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Toxic Effects of Lead in the Developing Nervous System: *In Oculo* Experimental Models

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Modern man is chronically exposed to lead in the biosphere at levels several orders of magnitude higher than the natural level that once existed. There is much concern about the possible adverse effects of this population-wide, low-level lead exposure, particularly on the developing organism, wherein the central nervous system may be one primary target. We have developed *in oculo* test systems, which permit temporal and spatial discrimination of possible effects of lead and other potential neurotoxic agents in the environment on the developing central nervous system as well as on different types of peripheral nerves in the adult. In one experimental protocol, defined areas of the fetal rat brain are grafted to the anterior chamber of the eye of adult rat recipients that are exposed to lead. Such grafts will become vascularized from the host iris and continue developing *in oculo*. Thus, grafted brain tissue and host brain will share circulation and therefore be exposed to similar amounts of lead. Studies of cerebellar grafts revealed that, although there was a normal gross cytological development in the presence of lead, there was a marked and permanent impairment of spontaneous discharge rates of the grafted Purkinje neurons as observed with electrophysiological techniques long after cessation of lead treatment. The host Purkinje neurons were not affected. A similar, although less dramatic, impairment of cerebellar function could be subsequently demonstrated in intact animals when newborn rats were given lead during the first 20 days of life and studied as adults. In other areas of the fetal central nervous system grafted to the eye (substantia nigra, cortex cerebri), lead caused disturbed growth. A screening technique for potentially harmful effects of heavy metals on autonomic and sensory nerve terminals in adult rats makes use of intraocular injections of agents to be tested. Morphological and histochemical changes of the innervation of the iris are then studied in whole mount preparations. Lead causes an adrenergic hyperinnervation of the iris. Again, similar but less pronounced hyperinnervations are seen *in situ* after perinatal lead exposure.

These studies demonstrate the usefulness of the intraocular grafts and the intraocular injection technique, and the necessity to use both structural and functional techniques in order to detect potential neurotoxic actions. The techniques have revealed hitherto unknown toxic actions of lead on cerebellar function.

Introduction

During the past decade, a considerable body of evidence has been collected that suggests a relationship between childhood lead exposure and various symptoms of minimal brain dysfunction. Thus, neonatal exposure to relatively low levels of environmental lead has been reported to cause hyperactivity, learning difficulties, and problems with motor coordination in both human and animal models (*1–7*). The relevance of these observations is accentuated by the prevalence of environmental lead in our cities together with the observation that a significant number of children from urban areas exhibit elevated blood lead levels (*8–12*). These data underscore the importance of understanding the conditions and mechanisms through which environmental lead can alter central nervous system (CNS) development.

Immature organisms are particularly susceptible to the adverse neurological effects of chronic lead exposure. As compared to adults, lead is absorbed and retained more readily perinatally (*13–17*), and the developing nervous system appears to be much more sensitive to the toxic effects of low-level lead exposure (*18–21*). This chapter reviews electrophysiological and histological investigations, which suggest that exposure to lead levels that are low enough to be without effects when administered to adult animals can cause persistent abnormalities in the brains of animals that are exposed perinatally. These studies will focus on the unique properties of homologous transplants of fetal brain regions into the anterior chamber of the eye of adult host rats as a method to delineate physiological and histochemical biomarkers for perinatal lead exposure.

In the following sections, we will present specific examples of how chronic perinatal lead exposure alters

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the physiological and histochemical parameters in the anterior chamber, and of how similar, albeit, more modest changes can be observed *in situ* after lead administration.

Results

Effects of Chronic Lead on Intraocular Transplants

One percent lead acetate in the drinking water was tolerated well by the recipient rats. Blood levels of 450 to 500 mg/L were elicited by this dose. There were no gross neurological disturbances. In a few experiments, 2% lead acetate was used. This lead concentration in the drinking water reduced the weight gain of recipient animals considerably. In general, lead treatment of the host had no adverse effects on the process of endothelial budding and vascularization of the transplants from the host iris. There were no petechial hemorrhages or delays of vascularization.

Substantia Nigra

Grafts of this area were tested in view of the possible involvement of dopamine neurons in hyperactivity states and the proposition that lead may cause hyperactivity in animals (*5,6,22–25*). Lead treatment (1%) caused a significant and pronounced delay of growth of the substantia nigra area during the second and third week after grafting, which corresponds approximately to the first 2 weeks of postnatal growth of this brain area *in situ*.

