, and deposit within tissues or cellular compartments. This category, called "conformational diseases," includes AD, PD, prion disease, and cataracts (Carrell and Lomas, 1997; Kopito and Ron, 2000).

A deficiency of GRP78 function is possible in AD, PD, and perhaps other conformational diseases as suggested by several studies. GRP78 levels are reduced in the brains of AD patients (Katayama et al., 1999). The formation of neuritic plaques from Aβ accumulation and aggregation in brain cognitive regions during aging is a hallmark of AD pathology (Selkoe, 2004). In vitro studies indicate that GRP78 facilitates the maturation of the amyloid precursor protein (APP), based on evidence that it binds to the amyloid β (Aβ) peptide and reduces Aβ protein secretion and formation of extracellular Aβ deposits (Yang et al., 1998; Kakimura et al., 2002; Kudo et al., 2006). Furthermore, Aβ42 peptides, which are believed to form the core of amyloid plaques, are generated and retained exclusively in the ER in neurons (Cook et al., 1997; Hartmann et al., 1997; Wild-Bode et al., 1997; Greenfield et al., 1999).

In vitro studies also demonstrate a protective role of GRP78 against oxidative stress. In familial Alzheimer's disease (FAD), a presenilin-1 (PS1) mutation downregulates the UPR by a failure of GRP78 upregulation, and cells become sensitive to conditions associated with ER stress. This pathological process is completely reversed in cultured neuroblastoma cells bearing PS1 mutants by overexpressing GRP78 (Katayama et al., 1999; Niwa et al., 1999). In rat hippocampal neurons in culture, GRP78 depletion by GRP78 antisense oligos results in enhanced neuronal death following glutamate and oxidative insults, while GRP78 induction by 2-deoxy-D-glucose protects neurons against excitotoxic and oxidative injury (Yu et al., 1999). In mouse WEHI7.2 lymphoma cells deficient in the induction of GRP78 expression, cells undergo apoptosis, even if the cells overexpress bcl-2 (McCormick et al., 1997). In contrast, induction of GRP78 expression renders the cell resistant to apoptosis (Yu et al., 1999; Reddy et al., 2003).

The above examination of the literature develops two related ideas: (1) that a deficiency in chaperone function from exposure to environmental contaminants could underlie age-related conformational diseases that exhibit protein aggregation, such as in AD and PD; and (2) that GRP78 deficiency may be a key component of these diseases. It is, therefore, of interest to identify environmental agents that disrupt GRP78 function. Pb has emerged as a neurotoxicant of interest in vitro studies, because it binds to GRP78 and impairs its function. Pb has also been linked with conformational diseases. These findings will be reviewed in the next sections.

Association of Pb with conformational diseases

Pb exposure in humans is associated with PD, AD, and cataract formation. Occupational exposure to Pb has been suggested as a risk factor in the development of Parkinson syndrome (Duckett et al., 1977; Kuhn et al., 1998). Epidemiological studies support the hypothesis that Pb plays a synergistic role with other heavy metals, especially manganese and copper in the incidence of PD (Gorell et al., 1997; 1999). The association of Pb with PD is supported by experimental in vivo data. Neonatal rats exposed to high Pb levels show damage to striatal dopaminergic neurons. Damage is not reversed after Pb treatment ceases and brain Pb levels decrease to control levels, though many of the behavioral and neurochemical abnormalities associated with high Pb exposure dissipate (Jason and Kellogg, 1981). Pb treatment also decreases dopamine levels in rat substantia nigra, though the decrease is probably not related to the physical loss of tyrosine hydroxylase (TH)-positive neurons (Tavakoli-Nezhad et al., 2001). Furthermore, as will be described in the following major section of this review on Pb and AD, a recent study has shown that fetal exposure to Pb prompts latent overexpression of APP and the production of the amyloid Aβ in the aging brain of rats (Basha et al., 2005). Epidemiological data also indicate Pb exposure as a risk factor for cataract development (Schaumberg et al., 2004). This conclusion is supported by experimental findings that Pb exposure alters post-translational processing of crystallins in Fisher 344 rats and induces cataract formation in lens organ cultures (Neal et al., 2005).

Interactions of Pb with GRP78

Our laboratory has identified interactions of Pb and GRP78 as a possible mechanistic link between heavy metal exposure and chaperone deficiency. We used proteomic strategies and cellular functional analysis to successfully identify GRP78 as a Pb-binding protein in astrocyte-like C6 rat glioma cells in culture (Qian et al., 2000). Astrocytes are the brain cell type that preferentially accumulates Pb (Holtzman et al., 1984; Tiffany-Castiglioni et al., 1989; Lindahl et al., 1999), and C6 cells likewise accumulate Pb ion in culture (de Vellis et al., 1987; Tiffany-Castiglioni et al., 1988; Qian et al., 1999). We first found that more than 90% of total cellular Pb extracted from Pb-exposed C6 cells is associated with a protein fraction having molecular weights of above 14 kDa (Qian et al., 1999). This finding suggests that Pb does not randomly target cellular components and that small molecules or polypeptides are probably not targets for Pb toxicity in glial cells. We then found that Pb specifically binds to GRP78 in silico, as demonstrated by Pb-affinity column chromatography. Neither bovine serum albumin (BSA) nor reduced glutathione (GSH) disrupt the binding, and GRP78 binds with higher affinity to Pb than to zinc or nickel (Qian et al., 2000).

