

Long-Term, Low-Dose Lead Exposure Alters the Gonadotropin-Releasing Hormone System in the Male Rat

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Lead is a male reproductive toxicant. Data suggest that rats dosed with relatively high levels of lead acetate for short periods of time induced changes in the hypothalamic gonadotropin-releasing hormone (GnRH) at the molecular level, but these changes were attenuated with increased concentration of exposure. The current study evaluated whether exposure to low levels of lead acetate over longer periods of time would produce a similar pattern of adaptation to toxicity at the molecular and biologic levels. Adult 100-day-old Sprague-Dawley male rats were dosed with 0, 0.025, 0.05, 0.1, and 0.3% lead acetate in water. Animals were killed after 1, 4, 8, and 16 weeks of treatment. Luteinizing hormone (LH) and GnRH levels were measured in serum, and lead levels were quantified in whole blood. Hypothalamic GnRH mRNA levels were also quantified. We found no significant differences in serum LH and GnRH among the groups of animals treated within each time period. A significant dose-related increase of GnRH mRNA concentrations with lead dosing occurred in animals treated for 1 week. Animals treated for more than 1 week also exhibited a significant increase in GnRH mRNA, but with an attenuation of the increase at the higher concentrations of lead with increased duration of exposure. We conclude that the signals within and between the hypothalamus and pituitary gland appear to be disrupted by long-term, low-dose lead exposure. **Key words:** gonadotropin-releasing hormone, hypothalamic-pituitary axis, lead acetate. *Environ Health Perspect* 110:871–874 (2002). [Online 19 July 2002] <http://ehpnet1.niehs.nih.gov/docs/2002/110p871-874sokol/abstract.html>

Human exposure to lead continues to be a serious public health problem (1). Although blood lead levels continue to decline in the U.S. population, specific population groups are disproportionately at high risk for lead exposure. In a recent survey conducted by the National Center for Environmental Health and the Centers for Disease Control and Prevention (CDC), 4.4% of the children surveyed had blood lead levels of at least 10 µg/dL (1). These results emphasized the importance of leaded paint in older homes as a continuing exposure source (1). Other exposure sources include lead in the workplace, in dust and soil, in folk remedies, in crystal or ceramic containers, and in hobby-related materials (1,2).

Lead exposure is associated with neurologic (3–6), growth (3,7), and reproductive defects (7–25). Lead exposure in men has been associated with abnormalities of spermatogenesis (9,11–13). An inverse relationship between blood lead and sperm concentration has been reported (14,15). Animal studies tend to support the conclusion that lead exposure disrupts the reproductive hormones, but clinical studies evaluating the relationship between lead exposure and reproductive hormone changes are inconclusive (8,11).

The reproductive axis is composed anatomically of the hypothalamus, pituitary, and testes in males and physiologically by gonadotropin-releasing hormone (GnRH), luteinizing hormone (LH), follicle-stimulating

hormone (FSH), testosterone, and inhibin (18). GnRH, a peptide hormone, is produced in the medial basal portion of the hypothalamus. GnRH stimulates the pituitary gland to produce and secrete LH and FSH. LH is primarily responsible for stimulating testicular Leydig cell secretion of testosterone. FSH acts on the Sertoli cells of the testes to stimulate spermatogenesis and inhibin.

In the animal model, lead has a primary toxic effect on the hypothalamic-pituitary unit, a primary effect on the testes, and acts at all levels of the reproductive axis. We and others have demonstrated, using the rat model, that lead exposure produces a dose-response suppression of serum testosterone and spermatogenesis accompanied by no significant changes in circulating gonadotropin levels (16,17). However, mRNA levels of GnRH in the hypothalamus and LH in the pituitary are increased with lead exposure (17–20). This reported endocrine disruption was noted at blood lead levels > 30 µg/dL after exposure times of 2–8 weeks. Limited data are available on the toxic effects of lead at lower exposure concentrations and for longer periods of exposure (21). Increasing the length of exposure to 8 weeks seems to allow the adult animal to normalize its responses (22). Other data suggest that the lead-exposed animal is able to adapt to the metal's toxic effects (20,22–25).

We are investigating the molecular mechanism(s) by which lead disrupts the reproductive

axis. We reported previously that exposure to lead acetate in relatively high dose ranges for 3 weeks increased mRNA levels of GnRH and LH as well as stored levels of LH (19). Consistent with the conclusion that the reproductive axis adapts to the toxic effects of lead was the finding that the increase in GnRH, mRNA, and β-LH concentration plateaued once the animal's blood lead levels reached 50 µg/dL.