Cerebellum

This area was chosen because it has been reported to be particularly vulnerable to lead intoxication. It is in the cerebellum of developing animals that hemorrhages first occur after very high-level lead intoxication (*26*). Two stages of prenatal development of the cerebellar bud (14 and 16 days of gestation) were chosen for grafting. There were no effects of 1% lead on cerebellar transplant growth at either of the two stages. A histological investigation revealed a typical trilaminar histological organization of the cerebellar cortex in the grafts (Fig. 1).

When the spontaneous electrical activity of Purkinje cells in the grafts was monitored, the activity was found to be normal in host animals that received sodium acetate in their drinking water (Fig. 2). In marked contrast, spontaneous discharge was absent in almost all Purkinje cells in grafts of hosts receiving 1% lead acetate in the drinking water, even though the recordings were performed up to 5 months after cessation of lead treatment (Fig. 3). Spontaneous activity of Purkinje cells was also studied in host cerebellum. No effects of the lead treatment could be detected on firing rates of host Purkinje neurons. In the lead-treated cerebellar grafts, the silent Purkinge neurons could be excited by mechanical stimulation by the electrode tip or following perfusion of penicillin. Thus, the Purkinje neurons had the capacity to fire action potentials but were not spontaneously active.

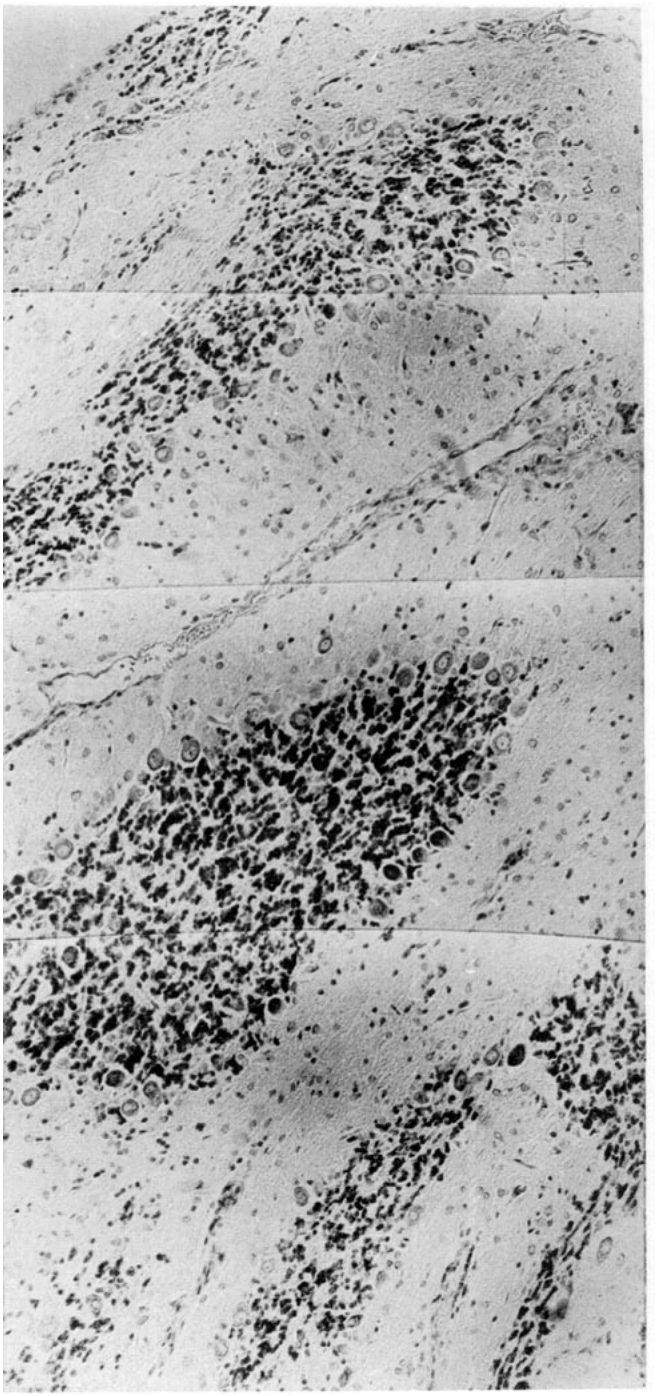

FIGURE 1. Cresyl-violet-stained section of a lead-treated cerebellar graft obtained from day 14 of gestation as seen after intraocular maturation. Note the high degree of organotypic organization. Two folia of cerebellar cortex can be seen with free surfaces covered by pial membrane.