We have extended the finding that Pb binds to GRP78 in silico to a functional analysis of GRP78 function in cell culture. We found that Pb induces GRP78 aggregation intracellularly in human CCF-STTG1 astrocytoma cells in culture (Qian et al., 2005). We constructed and expressed a chimeric protein for GRP78 and enhanced green fluorescence protein (EGFP) in cultured CCF-STTG1 cells by cloning GRP78 DNA into an EGFP-containing vector and transfecting the cells. The expression of GRP78–EGFP was driven by the promoter of human cytomegalovirus (CMV) in the vector. The chimeric protein was used to monitor GRP78–EGFP retention by fluorescence microscopy. Control cells transfected with non-chimeric EGFP vectors expressed EGFP that was trapped intracellularly. We found that Pb induces compartmentalized redistribution or aggregation of the GRP78–EGFP fusion protein and fails to aggregate EGFP (Qian et al., 2005). Furthermore, depletion of GRP78 proteins by siRNA sensitizes cells to Pb treatment, as shown by increased intracellular levels of reactive oxygen species (ROS). These findings indicate a deleterious functional interaction between Pb and GRP78. One of the proteins chaperoned by GRP78 in astrocytes is IL-6. Therefore, Pb binding to GRP78 may plausibly impair its chaperone function, which is the subject of current studies in our laboratory.

IL-6 is a pleiotropic cytokine involved in disease progression and inflammatory responses to environmental insults. IL-6 is secreted by astrocytes into the extracellular space and targets other cells via an IL-6 receptor complex composed of the IL-6 receptor and glycoprotein (gp)130 (Gruol and Nelson, 1997; Taga and Kishimoto, 1997). GRP78 serves as a molecular chaperone in the secretion of IL-6 from astrocytes (Hori et al., 1996). Its levels are normally low in the CNS but become elevated with CNS injury and inflammation (Gruol and Nelson, 1997). Elevated levels of IL-6 and other cytokines are detected in the cerebrospinal fluid (CSF) of patients with neurodegenerative diseases, including AD and PD (Zhao and Schwartz, 1998; Teismann et al., 2003). Microglia also secrete pleiotropic cytokines such as IL-6, but the relative contributions of astrocytes and microglia are unknown. However, astrocytes significantly outnumber microglia, which are the least numerous of the glia (Hanisch, 2002; Nakajima and Kohsaka, 2004). Data from transgenic mice overexpressing IL-6 indicate that IL-6 elevation in inflammatory responses leads to astrocytosis, neurodegeneration, and angiogenesis, suggesting a direct pathogenic role of IL-6 in inflammatory CNS diseases (Campbell et al., 1993). On the other hand, in keeping with its pleiotropic roles, IL-6 also plays a protective role for neurons in the CNS. For example, in human SY5Y neuroblastoma cell cultures, IL-6 treatment reduces cell death caused by hydrogen peroxide exposure (Bissonnette et al., 2004). IL-6 rescues neurons in rat organotypic hippocampal slices treated with NMDA (Pizzi et al., 2004). Furthermore, IL-6 knockout mice (IL-$6^{-/-}$) infected with Theiler's virus or lesioned with neurotoxicant *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) exhibit increased deaths and neurological deficits (Bolin et al., 2002; Pavelko et al., 2003; Penkowa et al., 2003). In addition, in transgenic mice with astrocyte-targeted IL-6 expression, i.e., the IL-6 gene with the promoter for glial fibrillary acidic protein (GFAP), IL-6 prevents neuroglial degeneration induced by aminonicotinamide (Penkowa et al., 2003). These findings implicate IL-6 secretion by astrocytes as an integral part of both neurodegenerative and neuroprotective processes.

Based on the previous finding that Pb strongly binds to GRP78 and induces GRP78 aggregation, Tiffany-Castiglioni and colleagues hypothesized that Pb blocks IL-6 secretion from astrocytes via binding to GRP78. Primary astrocyte cultures from the neonatal rat were studied. We established that an IL-6–EGFP chimera, constructed as previously described for GRP78–EGFP, retained its secretory properties in transfected primary cultures (Qian et al., 2007). Pb at 1 to 50 μM significantly reduced IL-6 level secretion by astrocytes. As shown in the photomicrograph Fig. 8A, intracellular IL-6–EGFP signal intensities increased in a concentration-dependent manner relative to Pb treatment. IL-6–EGFP signal intensity was measured and quantified in individual cells by densitometric analysis and was significantly increased in Pb-treated cells in a concentration-dependent manner (Fig. 8B). Furthermore, extracellular IL-6–EGFP levels, as detected by an immunochemical dot-blot analysis with an antibody to GFP, significantly decreased in a concentration-dependent manner relative to Pb exposure (Fig. 8C). Image analysis of fusion fluorescence proteins constructed for GRP78 and IL-6 in co-transfected cells showed that IL-6 and GRP78 were co-localized in live astrocytes, thus confirming the chaperone role of GRP78 for IL-6 previously reported (Hori et al., 1996). In addition, though both fluorescent chimeric IL-6 proteins aggregated in Pb-treated astrocytes, IL-6 did not bind to Pb in a Pb affinity column, indicating that Pb interacted with GRP78 alone. Furthermore, GRP78 depletion with double-strand RNA interference technique (dsRNAi) increased IL-6 retention in astrocytes, indicating that Pb indirectly blocked IL-6 secretion by targeting GRP78 (Qian et al., 2007). These data support the hypothesis that Pb binding to GRP78 decreases IL-6 secretion and thus provide evidence for a significant chaperone deficiency in Pb-exposed astrocytes in culture.

