Postnatal Inorganic Lead Exposure Reduces Midbrain Dopaminergic Impulse Flow and Decreases Dopamine D1 Receptor Sensitivity in Nucleus Accumbens Neurons

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ABSTRACT
Lead treatment via drinking water for 3 to 6 weeks at 250 ppm was found to significantly decrease the number of spontaneously active dopamine (DA) neurons in both substantia nigra and ventral tegmental area that were recorded using standard extracellular electrophysiological recording techniques. Lead exposure did not affect the discharge rate or discharge pattern of these DA neurons. No significant increase in the number of tyrosine hydroxylase immunopositive cells was detected in lead-treated animals relative to controls even though the length of lead exposure was extended beyond that of the electrophysiological studies. The significant lead-induced decrease in spontaneously active cells observed in the electrophysiological studies was, therefore, not due to cell death. An acute drug challenge with the DA receptor agonist apomorphine at a dose known to hyperpolarize midbrain DA neurons (50 µg/kg i.v.) was used to determine whether hyperpolarization would normalize the number of spontaneously active DA neurons. The results suggest that depolarization inactivation was most likely not the cause for this lead effect. The D1 receptor agonist SKF-38393 [1-phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine-7,8-diol] was iontophoretically applied to type I nucleus accumbens (Nacb) neurons. The results demonstrated that type I Nacb neurons have a significantly lower basal discharge rate in lead-treated animals relative to controls and that the Nacb DA D1 receptors were significantly less sensitive to SKF-38393 in the lead-treated animals. Therefore, lead exposure decreases DA neuron impulse flow presynaptically and decreases DA D1 receptor sensitivity postsynaptically in the nucleus accumbens.

Knowledge of the neurotoxic effects of lead has a long history dating back well before the industrial revolution. Since the industrial revolution, lead exposure has become more widespread. Lead exposure occurs from lead-based paint in old buildings, leaded gasoline emissions, and other industrial emissions. The problem of lead exposure was extended beyond that of the electrophysiological studies. The significant lead-induced decrease in spontaneously active cells observed in the electrophysiological studies was, therefore, not due to cell death. An acute drug challenge with the DA receptor agonist apomorphine at a dose known to hyperpolarize midbrain DA neurons (50 µg/kg i.v.) was used to determine whether hyperpolarization would normalize the number of spontaneously active DA neurons. The results suggest that depolarization inactivation was most likely not the cause for this lead effect. The D1 receptor agonist SKF-38393 [1-phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine-7,8-diol] was iontophoretically applied to type I nucleus accumbens (Nacb) neurons. The results demonstrated that type I Nacb neurons have a significantly lower basal discharge rate in lead-treated animals relative to controls and that the Nacb DA D1 receptors were significantly less sensitive to SKF-38393 in the lead-treated animals. Therefore, lead exposure decreases DA neuron impulse flow presynaptically and decreases DA D1 receptor sensitivity postsynaptically in the nucleus accumbens.

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ABBREVIATIONS: DA, dopamine; VTA, ventral tegmental area; SN, substantia nigra; HAL, haloperidol; ISI, interspike interval; SKF-38393, 1-phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine-7,8-diol; TBS, Tris-buffered saline; NGS, normal goat serum; kHz, kilohertz; TH, tyrosine hydroxylase; ANOVA, analysis of variance; LSD, least significant difference; ANCOVA, analysis of covariance; APO, apomorphine; Nacb, nucleus accumbens.
glutamatergic are affected by lead (e.g., see Shao and Suszkiew, 1991; Cory-Slechta, 1997; Guijarte, 1997; Braga et al., 1999; Lasley and Gilbert, 2002). It is likely that there are numerous targets of lead that are associated with various neurotransmitter mechanisms and that these various mechanisms participate in exposure level-dependent pathological responses once specific thresholds have been reached.

The effect of lead on the dopaminergic system has been reported to occur at clinically relevant blood levels (Kala and Jadhav, 1995a; Cory-Slechta, 1997; Tavakoli-Nezhad et al., 2001; Lewis and Pitts, 2004) in the rat. Many neurochemical studies have reported alterations in DA release, DA turnover, and changes in DA receptor numbers (Nation et al., 1989; Lasley, 1992; Kala and Jadhav, 1995a,b; Pokora et al., 1996; Cory-Slechta, 1997; Zuch et al., 1998). The midbrain DA system is involved in cognition, attention and motor function, and has been shown to be an important participant in many neurological and psychiatric disorders. The midbrain DA system may play a role in the etiology of attention deficit disorder, and as indicated above, lead exposure is a probable risk factor in this disorder (Minder et al., 1994; Tuthill, 1996; Canfield et al., 2003b). It has also been suggested that lead intoxication may be a risk factor in Parkinson’s disease (Kuhn et al., 1998), a disease associated with the relatively selective loss of DA-containing neurons in the substantia nigra.