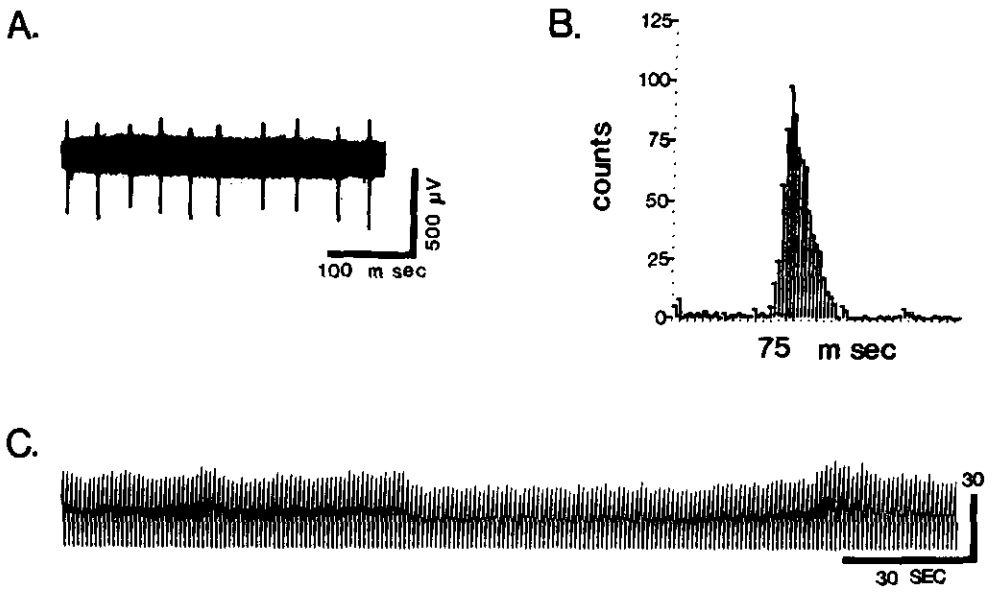

FIGURE 2. Spontaneous discharge from a graft Purkinje cell in a sodium acetate-treated animal. (*A*) Action potential record photographed from the oscilloscope. (*B*) Interspike interval histogram with prominent model peak indicating regularity of discharge. Abscissa calibration is for full scale. (*C*) Ratemeter record again showing sustained regular discharge.

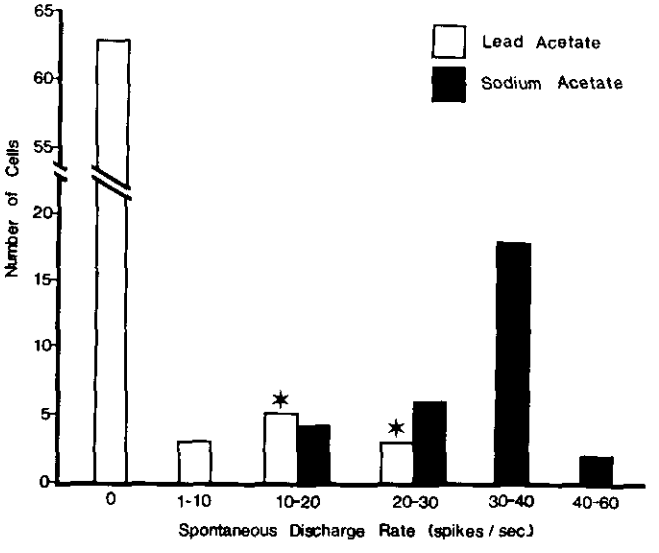

FIGURE 3. Histogram showing the distribution of spontaneous discharge rates of Purkinje neurons from lead acetate-treated and sodium acetate-treated cerebellar grafts. There is a marked difference between the two groups which is statistically significant ($p < 0.001$). Note that the majority of cells encountered in lead-treated grafts were silent. (*) denotes cells which were all derived from one graft, the only lead-treated graft containing Purkinje neurons with sustained, spontaneous discharge.