The current study was designed to determine whether exposure to lower levels of lead acetate over a longer time would produce a similar pattern of adaptation to toxicity at the molecular level in the male rat, and whether changes in GnRH mRNA are mirrored by changes in circulating levels of GnRH.

Materials and Methods

Adult male Sprague-Dawley rats, 92 days of age upon arrival at the laboratory, were purchased from Charles River Laboratories (Portage, MI) and housed for 8 days before being randomly distributed into study groups of 12–16 rats. The rats were housed in polycarbonate cages suspended on stainless-steel racks in a room with a 12 hr:12 hr light:dark cycle and controlled temperature. Animals were allowed free access to lead-free Purina (Ralston-Purina, St. Louis, MO) laboratory chow and were maintained in accordance with the standards set forth in the Animal Welfare Act. Acid-washed amber-glass water bottles, equipped with neoprene stoppers containing stainless dipper tubes with ball bearings, were used to dispense deionized water containing either no lead acetate (PbAc) or 0.025, 0.05, 0.1, 0.3% PbAc (Fisher Scientific, Springfield, NJ). We added

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We thank the staff of the Air and Industrial Hygiene Laboratory of the California Department of the Health Services for expert blood lead analysis and G. Wolf and J. Delaney for scientific advice and technical assistance.

This research was supported by grant R01ES037649 awarded to R. Z. Sokol and by grant P30 ES07048 awarded to the University of Southern California by the National Institute of Environmental Health Sciences.

Received 22 June 2001; accepted 15 February 2002.

1 mL 5 N HCl to all water bottles (including controls) to preclude precipitation of insoluble PbAc salts. We selected the doses of lead on the basis of previously published studies (19,22). Animals were weighed, and the volume of water consumption was measured at the beginning of study weeks 1, 4, 8, and 16.

The animals were killed by decapitation after 1, 4, 8, or 16 weeks of treatment. Hypothalami were dissected and snap frozen in liquid nitrogen and stored at -70°C until processing for RNA and DNA studies. Trunk blood was collected and lead levels measured. Serum was separated and frozen for the measurement of GnRH and LH levels. Care and treatment of animals was conducted in accordance with established guidelines and approval of the University of Southern California Animal Use Committee and internal review board.

Measurement of blood lead and hormones. Lead analysis was performed by the Air and Industrial Hygiene Laboratory of the California Department of Health Services as described previously (26).

We used the ultra-sensitive DELFIA rat LH assay (Wallac Oy, Turku, Finland) to measure LH in rodent serum (27). Sensitivity was 0.03 ng/mL, and the intra- and interassay coefficients of variations at 33.1 ng/mL were 3.4% and 16.0%, respectively.

We determined the GnRH concentrations in plasma by radioimmunoassay using reagents (^{125}I -labeled GnRH and GnRH standards and antiserum) from Phoenix Pharmaceuticals, Inc. (Mountain View, CA). The kit standard was compared to a standard curve generated using GnRH peptide supplied by the National Institute of Diabetes and Digestive and Kidney Diseases (National Institutes of Health, Bethesda, MD). Plasma was extracted using acetic acid and Bond Elut LRC-C18 columns (Varian, Harbor City, CA). Intra- and interassay variations were 10% and 18%, respectively. Sensitivity of the assay was 0.1 pg/mL.

Preparation of RNA and DNA. Total RNAs were extracted from frozen hypothalami ($n = 3$ per treatment group) by homogenization

in guanidium isothiocyanate according to established protocols (28). The concentrations of purified RNA and DNA product were determined by optical density at wavelength 260 nm (Beckman DU 640, Columbia, MO).

Northern blot performance. Isolated total RNA (30 μg per lane) was electrophoresed separately using formaldehyde 1.2% agarose gels in MOPS buffer and transferred via capillary action onto Nylon membranes (MSI, Westboro, MA).

An *EcoRI/Hind III* fragment (0.396 kb) of the rat GnRH cDNA insert and an *EcoRI/Hind III* fragment (0.7 kb) of the rat β -actin cDNA insert were excised and purified through a 1% Sea Plaque low melting agarose (FMC BioProducts, Rockland, ME). Both cDNA fragments as probes were labeled with [α - ^{32}P]-dCTP (NEN Life Science Products, Inc., Boston, MA) using a random primer labeling kit (Life Technologies, Bethesda, MD). (The cDNA probe was supplied by J. L. Robertson, Mount Sinai School of Medicine, New York.)

