Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 9915–9920, September 1996
Neurobiology

Low lead levels stunt neuronal growth in a reversible manner

(retinal axons/lead toxicity/visual system development)

HOLLIS T. CLINE*†, SONIA WITTE‡, AND KEITH W. JONES§

*Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724; ‡Department of Physiology and Biophysics, University of Iowa, Iowa City, IA 52245; and §Brookhaven National Laboratory, Upton, NY 11973

Communicated by James D. Watson, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, April 19, 1996 (received for review March 21, 1996)

ABSTRACT **The developing brain is particularly susceptible to lead toxicity; however, the cellular effects of lead on neuronal development are not well understood. The effect of exposure to nanomolar concentrations of lead on several parameters of the developing retinotectal system of frog tadpoles was tested. Lead severely reduced the area and branchtip number of retinal ganglion cell axon arborizations within the optic tectum at submicromolar concentrations. These effects of lead on neuronal growth are more dramatic and occur at lower exposure levels than previously reported. Lead exposure did not interfere with the development of retinotectal topography. The deficient neuronal growth does not appear to be secondary to impaired synaptic transmission, because concentrations of lead that stunted neuronal growth were lower than those required to block synaptic transmission. Subsequent treatment of lead-exposed animals with the chelating agent 2,3-dimercaptosuccinic acid completely reversed the effect of lead on neuronal growth. These studies indicate that impaired neuronal growth may be responsible in part for lead-induced cognitive deficits and that chelator treatment counteracts this effect.**

Children exposed to lead, even at concentrations that were once considered low, have learning disabilities and behavioral problems (1–3), including deficits in visual system function (4, 5). Two of the major questions regarding lead toxicity concern the limits of exposure that cause neurological damage in children and the reversibility of the damage following transient exposure to lead. The Centers for Disease Control (6) recently lowered the level of blood lead considered harmful to 10 μg per 100 ml of blood (0.48 μM); however, the issue of a threshold level for lead neurotoxicity remains controversial (7, 8).

Calcium disodium-EDTA, a commonly used chelation agent, reportedly causes a redistribution of lead to the brain (9). This does not occur following treatment with 2,3-dimercaptosuccinic acid (DMSA; ref. 10), an orally active lead chelating agent, which recently received Food and Drug Administration approval. Although DMSA lowers body lead burden (11–13), its ability to ameliorate behavioral deficits in lead-exposed children has not been demonstrated. DMSA is currently the subject of a double blind clinical study to test its ability to reverse the effect of lead on cognitive function.

We tested the effect of lead exposure in nanomolar concentrations on the following aspects of the development of the visual projection in frogs: neuronal growth, synaptic transmission, and the maintenance of topographic retinotectal projections. We also assessed the ability of the chelating agent DMSA to reverse the effect of lead exposure on neuronal growth and compared this to the effect of simply removing the lead source from the animal.

MATERIALS AND METHODS

Elvax Preparation and Surgical Implantation. Elvax was prepared and implanted over the optic tectum of *Rana pipiens* tadpoles as described (14). Elvax40P (DuPont) was prepared with stock solutions of 10^{-4} M–10^{-8} M lead chloride in 0.1 M NH_4Cl (pH 6.0; 10 μl of stock per ml of Elvax) and 10 μl of 0.1% fast green prepared with nanopure water. One piece of Elvax of ≈250 $\mu m^2 \times 30$ μm was surgically implanted under the dura mater over the pia on the dorsal surface of each tectal lobe of *R. pipiens* tadpoles (Taylor and Kollros stage V) under MS222 anesthesia. Each implanted piece of Elvax weighs ≈1 mg and contains about 0.1 μl of the stock $PbCl_2$ solution. For the Elvax prepared from 10^{-4} M stock, each milligram of Elvax contains 2.4 ng of lead. Each animal is implanted with two pieces of Elvax. Therefore, the maximal lead exposure per animal over the 6 weeks course of the experiment is ≈5 ng. Stage-matched control animals were implanted with Elvax prepared with vehicle only. For DMSA-Elvax, stock solutions were prepared in 0.1 M phosphate buffer (pH 7.4). Previous experiments have shown that this vehicle does not alter neuronal morphology (14).

Animals were maintained in Nalgene plastic tubs in 0.5% Instant Ocean (Aquarium Systems, Mentor, OH) and fed boiled romaine lettuce ad libidum. Tubs were washed twice a week with 70% EtOH and rinsed in double distilled water. Lead levels were undetectable in the water by atomic absorption spectroscopy (AAS; detection limit of 0.5 ppb, for these samples).

Lead Measurements. Synchrotron radiation-induced x-ray emission (SRIXE) was used to measure the lead in 1 μm-thick plastic sections of tadpole brains. SRIXE is particularly well-adapted to this type of measurement because of its low detection limits and multielement sensitivity (15–18). It can have a spatial resolution of <10 μm and detection limits as low as 1 ppm by weight for data accumulation periods of 300 sec. We were able to detect even lower levels by using longer analysis times and summing the spectra from different tissue sections of optic tectal tissue.

The work was done at the X26A x-ray microprobe beamline of the Brookhaven National Laboratory Synchrotron Light Source as described (17, 19). Briefly, the continuous x-ray energy spectrum from the synchrotron source is used. X-rays with energies below ≈4 keV are strongly attenuated by passage of the beam through a total of 508 μm of beryllium windows. The beam is defined by a tantalum collimator with an aperture size of 8 μm. The specimen is placed at a 45° angle to the incident beam. The beam position is measured using a microscope at 135° to the beam for normal viewing of the position of a fluorescent spot on a scintillator. This enables placement of the beam on any portion of the specimen to an accuracy of 1 μm. Fluorescent x-rays were detected using an energy-dispersive Si(Li) x-ray detector with an energy resolution of

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: DMSA, 2,3-dimercaptosuccinic acid; AAS, atomic absorption spectroscopy; SRIXE, synchrotron radiation-induced x-ray emission; HRP, horseradish peroxidase; RGC, retinal ganglion cell; NMDA, *N*-methyl-D-aspartate; VSCC, voltage-sensitive calcium channel.