Hippocampus

This area has been specifically implicated in lead toxicity because of its suggested role in learning and the high concentration of zinc and exogenous heavy metals in this region (*27–29*). There was a slight but permanent impairment of growth of the hippocampal area in animals receiving 1% lead in their drinking water. The effect was seen at two different stages of development.

Cortex Cerebri

In this region, effects of lead were complex and dose-dependent. While 2% lead acetate caused a permanently decreased growth of parietal cerebral cortex, 1% lead caused a small but significant augmentation in the growth of this cortical area taken from donors with a CRL of 18 to 22 mm. This increase was, however, not seen at all developmental stages.

Effects of Postnatal Lead Exposure on Cerebellar Purkinje Neuron Discharge *In Situ*

In view of the remarkable hypoactivity of cerebellar Purkinje neuron discharge seen in the intraocular cerebellar grafts that matured in host animals receiving lead in their drinking water, experiments were designed to see if the results could be generalized to the developing brain in an intact organism. The mean spontaneous firing rate of cerebellar Purkinje neurons was found to be significantly lower in adult animals that received 8 mg PbAc/kg body weight during their first 20 days of life than in animals that received either 1 mg PbAc or 8 mg NaAc/kg body weight (Fig. 4). Moreover, the distribution of the firing rates of the Purkinje cells differed; there was a preferential loss of faster firing cells in the 8 mg PbAc/kg body weight group.

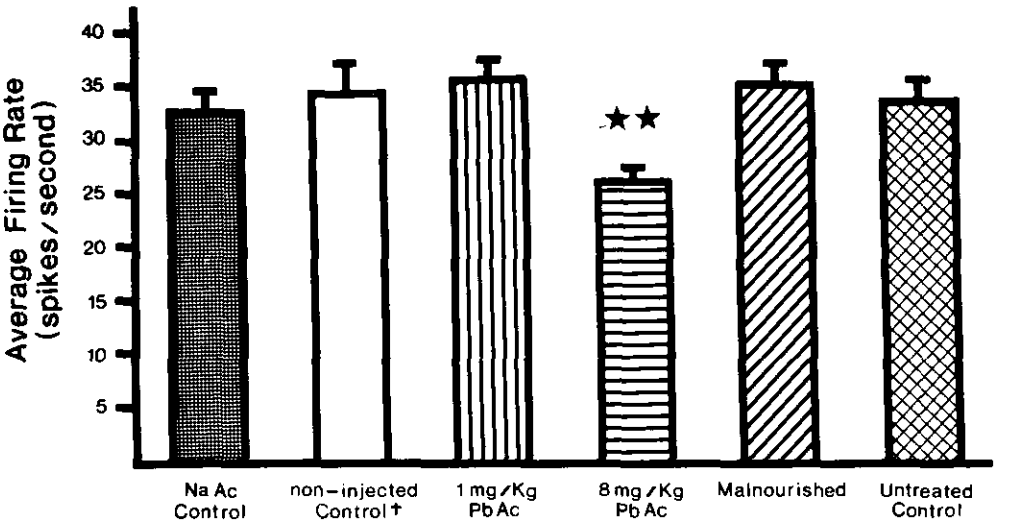

FIGURE 4. *Bar graph showing mean discharge rate ± SEM of cerebellar Purkinje neurons from animals injected postnatally with NaAc, 1 mg PbAc/kg, 8 mg PbAc/kg, and noninjected controls. In addition, the mean firing rates of Purkinje neurons from malnourished animals and from animals concomitantly raised in normal-sized litters are illustrated. The rates in 8 mg PbAc/kg animals are significantly different from those of NaAc animals ($p < 0.01$).*

Intraocular Injections of Potentially Neurotoxic Agents: Effects of Heavy Metals on Iris Adrenergic Nerves

Adrenergic nerve density of the iris was affected differentially by the various metal ions. Lead, tin, aluminum, and manganese caused a moderate adrenergic hyperinnervation (Fig. 5). Mercury and copper caused degeneration of adrenergic nerves, whereas cadmium, cobalt, iron, nickel, chromium, zinc, and thallium did not change the adrenergic nerve density. Lead-induced hyperinnervation was seen using 5 μL injections of 1.4 to 42 mM lead solutions. The increased fiber density was maintained for the entire period (8 weeks) of the study. The mercury-induced degeneration was dose-dependent and rapid. Neuropathological changes were seen 24 hr after injection . Slight degenerative changes were detected after injection of as little as 0.05 mM solutions. Following degeneration induced by 3.5 mM $HgCl_2$, the remaining adrenergic fibers proliferated enough in 2 weeks to cause the mean nerve density to recover approximately 80%.