Future directions

AD, PD, and Pb-induced neurobehavioral and developmental dysfunctions are recognized to have environmental determinants. This recognition is based upon a large body of work demonstrating that Pb damages the developing nervous system, as well as a growing body of work that links Pb exposure to late stage neurodegenerative diseases.

One limitation of this body of work is the recognition, discussed earlier in the review, regarding the examination of a single risk factor, i.e., Pb, as having effects that, in fact, are due to multiple factors acting simultaneously.

GRP78 stands out as having pivotal roles in the maintenance of cell health, not only as a master regulator in quality control of protein folding, but also in protecting the cell from environmental insults. Furthermore, GRP78 is implicated in some of the molecular events in the pathogenesis of neurodegenerative diseases. These characteristics make GRP78 an attractive and plausible mechanistic target for environmentally induced dysfunction in neural cells. GRP78 and Pb have several known

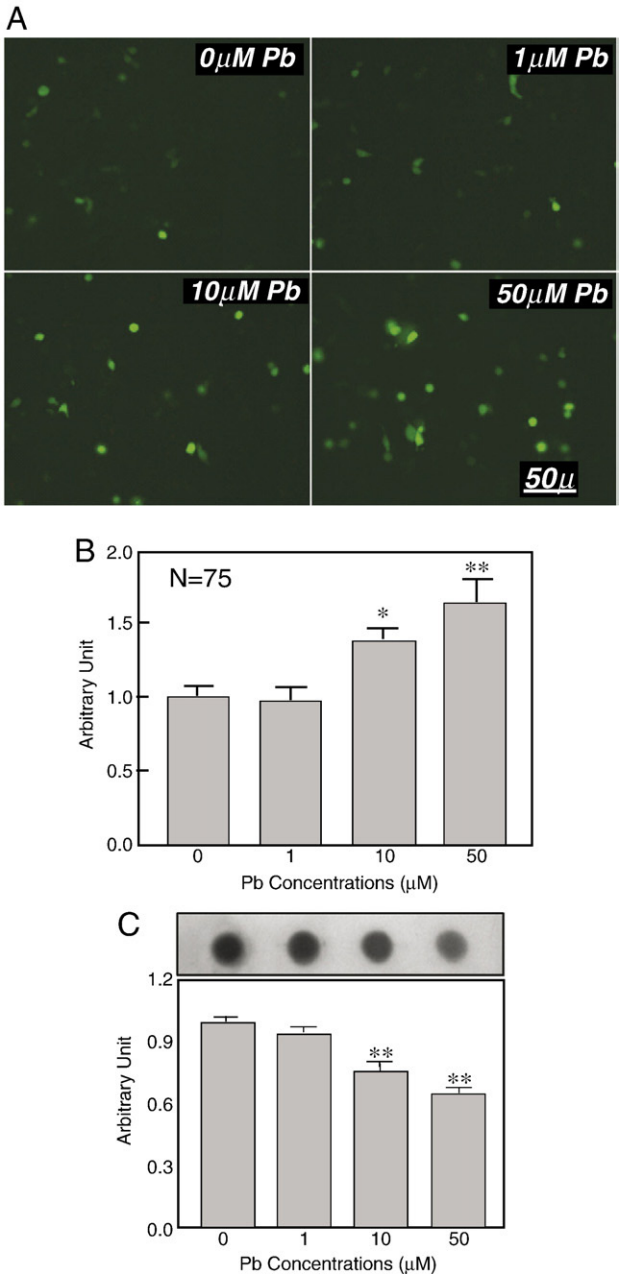

Fig. 8. Pb-induced IL-6–EGFP retention in rat astrocytes. (A) Astrocytes were transfected with IL-6–EGFP plasmids for 24 h and then treated with 0, 1, 10, or 50 μM Pb acetate for another 24 h. Intracellular IL-6–EGFP signals were randomly captured with fluorescence microscopy through the FITC channel (magnification: 20×). (B) IL-6–EGFP signals for transfected cells in panel A were from 6 images of each treatment ($n=75$). (C) Extracellular IL-6–EGFP signals were detected by dot-blot analysis with GFP antibody and quantified with ImageJ ($n=4$). Data in panels B and C represent mean±SE (*$p<0.05$ differs from the control; **$p<0.01$ differs from the control).

interactions that should permit clear-cut additional testing of the hypothesis that the environmental neurotoxicant Pb produces a deficiency in chaperone function that compromises protein secretion. In future studies, this approach can be expanded to other chaperones and the molecules they chaperone, other cell types, such as neurons and microglia, and other toxicants such as the parkinsonigenic pesticide paraquat.