Hybridization of blots with a ^{32}P -GnRH probe was performed in a NaPO_4 buffer with 7% SDS, 1 mM EDTA, and 1 mg/mL bovine serum albumin at 68°C for 18–20 hr. Blots were washed in 1% SDS, 50 mM NaCl, 1 mM EDTA, and exposed to X-ray film at -70°C with intensifying screen for about 4–5 days. We confirmed equivalent RNA loading by rehybridization of the blots for the β -actin probe as an internal standard.

Densitometric analysis. We analyzed autoradiograms for the relative intensity of signals by densitometry (BioRad Imaging Densitometer GS-670) using BioRad Molecular Analyst PC Version 1-1 (Life Sciences Group, Hercules, CA).

Statistical analysis. We analyzed mean data by analysis of variance and performed post-hoc comparisons with the Newman-Keuls test. Pearson's correlation coefficient was used to evaluate the linear relationship between lead levels and the amount of GnRH transcripts. Significance level was $p < 0.05$. Values are means \pm SEM. We analyzed data

using the commercially available software package SPSS (SPSS, Inc., Chicago, IL).

Results

Body weights and water consumption. Although there was a trend toward lower body weights with higher doses of lead in the animals treated for 8 and 16 weeks, rare significant differences in body weights at the time of sacrifice between lead-treated animals and their respective control groups were observed in any experimental group (Table 1). All lead-treated animals consumed less water than did their respective controls ($p < 0.001$; Table 1).

Circulating lead, GnRH, and LH levels. All control animals had mean blood lead levels consistently $< 3 \mu\text{g/mL}$. All lead-treated animals had blood lead levels greater than those of controls ($p < 0.001$; Table 2).

No significant differences in plasma GnRH and LH were found among animals treated with increasing doses of lead acetate for any time period studied (Table 2).

Northern blot analysis. A significant, steady dose-response increase of GnRH mRNA concentrations with lead dosing was seen in animals treated for 1 week ($p < 0.01$; Figure 1). Animals treated for more than 1 week also exhibited a significant increase in GnRH mRNA, but with an attenuation of the increase at the higher concentrations of lead with increased duration of exposure (Figure 2). Correction of loading by use of the standard probe β -actin indicated that the concentrations of GnRH mRNA were not dependent on the amount of RNA loaded per lane (Figure 2). Correlation between lead blood levels and expression of mRNA GnRH levels for doses ranging from 0 to 0.1% was significant ($r = 0.9$, $p < 0.05$).

Discussion

The data presented here show that low doses of lead for long periods of time alter the rat hypothalamic-pituitary axis in a manner similar to that previously reported at higher doses for shorter periods of time. These

Table 1. Water consumption and body weights in control and lead-exposed male rats (mean \pm SEM; $n = 10$ –16).

Weeks of exposure	Measurement	Percent PbAc in water					p-Value
		0	0.025	0.05	0.1	0.3	
1	Water consumption (g/animal/day)	41.21 \pm 0.77	37.12 \pm 0.86*	37.80 \pm 0.89*	32.81 \pm 0.63*	27.80 \pm 0.49*	0.001
	Weight (g)	408 \pm 2	403 \pm 2	401 \pm 2	401 \pm 1.8*	404 \pm 1.7	0.109
4	Water consumption (g/animal/day)	43.14 \pm 0.97	37.18 \pm 1.10*	37.38 \pm 1.17*	34.90 \pm 0.84*	28.89 \pm 0.51*	0.001
	Weight (g)	501 \pm 4	493 \pm 5	490 \pm 3	489 \pm 4	487 \pm 3*	0.015
8	Water consumption (g/animal/day)	40.34 \pm 1.02	36.24 \pm 1.39*	37.65 \pm 1.05*	35.19 \pm 1.04*	30.96 \pm 0.49*	0.001
	Weight (g)	578 \pm 7	572 \pm 8	566 \pm 6	564 \pm 7	559 \pm 5	0.052
16	Water consumption (g/animal/day)	37.91 \pm 1.52	33.76 \pm 1.39*	33.48 \pm 1.69*	33.04 \pm 1.10*	27.58 \pm 0.68*	0.001
	Weight (g)	694 \pm 11	680 \pm 15	668 \pm 11	672 \pm 13	661 \pm 9	0.083

* $p < 0.05$.

results agree with previous findings that indicated a significant positive correlation between lead dose and expression of mRNA GnRH levels in the hypothalamus (17,19). Furthermore, the data support the hypothesis that lead exposure initially induces an increase in intracellular levels of GnRH mRNA in a dose-related manner, but with an attenuation in message production at higher levels of blood lead. The attenuation of the increase in GnRH mRNA levels with a greater dose of lead exposure without a significant change in the levels of plasma GnRH and LH support the conclusion that the male Sprague-Dawley rat adapts to the toxic effects of lead on the hypothalamus and that alterations in GnRH production at the molecular level do not translate to increases in circulating GnRH or LH levels.