In Situ Adrenergic Hyperinnervation after Chronic Perinatal Lead Exposure

In an effort to establish whether lead-induced adrenergic hyperinnervation could also be seen *in situ*, rat pups were exposed to lead or sodium acetate postnatally for 20 days. Cortical smears were subsequently taken from animals after maturation, and the density of noradrenergic terminals was compared in the two groups by fluorescence histochemical measurements. As shown in Figure 6, all three cortical regions sampled showed increased norepinephrine varicosities in the lead-treated animals.

Discussion

In this paper, we have reviewed different methods that are useful in detecting potentially neurotoxic actions of lead. Combining morphological, histochemical, and electrophysiological techniques, several new aspects of toxicity have been revealed. In particular, lead exposure during development of the cerebellum causes permanent depression of the spontaneous firing of cerebellar Purkinje neurons. In order to evaluate these and other results, a discussion of the various techniques involved is necessary.

Intraocular Grafting of Brain Tissue in Rodents

Virtually any area of the developing central nervous system will survive intraocular grafting and continue development in the anterior chamber of the eye, provided that an optimal stage of development has been found (*30*). Development *in oculo* is usually surprisingly organotypic in terms both of structural and functional organization of the grafted brain areas. It thus becomes possible to study development of defined areas of the central nervous system in complete isolation from the rest of the brain. One can differentiate between direct toxic effects on developing brain tissue and indirect effects caused by toxic effects in other areas of the central nervous system or elsewhere in the organism. This is particularly advantageous in studies of chronic low-level lead exposure, since host animals can be given lead, e.g., via the drinking water, in concentrations that are