Understanding the impact of lead exposure on the electrophysiological activity of the midbrain DA-containing neurons in the ventral tegmental area (VTA), substantia nigra (SN), and on target neurons in the nucleus accumbens is a necessary and integral step toward understanding the mechanism of the lead-induced neurochemical changes. However, to date, there has been only one report examining the effect of lead on the activity of single midbrain DA neurons by Tavakoli-Nezhad et al. (2001). The present study is an extension of the previous study by Tavakoli-Nezhad et al. (2001) and further examines the effects of postnatal lead exposure on both pre- and postsynaptic components of the midbrain DA system using electrophysiological and immunohistochemical techniques.

Materials and Methods

Animals and Exposure Protocol. Animals were housed in facilities operated by the Department of Laboratory Animal Resources at Wayne State University and maintained according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male Sprague-Dawley weanling rats (21 days old) were obtained from Charles River Laboratories, Inc. (Wilmington, MA). The animals were housed in facilities operated by the Department of Laboratory Animal Resources at Wayne State University. The animals were maintained in a room with constant temperature and humidity and a 12-h light/dark cycle. The subjects received access to food and water ad libitum. Beginning 22 days after birth, subjects were provided drinking water treated with lead- or sodium-acetate at exposure levels of 250 ppm for a period of 3 to 6 weeks. The treatment for electrophysiological studies extended from postnatal day 22 up to the day of electrophysiological recording with a minimum possible exposure period equaling 3 weeks and a maximum possible exposure period equaling 6 weeks. The treatment for immunohistochemical studies was longer than that of the electrophysiological studies and lasted for 7 to 13 weeks.

Extracellular Electrophysiological Recording and Neuron Identification. The rats were anesthetized with either chloral hydrate (400 mg/kg i.p.) for studies of midbrain DA neurons or urethane (1.25 g/kg i.p.) for studies of nucleus accumbens neurons (under iontophoresis: Nucleus Accumbens Neurons) and placed in a stereotaxic apparatus. Body temperature was maintained at 37 ± 1°C with a heating pad. Single-unit extracellular recordings were made from spontaneously active DA neurons with single-barrel glass micropipettes (1.5-mm diameter) broken to achieve a tip diameter of approximately 1 to 2 μm (five-barrel pipettes were used for iontophoresis, see below). The pipettes were filled with a 2 M NaCl solution containing 2% pontamine sky blue dye. Electrode impedance typically measured 1.8 to 2.5 megs at 15 Hz in physiological saline.

Extracellular signals were amplified by a high impedance amplifier (EX1 differential amplifier; Dagan, Minneapolis, MN) with bandpass settings of 300 Hz to 3 kHz. The amplified signal was then sent to an analog oscilloscope, an audiomonitor, a window discriminator (WD-2; Dagan), and via a digital interface (M-100; Modular Instruments, Inc., Southeastern, PA) to a Pentium computer. Two animals were recorded on a given experimental day, one lead- and one sodium-treated.

Midbrain DA neurons were identified by well established electrophysiological criteria (Grace and Bunney, 1980; Bunney et al., 1991). The electrophysiological characteristics used to identify midbrain DA neurons included the following: 1) biphasic positive-negative action potential (Fig. 1A) located in the ventral midbrain approximately 6.5 to 8.5 mm below the brain surface (see other coordinates relative to lambda and sagittal suture below), 2) a very long duration action potential (range: 2.0 to 4.0 ms) with an initial segment-somatodendritic break on the ascending limb (Fig. 1), 3) a discharge pattern consisting of either an irregular single-spike mode or burst mode with each burst containing three to eight spikes of decreasing amplitude and increasing duration, and 4) a discharge rate of approximately 0.5 to 10 spikes per second (mean = 4.0). The midbrain DA neurons can be easily distinguished from neighboring non-DA neurons (e.g., the GABAergic reticulata neurons of the SN). The

![Fig. 1. Panel A depicts an extracellular voltage trace of a single action potential recorded from a nigral DA neuron. Note the wide positive-negative going action potential and the initial segment-somatodendritic break on the ascending limb. The cell was recorded with amplifier bandpass settings of 300 Hz to 3 kHz. The calibration bar represents 1 ms (horizontal) and 250 μV (vertical) and 250 Hz to 3 kHz. The calibration bar represents 1 ms (horizontal) and 100 μV (vertical).](image-url)
non-DA neurons of the SN and VTA generally have a much more narrow spike width (<1.7 ms) that lacks an initial segment–soma
todendritic break, a higher average discharge rate, and different
discharge patterns (Grace and Bunney, 1979).