†To whom reprint requests should be addressed at: Cold Spring Harbor Laboratory, 1 Bungtown Road, Cold Spring Harbor, NY 11724-0100. e-mail: cline@cshl.org.

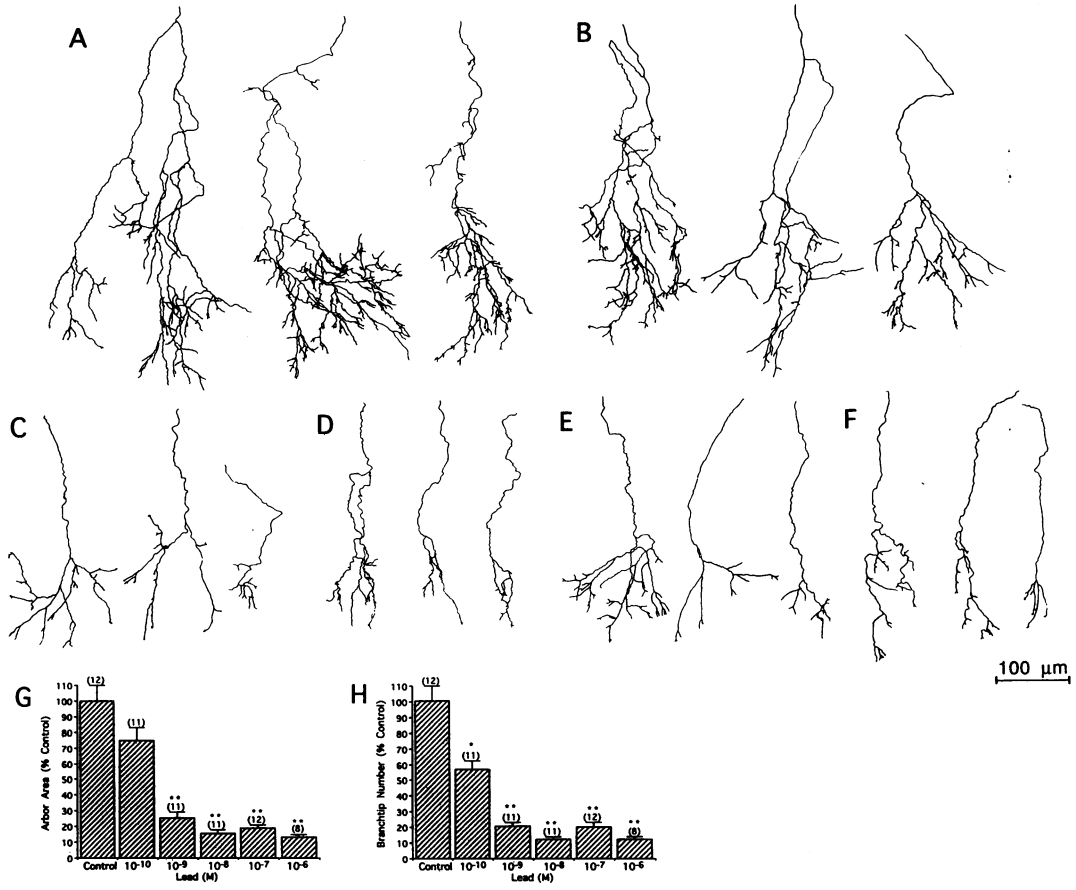

FIG. 1. Nanomolar concentrations of lead impair the growth of retinal axons. Examples of retinal axon arbors from controls (*A*) and tadpoles treated with estimated lead concentrations of 10^{-10} M (*B*), 10^{-9} M (*C*), 10^{-8} M (*D*), 10^{-7} M (*E*), and 10^{-6} M (*F*). For each group of axons, the arbor on the left is the largest of the group drawn, the one on the right is the smallest and the middle arbor is the one closest to the mean. Lead treatment reduces the area (*G*) and numbers of branchtips (*H*) in retinal axon arbors. Sample size, shown in parentheses above the bars in the graphs, refers to the numbers of axon arbors. For *G* and *H*: *, $P < 0.002$ and **, $P < 0.0005$, by a two-tailed *t* test.

≈160 eV. Detection limits for the apparatus is 10 fg for Pb, using L-x-ray detection and 10 fg for Zn using K-x-ray detection (18).

Plastic sections (1 μm thick) through the optic tectum were floated onto 7.6 μm-thick polyimide foils. Specimens were irradiated for ≈5500 sec. The resulting spectra were summed in the two groups and analyzed for lead and zinc content. The lead spectra were normalized to the zinc readings to control for inhomogeneities in sample thickness. The Pb/Zn ratio in the samples treated with lead was twice that in the control untreated sample. An estimate of the concentration of lead gave a value of ≈200 ppb (dry weight) for the lead-treated specimens and ≈100 ppb for the control specimens. This estimate is based on the comparison with a standard of known areal density, section thickness of 1 μm, and dry density of 1 g/cm^3 for the tissue. Uncertainties in the values for specimen thickness and density make this only a qualitative estimate; however, these uncertainties do not play a role when considering the Pb/Zn ratio. It is important to realize that the value for the control also serves as an upper limit on background lead irradiation from the shielded room in which the measurement was performed, as well as the lead content of the specimen. Consequently, these values are likely to be overestimates of the lead content of the tissue. The data collection and analysis were done blind to the treatment of the specimens.