Pb and Alzheimer's disease

The preceding section described the chaperone deficiency hypothesis as an explanation for the effects of Pb at the cellular level on protein secretion and aggregation that can then impact cell survival and function. Chaperone deficiencies are implicated in a number of CNS diseases including AD, a progressive neurodegenerative disorder whose clinical manifestations appear in old age. The hallmark pathological features of AD (amyloid plaques and associated proteins) are present in normal aging individuals, suggesting that AD may result from the acceleration of normal age-related processes in the brain. The major constituents of senile plaques are 39 to 42 amino acid peptides, snipped from a larger protein called the amyloid precursor protein (APP) (Glenner and Wong, 1984; Masters et al., 1985; Selkoe et al., 1986; Goldgaber et al., 1987; Robakis et al., 1987; Tanzi et al., 1987). Of these, the Aβ form that is comprised of 42 amino acids is considered the most amyloidogenic. Between 5% and 10% of AD is of familial origin and may involve mutations in genes associated with APP biosynthesis and proteolytic processing (Haass et al., 1994; Suzuki et al., 1994; Eckman et al., 1997; Ancolio et al., 1999). The genetics of AD have revealed that early onset AD (at <60 years of age) is associated with APP or the presenilins (1 and 2), while susceptibility to late onset-AD is linked to apolipoprotein E (ApoE, Bertram and Tanzi, 2004) and potential interactions with the environment. This suggests that the environment and other nongenetic factors are causally related to the onset of the more common sporadic forms (>95%) of AD.

Even though it is suggested that environmental influences may drive AD pathogenesis in sporadic AD cases, it is not clear when this may occur. Therefore, it is important to identify environmental triggers and to pinpoint the period during which such factors pose the greatest risk. Twin studies often used to confirm the inheritance pattern of a disease have shown poor concordance in neurodegenerative diseases such as AD and PD (Gatz et al., 1997, 2005; Raiha et al., 1997). The negative findings of such studies along with the sporadic nature of late-onset AD suggest a strong role for the environment. The rising incidence of AD suggests that a long latent period passes before overt cellular damage and cognitive defects are observed.

Environmental and occupational exposure to myriad chemicals occurs throughout human lifetimes. Many of these chemicals are benign, but some, such as Pb, pose a significant heath risk, as has been described in the three previous sections. Further, Pb has been shown to pose the greatest risk to children, and a large number of the currently aging population has been significantly exposed during childhood (Basha et al., 2005; Zawia and Basha, 2005; Bolin et al., 2006).

Pb exposure has been overwhelmingly viewed as an environmental risk facing children, leaving them with residual cognitive and behavioral deficits that persist well into their young adult life. The ability of developmental exposure to Pb to impact disease processes whose symptoms do not appear until old age was not considered (Basha et al., 2005; Zawia and Basha, 2005). Therefore, it is no surprise that few epidemiological studies have

focused on Pb exposure and its connection to neurodegenerative diseases. In 1999, a population-based case–control study found that chronic occupational exposure to Pb, as well as to other metals, was associated with PD (Gorell et al., 1999). Kamel et al. (2002) evaluated the relationship between Pb exposure and amyotrophic lateral sclerosis (ALS). They found that risk for ALS was associated with elevations in both blood and bone Pb levels, suggesting that Pb exposure played a role in the etiology of ALS. While these studies provided hints as to the possible connection between Pb exposure and neurodegenerative disease, more convincing evidence was provided by the work published by Stewart et al. (2002), which revealed the occupational exposure to Pb as a risk factor for AD. In this seminal work, Stewart et al. (2002) looked at tibia bone Pb levels in 529 former organolead workers and the relationship of Pb bone level to the ApoE genotype, a known risk factor for AD. They concluded that the persistent CNS effects of Pb are more toxic in individuals with at least one ApoE €4 allele (Stewart et al., 2002). The link between past adult Pb exposure and neurodegeneration was further established by this group using brain MRI imaging (Stewart et al., 2006) and was consistent with their previous work showing an association between Pb exposure and longitudinal cognitive decline. While these studies evaluated adult occupational Pb exposure, it is not known if the workers they studied had been previously exposed to Pb as children.