Previous published studies in other systems support the hypothesis that adaptation to lead toxicity occurs with prolonged exposure. Increased duration of exposure to lead does not produce more profound toxic effects on circulating testosterone, sperm concentration, and sperm production rate in animals whose dosing began at the beginning of the pubertal period (22). Castrated, lead-treated male rats are able to respond to castration with an increase in LH, indicating an adaptation of the hypothalamic-pituitary axis to the toxic effects of lead (23). At lower exposure levels, a significant disruption in the reproductive axis occurs at puberty in pups exposed from gestation through the onset of puberty (9,20,29) or in adulthood after exposure during sexual differentiation (30). However, animals whose exposure continues to adulthood do not manifest as profound an alteration in the hypothalamic-pituitary-gonadal axis (20). Studies in monkeys also suggest that chronically exposed animals may develop compensatory mechanisms. In the female monkey, lead exposure during adulthood induced menstrual irregularities (31), but chronic dosing from infancy throughout adulthood did not alter menstrual cycles (32).

Chronically lead-exposed male monkeys revealed only subtle signs of reproductive toxicity (24).

A similar tolerance has been reported in studies evaluating the renal toxicity of lead. Hitzfield and Taylor (33) reported the development of resistance to lead in a rat kidney cell line. Kidney cells pre-exposed to lead showed a higher rate of protein synthesis than nonadapted cells. They suggested that changes in protein synthesis after lead exposure appeared to be a potent parameter in the development of this resistance. Clinical studies also suggest an adaptation to lead's toxic effects on the kidney (34). However, clinical studies evaluating the relationship between duration of exposure and reproductive toxicity have not been published.

Although lead exposure alters the production of GnRH in the hypothalamus at the molecular level, circulating GnRH and LH levels are not altered significantly. Previous studies evaluating higher exposure levels for shorter periods of time reported similar results (19,20,24,35). The signals within and between the hypothalamus and pituitary gland appear to be disrupted by lead exposure, but elevations of GnRH mRNA levels do not automatically translate into increased blood levels of GnRH, at least in the lead-intoxicated rat. GnRH is secreted from the hypothalamus in a pulsatile fashion and in turn stimulates LH biosynthesis and secretion (36). Our failure to document a corresponding change in serum GnRH levels may be due to the fact that we measured only one serum sample at the time of sacrifice. However, a more likely explanation is that lead interferes with the release of GnRH from the nerve terminal in the median eminence. Autoradiographic studies indicated that ^{210}Pb accumulated in the greatest concentration in the median eminence (37). Bratton and coworkers (38) investigated the effects of lead on norepinephrine and prostaglandin E_2 (PGE_2)-induced GnRH release from the adult male rat median eminence incubated *in*

vitro. Lead did not alter basal GnRH release, but it did block norepinephrine-induced GnRH release in a dose-related manner. Conversely, lead had no effect on the PGE_2 -induced release of GnRH, but did block the norepinephrine-stimulated release of PGE_2 . Therefore, lead may disrupt GnRH release at the hypothalamic level by interfering with PGE_2 synthesis or release. *In vivo* studies evaluating the effects of lead exposure on noreadrenergic stimulation of GnRH release support this conclusion. Lead exposure at high doses interferes with naloxone-mediated release of LH (18) and acts synergistically to inhibit catecholamine synthesis by the tyrosine hydroxylase inhibitor α -methyl-*p*-tyrosine (23). Because the hypothalamic-pituitary axis is a dynamic system and the actions of lead at these central nervous system sites are not completely suppressive (23), adequate amounts of GnRH are released to maintain LH synthesis and secretion resulting in steady-state serum levels. As exposure time increases, adaptation at the molecular level intensifies.