Lead content in whole brains and eyes was determined by AAS at the end of the exposure period at the University of Iowa Hygienic Laboratory with a Perkin–Elmer atomic absorption spectrometer (model 4100ZL) equipped with a transverse heated graphite analyzer, a microsampling unit, and Zeeman background correction. Brains and eyes were dissected, frozen on dry ice, and sent to the University of Iowa Hygienic Laboratory for analysis. Two independent sets of samples, comprised of tissue from 8–10 animals, were pooled and analyzed for each lead concentration tested. Frozen tissue was digested in ultrapure nitric acid and read in duplicate using standard in 0.5% nitric acid. All samples had spiked recoveries ranging from 80% to 104%. Lead could only be detected in tissues exposed to the highest lead levels tested, 50 ng lead, made from stock solutions of 10^{-4} M $PbCl_2$ in Elvax. There was no detectable lead in the saline, nor in samples from Elvax prepared with NH_4Cl or 10^{-6} M $PbCl_2$.

Retinal Axon Arbor Morphology. Minutin pins tipped with horseradish peroxidase (HRP) were inserted at several sites in the retina under MS222 anesthesia as described (14). Two days later, animals were terminally anesthetized and their brains were processed for HRP histochemistry. The optic tecta were cut away from the brain, flattened between coverslips, and fixed. Each optic tectum was assigned a number, and the remainder of the data collection and analysis was performed blind to the treatment. Labeled class 3 retinal ganglion cell (RGC) axons, which are located 100–200 μm below the pial surface, were drawn with a camera lucida using a ×63 oil immersion lens. Branchtips were counted from the drawn arbors. Arbor areas were determined from the number of pixels covered by a scanned image of the arbor using the Universal Imaging (Media, PA) IMAGE 1 software. Once the blind was broken, it was clear that exposure to lead does not appear to interfere with HRP labeling of RGC axons. Comparable numbers of labeled axons and staining intensity were achieved in the control and experimental animals. Axons from three to seven animals per treatment group were analyzed.

Retinotectal Topography. Animals were implanted with lead-Elvax prepared with 10^{-3} M, 10^{-4} M, and 10^{-5} M $PbCl_2$ stock (two animals for each concentration). Two months later, the optic tectum was exposed and the position of the Elvax over the tectum was verified. HRP (20% in water) was injected focally into the rostromedial optic tectum using a General Valve (Fairfield, NJ) Picospritzer. After 5 days, the brains and retinae were processed for HRP histochemistry. The tecta and

retinae were flattened between coverslips and fixed. The area of the retina in which HRP-labeled RGCs were located was measured using Universal Imaging IMAGE 1 software and compared with stage-matched, sham-operated tadpoles. No differences were seen in the labeled areas for the three lead concentrations, so the data were pooled.

Electrophysiology. Animals between stages V and X were anesthetized in 0.05% MS222. Brains including ≈3-mm optic nerve were removed and placed in a recording chamber under constant perfusion with saline solution (100 mM NaCl/2 mM KCl/2.5 mM $MgCl_2$/1.5 mM $CaCl_2$/5 mM glucose, buffered to pH 7.4 with bicarbonate and CO_2). The dura overlying the optic tectal lobes was removed and the brain was split along the dorsal midline and secured with minutin pins. The optic nerve was stimulated through a glass suction electrode and excitatory postsynaptic currents were recorded from tectal neuronal cell bodies using patch pipettes of 7–10 MΩ resistance filled with 100 mM cesium gluconate, 0.2 mM EGTA, 5 mM $MgCl_2$, 4 mM Na-ATP, 0.3 mM GTP, 40 mM Hepes (pH 7.2) with CsOH. Concentrated solutions of lead chloride in 0.1 M NH_4Cl were added to the perfusion solution to give the final concentrations listed in the text and figures. The highest concentration of NH_4Cl in the perfusion solution was 1 mM. Addition of NH_4Cl to the perfusion solution did not significantly change the amplitude of synaptic responses.

RESULTS

Lead was delivered to the brains of *R. pipiens* tadpoles using implants of the slow release plastic polymer, Elvax, which releases low molecular weight compounds at a constant low rate over a period of several months (14, 20). Elvax has been used extensively to provide controlled and sustained release of a wide variety of bioactive molecules, including neurotransmitters, receptor and channel antagonists, enzymes, polypeptides, steroid hormones, and growth factors (20–25). It provides the advantage of direct exposure of the brain to lead or DMSA, thereby eliminating concerns of changes in uptake, clearance, or distribution during the experiment. Lead application by Elvax also minimizes the amount of toxic lead in the laboratory and environment.

Lead content in the optic tectum of animals exposed to 5 ng of lead for 6 weeks was measured using SRIXE. SRIXE is a quantitative method to analyze trace elements in tissue with spatial resolution <10 μm and detection limits as low as 1 ppm by weight (15–18). SRIXE is ideal for lead detection in small tissue samples. By repeatedly scanning sections of tadpole optic tectum (see *Materials and Methods*), we were able to detect even lower lead levels of 200 ppb (dry weight) lead in tadpoles exposed to 5 ng of lead in Elvax. We were unable to detect lead in tissue exposed to 5 ng of lead in Elvax using AAS due to the small mass of the sample and low lead levels.