Nucleus accumbens neurons were studied under urethane anesthesia as described because chloral hydrate appears to suppress the activity of spontaneously active neurons more than urethane. Nucleus accumbens neurons were found approximately 7.5 mm below the cortical surface. Stereotaxic coordinates for recording nucleus accumbens neurons were as follows: 10.2 to 10.8 mm anterior relative to lambda and 0.9 to 1.6 mm lateral to midline. Type I neurons were identified by their negative-positive extracellular waveforms, respectively (Shen et al., 1992) (Fig. 1B).

**Cells Per Track Assay: Midbrain DA Neurons.** To systematically sample midbrain DA neuron activity, stereotaxic coordinates (see below) were used to define a block of tissue (e.g., see Chiodo and Bunney, 1983; White and Wang, 1983; Tavakoli-Nezhad et al., 2001). Each neuron located was monitored for approximately 3 to 5 min to assess discharge rate and discharge pattern. For each of the nuclei (VTA or SN), mean values were calculated for the number of cells per track and for the average firing rate. The average firing rate was calculated as the mean of all VTA and the mean of all SN DA neurons for each rat.

From the age of 22 days old to 9 weeks old, rats were chronically exposed to either lead- or sodium-acetate via drinking water. In addition, a third group was given haloperidol (HAL) injections (0.5 mg/kg/day i.p.) between 8:00 and 10:00 AM each day over the same time period. To test if depolarization inactivation contributed to changes in the spontaneous activity of DA neurons after lead exposure, apomorphine (50 µg/kg i.v.) was administered to sodium-, lead-, and HAL-treated groups as a “hyperpolarizing” challenge. The HAL-treated group was given a morning injection of haloperidol approximately 2 to 3 h before surgical preparation for the cells per track assay. The cells per track assay was performed before and after apomorphine. In this cell per track study, the number of spontaneous active DA neurons was counted in 12 electrode tracks (six in SN and six in VTA) in a given subject before apomorphine challenge. An additional 12 electrode tracks (six in SN and six in VTA) was then recorded after apomorphine challenge.

Two basic sets of region-targeted coordinates were used in the apomorphine challenge study, which corresponded to the two areas of the midbrain studied, the VTA and SN. The specific coordinates used within a given targeted region (VTA or SN) were also systematically varied in relation to the timing of the apomorphine challenge with the specific coordinates used for a midbrain nucleus changing after apomorphine administration. The coordinates used for the VTA were as follows: 2.8 and 3.2 mm anterior to lambda and 0.4, 0.6, and 0.8 mm lateral to sagittal suture after apomorphine, and 3.0 and 3.4 mm anterior to lambda and 0.4, 0.6, and 0.8 mm lateral to sagittal suture after apomorphine. The coordinates used for the SN before apomorphine were 2.8 and 3.2 mm anterior to lambda and 2.0, 2.2, and 2.4 mm lateral to sagittal suture, and after apomorphine, the SN coordinates were 3.0 and 3.4 mm anterior to lambda and 2.0, 2.2, and 2.4 mm lateral to sagittal suture. Note that the anterior coordinates change according to the status of the apomorphine challenge, not the lateral coordinates. Finally, for a given region (VTA or SN), the sequence of the anterior coordinates that related to the apomorphine challenge was reversed for one half of the animals studied. For example, one half of the animals used 3.0 and 3.4 mm anterior to lambda before apomorphine and 2.8 and 3.2 mm anterior to lambda after apomorphine. The number of spontaneously active cells, discharge rate, and percentage of bursting cell were calculated in both regions with the presence and absence of apomorphine being treated as a within-subjects factor (see Data Analysis below for more details).

**Discharge Pattern Analysis: Midbrain DA Neurons.** DA neurons were classified as bursting or nonbursts in each cell per track experiment using computer software. Briefly, interspike interval (ISI) histograms consisting of 500 consecutive active potentials were collected from DA neurons as previously described (Tavakoli-Nezhad et al., 2001). To distinguish bursting cells from nonbursting cells, the following criteria were used: 1) bursts were defined to begin within any ISI less than 80 ms and to terminate with the first ISI greater than 160 ms, and 2) a minimum of two, three-spike bursts were encountered within the sample of 500 consecutive action potentials (see Zhang et al., 1996). The mean value was calculated for the proportion of bursting cells found in each animal, and this value was used in inferential statistical tests.