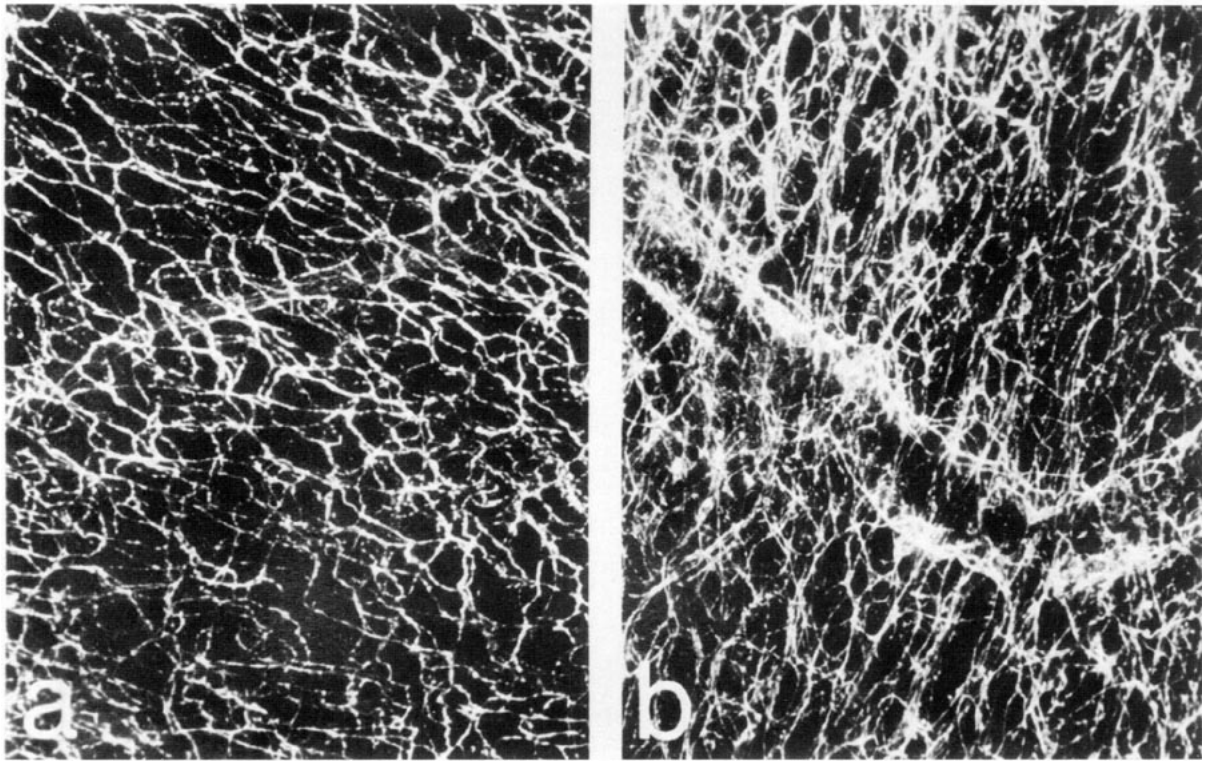

FIGURE 5. Effects of lead injected into the anterior chamber of the eye in a 5-μL volume on sympathetic adrenergic nerves in the rat iris. Illustrated are fluorescence microphotographs from representative areas of iris whole mounts processed for visualization of catecholamines according to Falck and Hillarp. (*A*) Sham-injected eye. Normal density of adrenergic ground plexus in dilator area of the iris. × 114. (*B*) Adrenergic hyperinnervation 14 days after injection of lead acetate. There is almost no decrease in fluorescence intensity (i.e., catecholamine content) of individual nerve fibers. An innervated blood vessel traverses from upper left to lower right. × 114.

well tolerated by the adult organism. Since the intraocular transplants will be effectively vascularized from the blood vessels on the anterior surface of the host iris, the developing fetal brain tissue and the adult host brain will share circulation and thus be exposed to similar blood lead levels, which permits comparative studies between graft and the corresponding area of the host brain. Repeated *in vivo* observations and measurements through the cornea of the host animals permit precise establishment of growth curves for different brain areas (*18*). Moreover, morphological and histochemical studies of the grafted tissues can be related to studies of electrophysiological activities within the grafts. In summary, we believe that intraocular grafting of fetal brain tissue is an efficient way of revealing regional and temporal neurotoxic effects in the central nervous system.

Postnatal Lead Exposure of Rat Pups

A common approach in studies of developmental neurotoxicity is to expose whole animals to the neurotoxic agents during early postnatal development. In the case of lead, this can be achieved either by administering the compound in the drinking water to the dam so that it will reach the pups via the milk, or pups can be treated directly with lead. We have used IP lead injections during the first 20 days of life. The experiments were carried out in order to determine if the marked effect of lead on electrical activity in cerebellar grafts would also be manifested in the intact organism. After the last lead injection, animals were allowed to mature. Electrophysiological recordings from cerebellum using standard techniques were performed when lead levels in cerebellum had returned to almost normal levels (*18*). While there are several problems in interpreting data from whole animal studies, the studies are necessary to validate findings from simpler test systems such as grafts or tissue culture experiments. In the present case, spontaneous activity of Purkinje neurons *in situ* was also reduced. The effect was considerably smaller in magnitude but statistically significant. Moreover, it seemed as if the fastest-firing Purkinje neurons were selectively affected, suggesting impairment of those nerve cells in the cerebellum, where the functional and energy demands are the highest. Some of the interpretational problems in whole animal studies can be overcome by using appropriate control groups. It is known, for example, that lead exposure causes retarded growth of rat pups. Therefore, malnourished controls, obtained by using oversized litters, should be included in order to differentiate between effects caused by malnutrition