To study the effect of lead exposure on the development of neuronal structure, we examined HRP-labeled retinal ganglion cell axon terminations (arbors) following 6 weeks of exposure of the optic tectum to lead-Elvax. Retinal axon arbors exposed to 5 ng–5 pg total lead in Elvax for 6 weeks were reduced in size to between 12% and 25% of the area of sham-operated control axon arbors. These axon arbors also had correspondingly fewer branchtips ($P < 0.0005$, two-tailed t test; Fig. 1). Exposure to 0.5 pg of lead in Elvax resulted in a significant decrease in branchtip number; however, arbor area was not significantly reduced compared with controls ($P < 0.002$). These data suggest a threshold for the effect of lead on these parameters of neuronal development. Exposures to increasing amounts of lead showed greater reductions in arbor area and branch number. The reduction in arbor area and branch number appears to level off at the higher lead concentrations tested, suggesting that the mechanism by which lead impairs neuronal growth becomes saturated. Despite the reduced arbor area and branch numbers, the majority of the branches are tipped by growth cones with wide lamellapodia and numerous filopodia (Fig. 1 *C–F*). Lamellar growth cones are more likely to be in a nonmigratory, exploratory mode, rather than rapidly extending (26). The increased prevalence of lamellar growth cones suggests the lead exposure may have altered the extracellular environment in the tectum (27, 28).

To estimate exposure levels of lead to the brain, we assumed that the lead is released into a volume equivalent to that of the brain. This is supported by two independent measures. First, we compared the total lead content of the brain and the eyes of animals in which Elvax was implanted over the brain, while the eyes had no contact with the Elvax. The lead content of the eyes (3.9 ± 0.38 μg per g) was comparable to the lead content of the brain (4.5 ± 0.46 μg per g) after 6 weeks of exposure of the brain alone to lead-Elvax containing 50 ng of lead. These data indicate that comparable amounts of lead are accumulated throughout the brain and eyes.

Second, we tested for a possible gradient of effective lead concentration originating from the Elvax implant by plotting the area of lead-treated arbors as a function of the distance of the center of the axon arbor from the Elvax implant in the tectum (Fig. 2). The Elvax was implanted over the dorsal optic tectum. Axons in the lateral tectum may be as far as 2 mm from

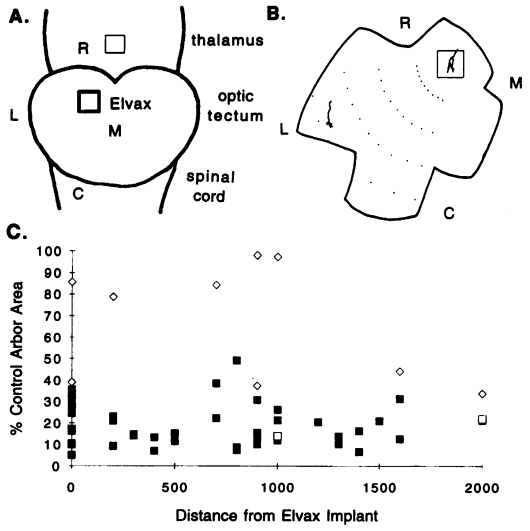

FIG. 2. Effective exposure levels of lead are uniform across the optic tectum. (*A*) Diagram of the dorsal midbrain region of the tadpole brain showing the caudal thalamus, the bilateral optic tectal lobes, and rostral spinal cord. The dark box in the left tectal lobe represents the position of one Elvax implant. The implant over the right tectum is not shown. The light box in the medial thalamus shows the position of an Elvax implant recovered from one animal. The data corresponding to the axons from this animal are shown in *C* in open squares. (*B*) Diagram of the left optic tectum that has been dissected from the brain and flattened. The dotted lines are shown at 500-μm intervals irradiating from the center of the Elvax implant, which is shown as the box. The two axons, the box, and the tectum are drawn to scale to show that axons immediately under the Elvax are not more severely effected by the lead than axons as far as 2 mm from the Elvax. R, rostral; M, medial; L, lateral; C, caudal. (*C*) No correlation exists between the size of the axon arbor and the distance of the axon from the Elvax implant. Data compiled from animals treated with lead-Elvax at 10^{-6} M–10^{-9} M are shown in filled squares. Data from animals treated with 10^{-10} M lead are shown in open diamonds. The open squares are data from two axons drawn from an animal in which the Elvax was recovered over the thalamus, at distances of 1 and 2 mm from the reconstructed axons. $R = -0.21$, $P > 0.55$ for all data pooled; $R = -0.05$, $P > 0.74$ for the lowest lead concentration tested. If a gradient in the effective lead concentration existed in the optic tectum, it would be most easily seen in these data, where the effect of lead on axon arbor area was the least dramatic of the concentrations tested. Nevertheless, axons that were the farthest from the implant are among the smallest axons in this group.

the lead source. The type 3 retinal axons analyzed here are ≈200 μm below the pial surface of the tectum. Therefore, axons may be as close as 200 μm and as far as 2000 μm from the Elvax implant. If lead were to diffuse out of the Elvax and be rapidly accumulated by neurons close to the implant (29), one might expect to see a greater effect of lead exposure on axons close to the Elvax compared with those farther away. Such an effect of distance on arbor size should be most easily seen with the lowest lead concentration. There is no correlation between the arbor area and the distance of the axon from the Elvax implant, even at the lowest concentration of lead-Elvax ($R = 0.05$, $P > 0.74$ by Spearman nonparametric test of association). Taken together, these data suggest that the effective lead levels that alter neuronal growth are comparable throughout the optic tectum. In addition, the data support the idea that lead is released into a volume equivalent to the brain cavity.

Assuming that all the lead in the Elvax is released into a closed volume equivalent to the brain volume of 25 μl (i.e., assume that no lead is cleared from the tissue during the experiment), then 5 ng of lead in Elvax would correspond to 1 μM extracellular exposure concentration. Based on this estimate, our data show that exposure to 1 nM lead significantly impairs neuronal growth. This value is likely to be an overestimate of the extracellular ionic lead concentration, because it does not take into consideration factors that are known to decrease the levels of toxic free lead. In particular, both the binding of lead to extracellular and intracellular proteins as well as the accumulation and precipitation of lead into intracellular organelles (29) reduces ionic lead concentrations several-fold (30).