Pb has been reported to accumulate in the bone of 80-year-old humans who had been exposed to Pb (Manea-Krichten et al., 1991) and in rats and mice (Massie and Aiello, 1992). Although in animals, this accumulation occurs primarily in glial cells, as has been discussed in the previous section (Lindahl et al., 1999; Qian et al., 2000), intercranial calcification (cerebral and cerebellar) in adult humans with chronic Pb exposure has been demonstrated using computed tomographic (CT) scans (Reyes et al., 1986). These individuals had been exposed to Pb for 30 years and, at 57 years of age, exhibited diminished visual acuity, peripheral neuropathy, and hypertension. These observations underlie the great need for more research in this area and suggest that the aging brain can be compromised by either concurrent or prior exposure to Pb. Momcilovic et al. (2001) found a 10-fold increase of Pb in the white matter of AD patients. The possibility that toxic levels of Pb in any form could result in the formation of Alzheimer's fibrillary tangles was implied in the findings of a patient who survived severe Pb encephalopathy at 2 years of age but died of severe mental deterioration at the age of 42 (Niklowitz and Mandybur, 1975). The brain revealed that numerous pyramidal cells of the forebrain grisea contained Alzheimer's neurofibrillary tangles while the remaining pyramidal cells of the hippocampi showed granulovacuolar degeneration. Many senile plaques were also observed, predominantly in the atrophic temporal cortex. Atomic absorption spectrophotometry disclosed a 10-fold increase of Pb in frontal and temporal cortices as compared to the control. A high concentration of Pb has also been reported in patients with diffuse neurofibrillary tangles with calcification (DNTC) (Haraguchi et al., 2001). Pb is thus suspected to play a role in the pathogenesis of DNTC, a form of presenile dementia.

Exposure to Pb and the developmental basis of AD

In a seminal study, Barker et al. (1989) demonstrated an inverse relationship between birth weight and the incidence of cardiovascular disease. The Barker hypothesis, also known as the Fetal Basis of Adult Disease (FeBAD), states that many adult diseases have a fetal origin (Barker et al., 1989; Barker, 1999, 2002, 2004). A large body of subsequent clinical and experimental data has supported this hypothesis and has shown that diseases of the cardiovascular system and HPA axis and diabetes can also be affected by nutritional imbalances during pregnancy (Valdez et al., 1994; Yarbrough et al., 1998). Diseases such as schizophrenia have also been linked to infection, fetal malnutrition, or hypoxia in early life (Boksa and El-Khodor, 2003; Dalman et al., 1999; Preti, 2003; Bilbo et al., 2005). These observations led to a new concept regarding some adult diseases that emphasizes the role of environmental factors acting in the preconceptual, fetal, and infantile phases of life (Gluckman and Hanson, 2004).

Chemical exposure during the early phases of life can also impact future disease processes. Behavioral studies following developmental exposure to environmental agents such as methylmercury (Rice, 1989), methylazoxymethanol (Lee and Rabe, 1992), and triethyltin (Barone et al., 1995) have provided evidence that delayed latent neurotoxicity is exhibited by animals following chemical exposure. Likewise, developmental exposure to pesticides has been shown to result in a loss of nigral dopamine neurons and in a decrease in dopamine content 9 months later (Thiruchelvam et al., 2002; Reeves et al., 2003). These data suggest that behavioral and neurodegenerative outcomes later in life can be influenced by developmental exposure to environmental agents that enhance the response to future chemical exposure as shown in Fig. 9.

Animal studies from the Zawia laboratory strongly suggest that exposure to Pb can be a risk factor that promotes the pathogenesis of AD (Basha et al., 2005). Exposure of rodents to Pb during the postnatal period (PND 1–20) results in a transient increase of APP mRNA expression during the first month after birth followed by a return to basal levels by 1 year and a subsequent rise in APP expression at 20 months of age. The upregulation in APP mRNA levels occurred concurrently with Pb exposure early in life; however, the late rise in APP mRNA expression at 20 months of age occurred when both blood and tissue concentrations of Pb in these aged animals were at background levels (Basha et al., 2005); thus, ruling out the possibility that Pb is stored in bones and leaches out during old age to stimulate the upregulation in APP mRNA expression. These findings indicate that reprogramming of gene expression in the aging rodent brain can occur after developmental exposure to Pb. Can exposure at any time in adulthood produce the same effects? To answer these questions, Zawia and colleagues monitored the levels of the APP mRNA, APP, and Aβ in developmentally exposed rats (Pb-E) as well as those exposed to Pb in old age. Only developmental exposure resulted in significant alterations in these biomarkers, and changes in APP mRNA expression were followed by elevations in APP and its amyloidogenic Aβ cleavage product (Basha et al., 2005). This affirmed that the