These findings of perturbed GnRH mRNA expression at low levels of lead exposure are of potential clinical importance. The CDC defines blood lead levels as elevated in children if they exceed 10 $\mu\text{g}/\text{dL}$ (1). Cognitive

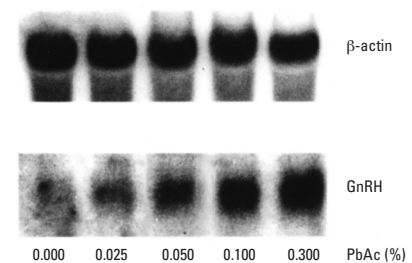


Figure 1. Example of a northern blot for the quantification of hypothalamic GnRH mRNA levels in control and lead-dosed animals. Autoradiograms depict GnRH mRNA levels in the medial basal hypothalamus of rats exposed to 0, 0.025, 0.05, 0.1, and 0.3% PbAc for 1 week ($p < 0.01$). The data are representative of 3 different northern blots on separate RNA samples collected for each lead dose.

Table 2. Blood lead and serum GnRH and LH levels in control and lead-exposed male rats (mean \pm SEM).

Weeks of exposure	Measurement ^a	Percent PbAc in water					<i>p</i> -Value
		0.0	0.025	0.05	0.1	0.3	
1	Lead ($\mu\text{g}/\text{dL}$)	< 2	12 \pm 2*	17 \pm 2*	20 \pm 1*	28 \pm 1*	0.001
	LH (ng/mL)	0.60 \pm 0.09	0.96 \pm 0.23	0.73 \pm 0.13	0.56 \pm 0.13	0.77 \pm 0.24	0.143
	GnRH (pg/mL)	20 \pm 4	14 \pm 1	16 \pm 2	15 \pm 1	13 \pm 1	0.095
4	Lead ($\mu\text{g}/\text{dL}$)	< 2	10 \pm 1*	15 \pm 1*	23 \pm 2*	35 \pm 4*	0.001
	LH (ng/mL)	0.86 \pm 0.19	1.09 \pm 0.24	0.80 \pm 0.12	0.96 \pm 0.15	0.65 \pm 0.11	0.273
	GnRH (pg/mL)	14 \pm 1	16 \pm 1	15 \pm 1	14 \pm 2	14 \pm 1	0.807
8	Lead ($\mu\text{g}/\text{dL}$)	< 3	14 \pm 2*	17 \pm 2*	24 \pm 1*	37 \pm 2*	0.001
	LH (ng/mL)	0.70 \pm 0.12	0.77 \pm 0.12	0.41 \pm 0.09	0.48 \pm 0.05	0.57 \pm 0.1	0.083
	GnRH (pg/mL)	19 \pm 1	16 \pm 2	16 \pm 1	16 \pm 1	15 \pm 1	0.436
16	Lead ($\mu\text{g}/\text{dL}$)	< 3	12 \pm 1*	18 \pm 2*	27 \pm 1*	42 \pm 3*	0.001
	LH (ng/mL)	0.87 \pm 0.14	0.75 \pm 0.12	0.65 \pm 0.09	0.68 \pm 0.06	0.63 \pm 0.09	0.466
	GnRH (pg/mL)	20 \pm 1	16 \pm 1	19 \pm 1	15 \pm 1	17 \pm 1	0.071

^aFor blood lead measurements, $n = 10$ –16; for LH, $n = 14$ –16; for GnRH, $n = 7$ –12, except week 1 and week 16 at 0%, $n = 5$. * $p < 0.001$.

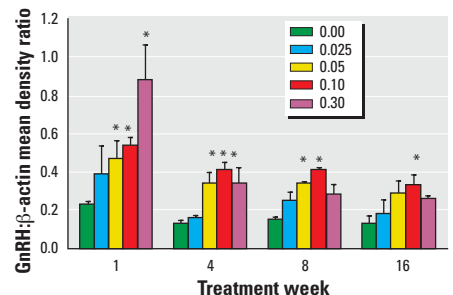


Figure 2. Summary of GnRH β -actin mean density ratios in control and lead-dosed animals over time. Data represent the mean \pm SEM for three hypothalami per dose group per time point. * $p < 0.01$ as compared to controls.

and behavioral development may be altered in children exposed to these low levels of lead (4,5,21). We report lead-induced reproductive abnormalities in male rats at lead levels as low as 10 µg/dL. A recent clinical study evaluating the impact of lead exposure on normal reproductive development suggests that boys with marginally increased blood lead levels may mature sexually at a later age and have smaller testicular volume than control subjects (39). The exact mechanism by which this occurs clinically remains to be elucidated, but our results using an animal model suggest that subtle disruptions in GnRH production and/or release may be involved.

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