The capacity of DMSA to reverse the effect of lead on retinal arbor growth was tested in the following experiment. Animals were treated for 6 weeks with 50 pg of lead (10^{-6} M lead chloride stock in Elvax). Some animals were processed immediately to assess the effect of lead on arbor morphology. The remaining animals were divided into four groups. In two groups, the lead-Elvax was removed and replaced with either DMSA-Elvax (10^{-5} or 10^{-4} M) or Elvax prepared with the phosphate buffer vehicle for 4 weeks. In the other group, the lead-Elvax was removed and the animals were left to survive with no further treatment for 4 weeks before retinal axon morphology was assayed to test the possibility that axon morphology could recover in the absence of lead or chelator. Axons from a final group of animals were treated only with DMSA for 4 weeks.

The results indicate that the effect of lead on arbor morphology can be reversed by subsequent treatment with DMSA for 4 weeks (Figs. 3 and 4). Axons that were exposed to lead followed by a 4-week period without DMSA treatment showed partial recovery of normal arbor morphology (Fig. 3*E*). Exposure to the phosphate buffer vehicle alone did not result in greater recovery of normal axon morphology than simple removal of the lead-Elvax. The axon arbors from animals that were exposed to lead and left for 4 weeks without further treatment are significantly smaller than controls ($P < 0.01$) and have fewer branchtips ($P < 0.0001$; Fig. 4). When compared with the lead-treated arbors from animals with no recovery period (Fig. 3*B*), they are significantly larger ($P < 0.0002$) and have more branchtips ($P < 0.001$). Treatment with chelator alone does not significantly alter arbor morphology (Fig. 3*D*), indicating that the chelator itself does not impair the cellular mechanisms controlling neuronal growth.

To test the effect of lead on synaptic transmission, whole cell patch clamp recordings of retinotectal synaptic currents were taken from an isolated brain preparation. Lead (10 μM–10 nM) was applied by perfusion while recording synaptic currents from normal tectal neurons held at −55 mV holding potential in response to electrical stimulation of the optic nerve. Lead (10 nM) blocked postsynaptic currents by 16.9 ± 3.4%, whereas 60% of the current was blocked by the addition of 10 μM lead in the perfusion solution (Fig. 5). The decrease in synaptic transmission at higher lead concentrations is most likely due to block of voltage-sensitive calcium channels (VSCCs) required for transmitter release, as has been observed in mammalian (31–33) and nonmammalian neurons (34). It is unlikely that the change in arbor morphology we observe is due to lead blocking VSCCs and synaptic transmission because tetrodotoxin, which indirectly blocks VSCCs and retinotectal synaptic transmission, results in enlarged retinal axon arbors (22).

The formation of accurate topographic sensory projections represents one aspect of the brain's capacity to develop organized projections between different regions of the brain. Based on extensive work on the development of topographic

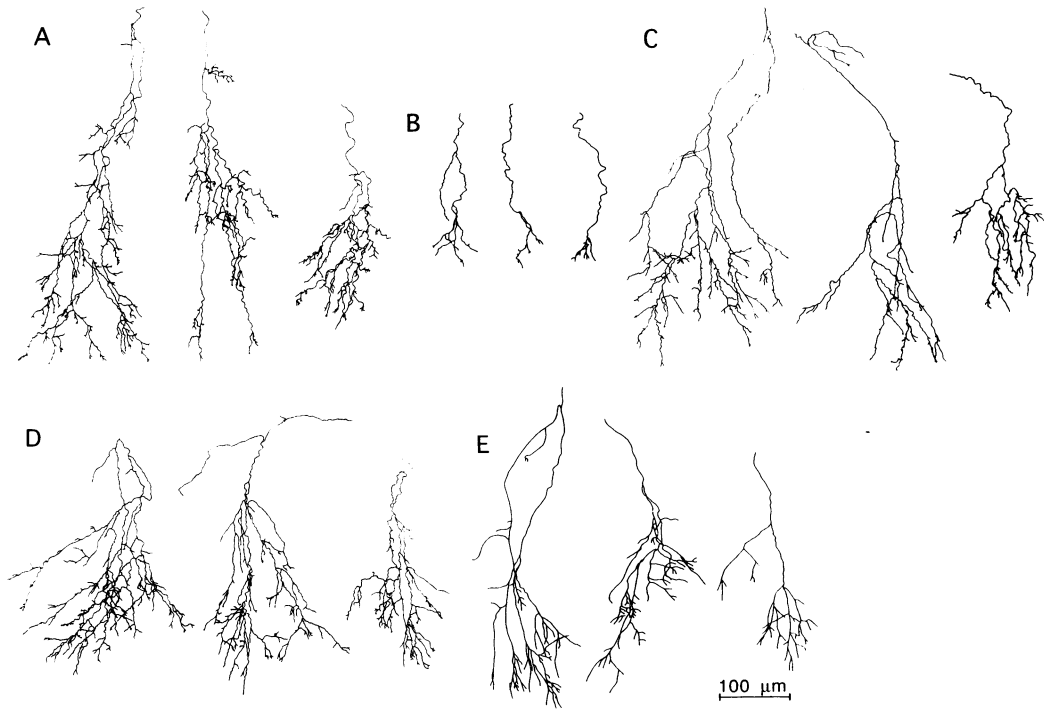

FIG. 3. DMSA treatment reverses the effect of lead exposure on arbor morphology. Examples of retinal axons after the following treatments: sham-operated controls (*A*), 10^{-8} M lead for 6 weeks (*B*), 10^{-8} M lead for 6 weeks followed by DMSA for 4 weeks (*C*), DMSA alone (*D*), and 10^{-8} M lead for 6 weeks followed by 4 weeks without lead or DMSA (*E*). For each treatment, the arbor on the left is the largest of the group drawn, the one on the right is the smallest, and the middle arbor is the one closest to the mean, except in *A* and *B*. For these cases, different examples were chosen than those in Fig. 1 to show a broader sample of the data.