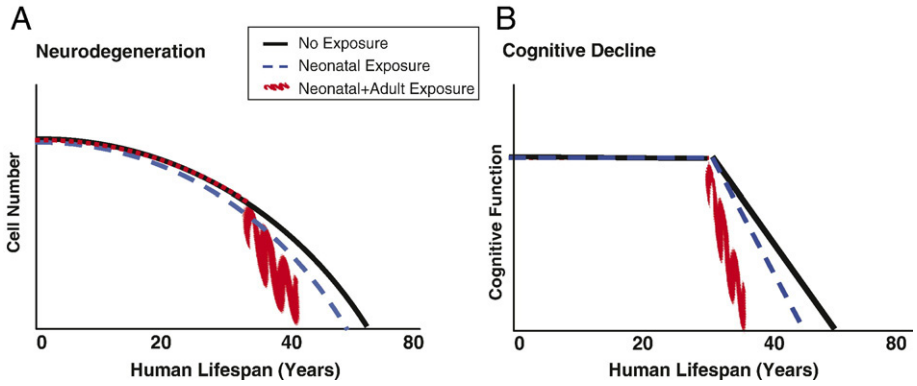

Fig. 9. Hypothetical model showing the relationship between chemical exposure, neurodegeneration, and cognitive decline. Cells are lost in a gradual manner early in adulthood; however, cognitive decline is not evident until the aging period. In panel A, a model of cellular loss in the brain is displayed. Cell loss in the human brain is known to begin as early as 20 years of age. In Alzheimer's disease (AD), the process of neuron loss may be accelerated, perhaps by chemical exposure early in life (dashed line). This acceleration of AD pathology by early exposure can be worsened if a repeated occupational exposure occurs in adulthood. In panel B, a possible curve of cognitive decline is shown. Humans generally begin to experience significant cognitive decline later in life at about 60 years of age. There is then a rapid drop in cognitive ability. In the AD brain, the decline begins earlier in life and is more accentuated later than in normal aging.

latent expression of APP mRNA was a programmed event that was set in motion by exposure to Pb early in life.

Rats served as a good model for feasible life span studies under controlled conditions and can show that environmental influences occurring during brain development predetermine the expression and regulation of APP later in life. Using rats, it was also possible to show that these molecular perturbations have biological consequences. Although the elevation of Aβ as a consequence of this developmental reprogramming would be expected to have deleterious consequences, Aβ deposition and plaque formation does not occur in rodents. In order to extrapolate these studies to humans, it would be necessary to show this in plaque-forming animals. Thus, current work in the Zawia laboratory is focusing on the frontal association cortex of Pb-exposed 23-year-old monkeys that exhibit human-like plaque pathology.

Aβ is also known to generate ROS in the aging brain; therefore, we also studied the lifetime 8-hydroxy-2′-deoxyguanosine (oxo^{8}dG) levels and the activity of the DNA repair enzyme 8-oxoguanine DNA glycosylase (Ogg1) in rats developmentally exposed to Pb. Oxo^{8}dG was transiently modulated early in life (PND 5) and was later elevated 20 months after exposure to Pb had ceased, while Ogg1 activity was not altered (Bolin et al., 2006). Furthermore, an age-dependent loss in the inverse correlation between Ogg1 activity and oxo^{8}dG accumulation was observed. The effect of Pb on oxo^{8}dG levels did not occur if animals were exposed to Pb in old age. These data suggest that oxidative damage and neurodegeneration in the aging brain may be impacted by developmental disturbances.

Epigenetic mechanisms

One way to achieve permanent changes or long-term alteration in gene expression is to alter the structural makeup of the DNA bases that determine the sequence-specific DNA binding of a transcription factor to the gene's promoter regions. Such chemical changes in the sequence would be retained in the DNA template for the life of a differentiated cell and control the ability of stimuli to activate such a gene. Evidence for the role of methylation in long-term regulation of gene expression has come from several studies. For example, maternal grooming changed the methylation pattern and expression of the glucocorticoid receptor (GR) in the hippocampus in rat offspring, resulting in permanent changes in their stress response (Weaver et al., 2004). Recently, Lillycrop et al. (2005) restricted the maternal diet and studied the expression of the GR and the perioxisomal proliferator-activated receptor (PPAR) in the offspring after weaning. They found a decrease in the methylation of these genes, which was consistent with their elevated mRNA expression.

Environmental agents could interfere with DNA methylation by disrupting the enzymes that conduct such reactions. Poirier and Vlasova (2002) found that the addition of cadmium to hepatic nuclear extracts inhibited DNA-methyltransferase. More recently, Takiguchi et al. (2003) reported that subchronic exposure to cadmium inhibited DNA-methyltransferase activity in cultured cells, while chronic exposure enhanced the activity of the DNA-methyltransferase. They also found that the level of DNA methylation was similarly changed and suggested that the action of cadmium on DNA methylation may be responsible for its carcinogenic properties. Recently, Ho et al. (2006) showed that early developmental exposure to estradiol and bisphenol A increased prostate gland susceptibility to adult-onset carcinogenesis through alterations in the DNA methylation pattern of multiple cell signaling genes. These findings, along with the work from the Zawia laboratory on AD, provide scientific evidence that early life exposure promotes disease during the aging process.

To determine whether developmental exposure to Pb interferes with DNA methylation patterns, the Zawia laboratory is examining the activity of DNA-methyltransferase in the tissues of 23-year-old primates that were exposed to Pb as infants. The activity of this methylating enzyme is selective for cytosine nucleotides in a CG sequence, which is base-paired to a methylated CG sequence (in the opposite orientation) on the other strand of DNA and is directly proportional to the abundance of