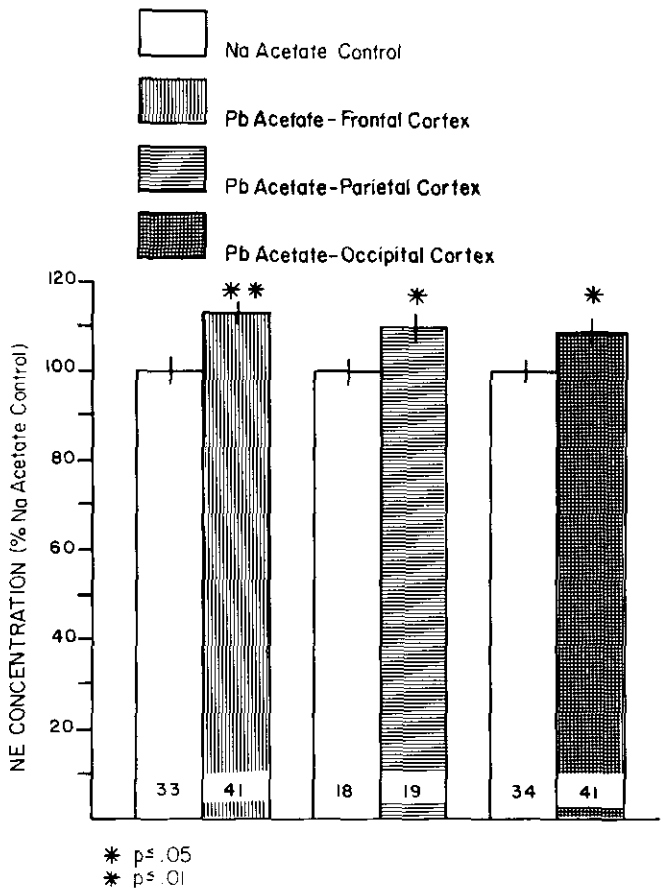

FIGURE 6. Cortical noradrenergic varicosities in animals treated neonatally with lead- or sodium acetate. Estimates were made by blind observers using Falck-Hillarp fluorescence histochemical techniques. Note elevation in lead-treated animals in all three cortical regions.

per se and more direct effects of lead on the central nervous system.

A Screening System for Detection of Neurotoxic Effects on Autonomic Nerves

The iris of the albino rat and mouse is an ideal site for studying autonomic nerve terminals. Stretch-prepared whole mounts of this tissue can be used to visualize entire two-dimensional networks of various thin and unmyelinated fiber types, as well as myelinated nerves, using appropriate histochemical and immunohistochemical staining methods. Moreover, catecholamine-containing CNS transplants *in oculo* will form two-dimensional networks that are far easier to study than the corresponding three-dimensional distributions in brain. We have applied Falck-Hillarp fluorescence histochemistry to this preparation to reveal possible neurotoxic actions of various compounds injected in microliter volumes into the anterior chamber of the eye. The technique is rapid and simple. Each animal provides one control and one experimental eye. Nerve densities can be quantitatively estimated by automatic image analysis.

Future Directions

We should now like to speculate on several future directions for studying the neurobiology of heavy metal toxicity. First, it must be recognized that a number of new techniques for cellular neurobiology have evolved to the point of permitting correlation of electrophysiological, anatomical, neurochemical, and behavioral abnormalities induced by heavy metal exposure. For example, computer-based image analysis, combined with cytochemical techniques (*31*) or receptor autoradiography (*32*), allows precise spatial definition of pre- and postsynaptic changes in identified transmitter systems. Furthermore, *in vivo* electrochemical techniques (*31*) can be used to measure monoamine transmitter release and reuptake dynamics in intact animals. Such measurements may be combined with electrophysiological protocols to monitor spontaneous and evoked synaptic release of the transmitter concomitantly with evoked changes in postsynaptic neuronal activity. With this type of combined approach, pre- vs. postsynaptic changes in transmitter function can be discerned *in vivo*. Applications of these techniques to neurotoxicological problems should yield unique multidisciplinary correlates of perinatal heavy metal exposure and thus a better understanding of the mechanisms of any toxic effects in man.

A second important future direction lies in comparison of the effects of exposure to environmental agents with the effects of well-defined selective neurotoxins. There are specific drugs that disrupt afferent fibers in the iris, such as noradrenergic afferents (DSP-4, 6-OHDA), cholinergic fibers (AF64A), or substance P pathways (capasaicin), to mention but a few. A precise analysis of how heavy metals interact with brain transplants, with and without the various neural inputs, should provide specific data on the mechanisms of neurotoxicity of these agents. Moreover, brain mechanisms that minimize deleterious effects of heavy metals could involve compensatory changes in various neurotransmitter inputs. Pharmacological or anatomical disruption of such inputs could then reveal hitherto unsuspected toxicities. These are but a few of the new approaches to neurotoxicological analysis that will challenge us during the next decade of investigation, approaches that can be initially applied to isolated single and multiple brain grafts *in oculo*, and subsequently extended to the intact animal.

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