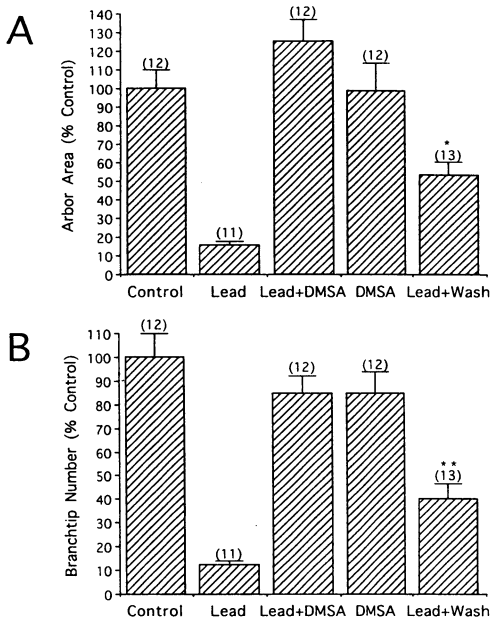

FIG. 4. Quantification of the effect of DMSA on arbors. DMSA reversed the effect of lead exposure on retinal arbor area (*A*) and numbers of branchtips (*B*). Sample size, shown in parentheses above the bars in the graphs, refers to the numbers of axon arbors. No difference was seen in the effect of treating with 10^{-4} M or 10^{-5} M DMSA, so the data from the two groups were pooled. (*A*) *, $P < 0.01$ compared with control and $P < 0.0002$ compared with the lead-treated arbors. (*B*) **, $P < 0.0005$ compared with controls and $P < 0.001$ compared with the lead-treated arbors by a two-tailed *t* test.

maps, many cellular mechanisms required for their development are known (35, 36). Development and maintenance of the topographic retinotectal projection in tadpoles requires retinotectal synaptic transmission, activation of postsynaptic *N*-methyl-D-aspartate (NMDA) receptors, and the migration of axon arbors within the tectal neuropil (35, 36). An assay of retinal topography in lead-treated animals therefore provides

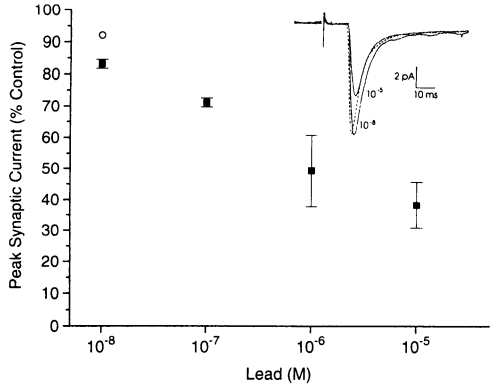

FIG. 5. Effect of increasing lead concentrations on retinotectal synaptic transmission. Whole cell recordings from tectal neurons were taken at a holding potential of −55 mV during electrical stimulation of the optic nerve. Peak amplitudes of evoked excitatory postsynaptic currents for each neuron were determined from the average of ≈500 sweeps for each concentration of lead. The means and standard error of averaged peak responses are shown. The open circle shows the peak synaptic current with 1 mM NH_4Cl in the saline recorded from two neurons. For 10^{-8} M, $n = 7$; for 10^{-7} M, $n = 6$; for 10^{-6} M, $n = 5$; and for 10^{-5} M, $n = 4$. (*Inset*) Examples of synaptic currents recorded under control conditions and in the presence of increasing concentrations of lead. The dashed line is control; the solid line is in the presence of the designated molar concentration of lead chloride. Averages of 500 sweeps.

an *in vivo* test of the integrity of these aspects of neural function. If lead blocks any one of them, then it would be detected as a disruption of retinal topography. Micromolar concentrations of lead block retinotectal synaptic transmission (Fig. 5). In other systems, micromolar concentrations of lead block the NMDA-type of glutamate receptor (37) and VSCCs (31, 33, 34) and thereby decrease or block synaptic transmission (32). To test whether the lead exposures that impair neuronal growth *in vivo* are sufficiently high to block synaptic transmission or NMDA receptor activity, we assayed the effect of lead exposure on the maintenance of the retinotectal topographic projection. If the lead exposures that alter retinal arbor morphology result in micromolar concentrations of lead *in vivo*, then this should block synaptic transmission and NMDA receptors and disrupt retinal topography. If the lead exposures that alter retinal arbor morphology have no effect on retinal topography, then this is consistent with the interpretation that submicromolar concentrations of lead *in vivo* impair neuronal growth.

No difference in retinotectal topography was seen between the controls and lead-treated tadpoles. The retinal area covered by the labeled RGCs was 4.25 ± 1.05% of the total retinal area in the control animals and 5.42 ± 0.91% in the lead-treated animals ($n = 6$ for each). These values are not significantly different. For comparison, blocking tectal cell NMDA receptors increases the retinal area covered by labeled RGCs to 20% of the total retinal area (14). These data indicate that the *in vivo* lead treatments used in these studies do not block NMDA receptors and do not grossly disrupt the patterns of retinotectal synaptic transmission which are required for the maintenance of retinal topography. This is consistent with the estimated concentration of lead in the tectum being lower than that required to block NMDA receptors (37). The data further suggest that these lead exposures do not impair the ability of the brain to develop topographic projections per se, even though the axonal projections that develop are deficient. Indeed, as has been shown in previous studies (38) treatments that decrease the size of the arbor do not necessarily disrupt retinal topography.