methyl groups on CG dinucleotides on the DNA (Poirier and Vlasova, 2002; Takiguchi et al., 2003). The CG content of the APP promoter is estimated to be 72%, and the rate of CpG dinucleotides is five times that observed in other eukaryotic promoters, suggesting that its expression would be subject to regulation by DNA methylation (Salbaum et al., 1989; Pollwein et al., 1992; Lukiw et al., 1994; Hoffman and Chernak, 1995; Querfurth et al., 1999). The Sp1 consensus sequence, 5′GGG-CGGG (lower strand, 5′CCCGCCC), contains CG dinucleotides and is present in several places on the APP promoter. Few studies have examined methylcytosine levels on the APP promoter and the published work provides a varied picture depending on the region of the promoter that was examined. An analysis by Milici et al. (1990) of CpG elements in the APP promoter region between −460 and −275 did not detect methylation of cytosines in healthy human brain tissue. On the other hand, Rogaev et al. (1994) found those regions of the human and primate APP promoter upstream of −500 displayed tissue and brain region-specific profiles of methylation, that crudely reflect APP expression patterns. More recently, Tohgi et al. (1999) and Nagane et al. (2000) found at least 13 potential methylation sites in the region −236 to −101 of the human APP promoter. 26% of these cytosines were more frequently methylated in healthy individuals between the ages of 35 to 70 years as compared to 8% in those aged 74 to 90 years. This age-related reduction in methylcytosine was more prominent (10-fold reduction) in some locations (−207 to −182) of the APP promoter that belong to 9- and 11-bp-long GC-rich elements, which are typical Sp1 DNA-binding sites (Tohgi et al., 1999). Furthermore, these investigators suggested that age-related demethylation of cytosines may have some significance in the Aβ deposition in the aged brain (Tohgi et al., 1999). It is plausible that developmental exposure to Pb could exacerbate the demethylation process of the APP promoter in old age, thus elevating its expression. Studies have shown that Sp1 DNA-binding is inhibited by methylation of cytosine in proximity to CpG sites in the consensus sequence (Clark et al., 1997; Mancini et al., 1999). The expression of Sp1 target genes, such as the epithelial gene *T1α* and *MAO B*, has also been shown to be regulated by increased methylation of CpG sites on or around Sp1 DNA-binding sites (Cao et al., 2000; Wong et al., 2003; Zhu et al., 2003).

The hypothesis to explain how metals such as Pb can disturb gene expression by inhibiting the methylation of CG dinucleotides in the APP promoter, thus increasing the responsiveness of the APP gene, is presented in Fig. 10. A more responsive APP gene during old age could react favorably to neurodegenerative stimuli that elevate Sp1, increasing the supply of APP which can be further processed to generate an over-abundance of Aβ. This illustration can also apply to other metals that may interfere with DNA methylation patterns on target regions of a promoter.

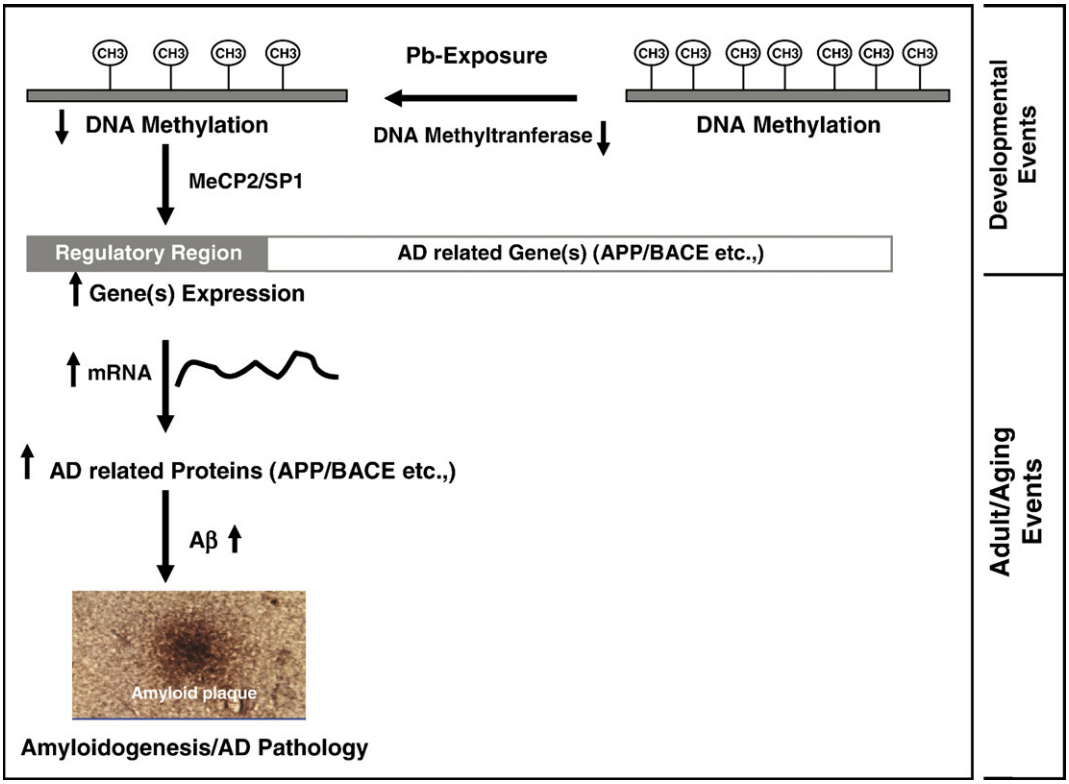

Fig. 10. Pb-induced disturbances to the methylation pattern of the APP gene and its relationship to amyloidogenesis. According to this model, the extent of DNA methylation during development sets the level of responsiveness of the amyloid precursor protein (APP) gene for life. Developmental exposure to Pb acts to inhibit DNA methylation patterns, thus setting the responsiveness of the APP promoter and the expression of the APP gene at a higher level. This higher transcriptional output produces more APP and results in an increase in beta-amyloid (Aβ) levels. Aβ accumulation and aggregation leads to the death of cells and the formation of more plaques in the brain.