DISCUSSION

The present data indicate that lead can cause gross morphological defects at submicromolar concentrations. The dramatic stunting of arbor morphology seen after 6 weeks is likely the cumulative result of a gradual decrease in arbor area and the number of branchtips. *In vivo* time-lapse imaging of growing RGC arbors has shown that branches are constantly added and retracted from the arbor (39). Only a fraction of the newly added branches are maintained and contribute to the net growth of the arbor. In principle, lead could reduce arbor area and branch number by decreasing the rate of branch additions or by increasing the rate of branch retractions (36). Based on the observation that retinal topography is not disrupted by lead exposure, lead does not appear to interfere with branch additions or arbor migration. We suggest that lead is more likely to increase the rate of branch retractions.

The effect of lead on neuronal morphology that we and others (40–45) observe may be due to impaired assembly or stability of the cytoskeleton (46). Our interpretation is consistent with the ability of lead to interfere with calcium-dependent events (47) including calcium-sensitive protein kinases (48–50), which have been implicated in neuronal growth in a variety of neurons (51–54), including frog retinal axons (38, 55).

The dramatic loss in axon arbor area and branchtip number necessarily means a reduction in the number and spatial distribution of the postsynaptic partners that each retinal ganglion cell axon contacts. Such a change in the normal degree of convergence and divergence of visual information in the optic tectum is consistent with the idea that the reported

impairment in visual processing following lead exposure (56, 57) may be at least partially due to defects in neuronal growth.

DMSA is required to accomplish full recovery of normal axon morphology following lead exposure, whereas simply removing the lead-Elvax results in partial recovery. This indicates that the mechanisms concerned with neuronal growth are affected even after the source of lead is removed from the animal. One possible reason is that lead is harbored in the optic tectum. The optic tectum of frogs stains intensely for Timms reaction, which classically reveals anatomical regions of high zinc content; however, regions rich in endogenous zinc also accumulate environmental lead (58). The data suggest that DMSA reduces lead content and permits recovery of neuronal morphology and are consistent with the idea that DMSA may decrease intracellular lead levels.

In summary, exposure to nanomolar lead stunts the growth of retinal ganglion cell axonal projections within the central nervous system, but it has no apparent effect on the topographic organization of the retinotectal projection. The integration of visual information is likely to be abnormal due to the reduction in the numbers of retinal afferents that contact each tectal neuron. This in turn may result in more generalized deficits in neural function. The magnitude of the effect of lead on neuronal morphology reported here is greater than previously reported in other systems and occurs at lower lead concentrations than previously tested. Previous studies have shown that amphibians and mammals employ similar cellular mechanisms during nervous system development (35). Therefore, impaired neuronal growth may contribute to lead-induced cognitive deficits in humans. Finally, treatment with DMSA is highly effective at reversing the effect of lead exposure on neuronal growth.

We thank Terry Bowman and Patricia Rose at the University of Iowa Hygienic Laboratory for performing atomic absorption spectroscopy and Patterson Nuessle for his assistance in performing the x-ray fluorescence measurements. This work was supported by the McKnight Foundation, the National Down Syndrome Society, the Patterson Fund (H.T.C.), and the U.S. Department of Energy under Contract DE-AC02-76CH00016 (K.W.J.).