Conclusions

These four new avenues of research further characterize the neurotoxic effects of Pb by identifying novel mechanisms of action. Of note in the work from the Cory-Slechta laboratory is the important point that Pb exposure does not occur in isolation, but in a mix of other pollutants and in a context of low SES, poor nutrition, and elevated stress levels. Inner-city minority children and adults are exposed to the co-occurring risk factors of Pb burden and heightened stress. Both these risk factors, via disparate mechanisms, act on the mesocorticolimbic system of the brain, which mediates higher-level cognitive function. The multi-hit hypothesis of Pb's action (Fig. 2) posits that concurrent insults to the brain overwhelm the brain's capacity to compensate for the effect of an individual hit. This laboratory has demonstrated that Pb has both permanent and dynamic effects on the HPA axis. Further, important gender differences exist in response to Pb, to stress, and to the combined insult, with females showing greater vulnerability as evidenced by changes in brain dopaminergic systems. The implications of this work include the possibility of Pb contributing to cardiovascular, neuroendocrine, and metabolic disorders and to Pb adversely affecting cognitive function through alterations in HPA axis function.

Just as Pb has been shown to impair learning, it has also been implicated in memory deficits. Work from the Gilbert laboratory used the rodent hippocampus to model alterations in physiology, neurochemistry, and structure induced by chronic developmental Pb exposure. By examining LTP, they found Pb-induced increases in threshold, decreases in magnitude, and shorter retention times of synaptic plasticity.

A dose–response analysis revealed a biphasic dose–response for glutamate release, LTP, and postsynaptic NMDA receptor alteration, suggesting the presence of more than one mechanism of Pb action. Other studies revealed that developmental Pb exposure decreases the maintenance of synaptic plasticity (i.e., reducing the durability of memory). Structural plasticity in the form of adult neurogenesis in the hippocampus is also impacted by Pb exposure. Fig. 6 shows that chronic exposure to Pb, along with other factors (organismal, environmental, and experiential) can modify neurogenesis in the adult brain and may point to a new approach for developmental neurotoxicological studies. This is in accord with the multi-hit hypothesis discussed above in terms of multiple risk factors acting simultaneously to have effects that may differ in intensity, timing, or character from effects produced by a single risk factor.

The following sections of this review examine the hypothesis that Pb affects protein expression, conformation, and accumulation in brain. The report from the Tiffany-Castiglioni laboratory builds upon the chaperone deficiency hypothesis, which is based on the observation that many neurodegenerative diseases exhibit intracellular accumulation of proteins in neurons and glia of the brain, due to failure of the UPR. They demonstrated that Pb binds strongly to the molecular chaperone GRP78 and provides a possible mechanistic link between heavy metal exposure and chaperone deficiency. They also showed that Pb induces GRP78 aggregation in culture and that Pb binding to GRP78 decreases IL-6 secretion. The implications of this work include the possibility that GRP78, and possibly other chaperones, have a pivotal role in protecting the cells from environmental insults. Additionally, as GRP78 is implicated in the pathogenesis of neurodegenerative diseases, it may be a target for environmentally induced dysfunction in neural cells. A role for Pb in various "conformational diseases" such as AD, PD, prion disease, and cataracts is suggested by this hypothesis.

Zawia and colleagues examined Pb-induced changes in protein expression, hypothesizing that Pb exposure in early life is associated the subsequent progression of amyloidogenesis in rodents. They based their research on the Fetal Basis of Adult Disease hypothesis, which states that many adult diseases have a fetal origin. Rodents exposed to Pb during the postnatal period showed a subsequent rise in APP mRNA expression in senescence, indicating that latent expression was a programmed event set in motion by exposure to Pb in early life. Furthermore, increased levels of oxidative DNA damage were also evident in rats following developmental Pb exposure. To explain this upregulation of APP, it is hypothesized that Pb exposure may modulate the DNA methylation patterns on the APP promoter as shown in Fig. 10, thereby upregulating the responsiveness of the APP gene. Furthermore, alterations in the methylation burden of cytosine residues on the DNA may also make the adjacent guanine residues susceptible to oxidative damage.

Taken together, this body of research extends earlier work identifying mechanisms of Pb neurotoxicity. Pb does not act alone in causing cognitive deficits, neurodegeneration, or other adverse health effects, but acts in concert with other hits or factors that may modify its effects. Importantly, Pb acts through a number of mechanisms to affect developmental processes, neurotransmission, and neurodegeneration.

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