1. U.S. Department of Health and Human Services (1990) *Toxicological Profile for Lead* (Centers for Disease Control, Atlanta, GA).
2. Fulton, M., Raab, G., Thomson, G., Laxen, D., Hunter, R. & Hepburn, W. (1987) *Lancet* **i,** 1221–1226.
3. Needleman, H. L., Schell, A., Bellinger, D., Leviton, A. & Allred, E. N. (1990) *N. Engl. J. Med.* **322,** 83–88.
4. Otto, D., Baumann, S. B., Robinson, G. S., Schroeder, S. R., Kleinbaum, D. G., Barton, C. N. & Mushak, D. (1985) *Toxicologist* **5,** 81.
5. Shannon, M. W. & Greaf, J. W. (1992) *Pediatrics* **89,** 87–90.
6. U.S. Department of Health and Human Services (1991) *Preventing Lead Poisoning in Young Children* (Centers for Disease Control, Atlanta).
7. Silbergeld, E. K. (1992) *FASEB J.* **6,** 3201–3206.
8. Bellinger, D., Leviton, A., Waternaux, C., Needleman, H. & Ribinowitz, M. (1987) *N. Engl. J. Med.* **316,** 1037–1043.
9. Chisolm, J. J. (1987) *Am. J. Disabled Child.* **141,** 1256–1257.
10. Aposhian, H. V. & Aposhian, M. M. (1990) *Annu. Rev. Pharmacol. Toxicol.* **30,** 279–306.
11. Smith, D. R. & Flegal, A. R. (1992) *Toxicol. Appl. Pharmacol.* **116,** 85–92.
12. Graziano, J. H., LoIacono, N. J. & Meyer, P. (1988) *J. Pediatr.* **113,** 751–757.
13. Graziano, J. H., LoIacono, N., J., Moulton, T., Mitchell, M. E., Slavkovich, V. & Concepcion, Z. (1992) *J. Pediatr.* **120,** 133–139.
14. Cline, H. T. & Constantine-Paton, M. (1989) *Neuron* **3,** 413–426.
15. Cholewa, A. L., Hanson, K., Jones, K. W., McNally, W. P. & Fand, I. (1986) *Neurotoxicology* **7,** 9–18.
16. Jones, K. W., Bockman, R. S. & Bronner, F. (1992) *Neurotoxicology* **13,** 835–842.
17. Jones, K. W. & Gordon, B. M. (1993) in *Microscopic and Spectroscopic Imaging of the Chemical State*, ed. Morris, M. D. (Dekker, New York), Vol. 16, pp. 303–344.
18. Jones, K. W., Berry, W. J., Borsay, D. J., Cline, H. T., Connor, W. C. J. & Fullmer, C. S. (1996) *J. X-Ray Spectrometr.*, in press.
19. Jones, K. W. (1993) in *Handbook of X-Ray Spectroscopy*, eds. Greiken, R. E. & Markowicz, A. A. (Dekker, New York), pp. 411–452.
20. Langer, R. & Folkman, J. (1976) *Nature (London)* **263,** 797–799.
21. Langer, R. (1990) *Science* **249,** 1473–1624.
22. Reh, T. & Constantine-Paton, M. (1985) *J. Neurosci.* **5,** 1132–1143.
23. Schlaggar, B. L. & O'Leary, D. D. (1993) *Perspect. Dev. Neurobiol.* **1,** 81–91.
24. Simon, D. K., Prusky, G. T., O'Leary, D. D. M. & Constantine-Paton, M. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 10593–10597.
25. Rabacchi, S., Bailly, Y., Delhaye-Bouchard, N. & Mariani, J. (1992) *Science* **256,** 1823–1825.
26. Godement, P., Wang, L.-C. & Mason, C. A. (1994) *J. Neurosci.* **14,** 7024–7039.
27. Regan, C. M. & Keegan, K. (1990) *Dev. Pharmacol. Ther.* **15,** 189–195.
28. Lagunowich, L. A., Stein, A. P. & Reuhl, K. R. (1994) *Neurotoxicology* **15,** 123–132.
29. Pamphlett, R. & Bayliss, A. (1992) *Acta Neuropathol.* **84,** 89–93.
30. Simons, T. J. B. (1985) *J. Membr. Biol.* **84,** 61–71.
31. Evans, M. L., Busselberg, D. & Carpenter, D. O. (1991) *Neurosci. Lett.* **129,** 103–106.
32. Wang, Y. & Quastel, D. M. J. (1991) *Eur. J. Physiol.* **419,** 274–280.
33. Reuveny, E. & Narahshi, T. (1991) *Brain Res.* **545,** 312–314.
34. Busselberg, D., Evans, M., L., Rahmann, H. & Carpenter, D., O. (1991) *J. Neurophys.* **65,** 786–795.
35. Constantine-Paton, M., Cline, H. T. & Debski, E. A. (1990) *Annu. Rev. Neurosci.* **13,** 129–154.
36. Cline, H. T. (1991) *Trends Neurosci.* **14,** 104–111.
37. Alkondon, M., Costa, A. C. S., Radhakrishnan, R. S., Aronstam, R. S. & Albuquerque, E. X. (1990) *FEBS Lett.* **261,** 124–130.
38. Cline, H. T. & Constantine-Paton, M. (1990) *Neuron* **4,** 899–908.
39. O'Rourke, N. A., Cline, H. T. & Fraser, S. E. (1994) *Neuron* **12,** 921–934.
40. Legare, M. E., Castiglioni, A. J., Jr., Rowles, T. K., Calvin, J. A., Snyder-Armstead, C. & Tiffany-Castiglioni, E. (1993) *Neurotoxicology* **14,** 77–80.
41. Reuhl, K. R., Rice, D. C., Gilbert, S. G. & Mellett, J. (1989) *Toxicol. Appl. Pharmacol.* **99,** 501–509.
42. Kiraly, E. & Jones, D. G. (1982) *Exp. Neurol.* **77,** 236–239.
43. Alfano, D. P. & Petit, T. L. (1982) *Exp. Neurol.* **75,** 275–288.
44. McConnell, P. & Berry, M. (1979) *Neuropathol. Appl. Neurobiol.* **5,** 115–132.
45. Lorton, D. & Anderson, W. J. (1986) *Neurobehav. Toxicol. Teratol.* **8,** 45–50.
46. Niklowitz, W. J. (1974) *Environ. Res.* **8,** 17–36.
47. Pounds, J. G. (1984) *Neurotoxicology* **5,** 295–332.
48. Markovac, J. & Goldstein, G. W. (1988) *Nature (London)* **334,** 71–73.
49. Murakami, K., Feng, G. & Chen, S. G. (1993) *J. Pharmacol. Exp. Ther.* **264,** 757–761.
50. Goldstein, G. W. (1993) *Neurotoxicology* **14,** 97–101.
51. Bixby, J. L. (1989) *Neuron* **3,** 287–297.
52. Cabell, L. & Audesirk, G. (1993) *Int. J. Dev. Neurosci.* **11,** 357–368.
53. Campenot, R. B., Walji, A. H. & Draker, D. D. (1991) *J. Neurosci.* **11,** 1126–1139.
54. Goshima, Y., Ohsako, S. & Yamauchi, T. (1993) *J. Neurosci.* **13,** 559–567.
55. Zou, D.-J. & Cline, H. T. (1996) *Neuron* **16,** 529–539.
56. Fox, D. & Katz, L. (1992) *Vision Res.* **23,** 249–255.
57. Bushnell, P. J., Bowman, R. E., Allen, J. R. & Marlar, R. J. (1977) *Science* **196,** 333–335.
58. Fjerdingstad, E. J., Danscher, G. & Fjerdingstad, E. (1974) *Brain Res.* **80,** 350–354.