**Tyrosine Hydroxylase Immunohistochemistry.** Since it was possible that some DA neurons recorded in electrophysiological experiments might be undergoing apoptotic or necrotic processes following lead exposure, animals used for immunohistochemical studies were treated for longer periods than that used in electrophysiological studies, 7 to 13 weeks rather than 3 to 6 weeks. The immunohistochemical experiments were designed to test the influence of lead on DA neuron viability.

Animals were given an overdose of chloral hydrate at the end of the exposure period, and just prior to complete cessation of respiration the thoracic cavity was opened surgically. A syringe needle was inserted into the left ventricle of the heart, the right atrium was severed, and the animal was then perfused with ice-cold phosphate-buffered saline until the blood exiting the right atrium ran clear. The perfusate was then changed to a chilled phosphate-buffered paraformaldehyde solution (4% prepared fresh each day). The brains were postfixed in phosphate-buffered saline for an hour and then stored in a 20-ml vial containing 10% sucrose in paraformaldehyde solution overnight at 4°C.

Tissue sections for lead-exposed and control animals were processed at the same time to minimize variation in the staining procedure. Forty-micron sections of midbrain were made on a freezing microtome, and the sections floated in chilled Tris-buffered saline (TBS). All sections were then incubated in TBS with 10% methanol and 3% hydrogen peroxide for 5 min to block endogenous peroxidase. This was followed by three 10-min rinses in TBS. The rinsed sections were incubated overnight in TBS/10% normal goat serum (NGS) to block nonspecific binding at 4°C. The next day the sections were rinsed with TBS and then incubated in a monoclonal primary antibody (1:2000) for tyrosine hydroxylase (TH) overnight at 4°C (in TBS/2% NGS with 0.3% Triton X-100). Following these 10-min rinses in TBS, the sections were incubated in the secondary antibody, goat anti-mouse IgG, for 3 h (in TBS/2% NGS with 0.3% Triton X-100). The incubation with secondary antibody is followed by the standard
TBS rinses. The sections were then incubated in mouse peroxidase-antiperoxidase for 1 h (TBS/NGS/Triton X-100) to tag the TH enzyme with peroxidase activity. This was followed by one 10-min rinse in TBS and two 10-min rinses in Tris buffer. The sections were next incubated with a diaminobenzidine solution until the dopamine-containing neurons become visible (approximately 15 min). The reaction was stopped by placing the sections in Tris buffer. Midbrain sections were then mounted on slides.

**Light Microscopy and Cell Counts.** Sections of the midbrain containing TH-positive (+) cells in the VTA and SN were examined using a light microscope equipped with an eyepiece grid. Slides were selected by specific anatomical criteria for each animal to represent three standard sections containing the midbrain DA neurons (Shen et al., 1999; Tavakoli-Nezhad et al., 2001), approximately 4.2, 3.6, and 3.0 mm anterior to the interaural line (Paxinos and Watson, 1986). These sections represented the anterior, middle, and posterior parts of the SN and VTA. Due to the distance between the selected sections (i.e., 0.6 mm), the same cells are not represented on these adjacent sections.

Sections were obtained from the brains of sodium- and lead-exposed animals that were age-matched. The anatomical features used to distinguish the SN and VTA in these selected sections included: basal cerebral peduncle, mammillary body, oculomotor nerve and roots, and medial lemniscus (e.g., see Paxinos and Watson, 1986). The division between VTA and SN was considered to be a ventral-dorsal line approximately 1.5, 1.2, and 1.2 mm lateral from midline corresponding to the 4.2, 3.6, and 3.0 mm sections, respectively (described above). The cells were counted at 200× magnification. The total number of SN and VTA TH (+) cells in a given midbrain section were counted in the two areas defined by the ventral-dorsal division described above. For each rat, TH (+) cells in the SN and VTA area of one side of the midbrain was counted in the three sections described above. The TH (+) counts for each nucleus were expressed in results as the average number of cells per section (i.e., average of three sections) corresponding to the SN or VTA. This procedure generated two mean cell counts per animal that corresponded to the SN and VTA regions, respectively.

**Determination of Blood Lead Level.** The blood lead level was measured by an electrolytic voltametric stripping method using a LeadCare blood lead testing system from ESA, Inc. (Chelmsford, MA). The lower limit of detection for this instrument is 1.4 μg/dl. The group of animals used to determine blood lead levels was not used in electrophysiological experiments. This cohort of animals was treated in a manner identical to that of animals used in electrophysiological studies.

**Data Analysis.** In the electrophysiological studies, the data from lead-exposed offspring were compared with sodium-exposed controls matched daily for their age and duration of treatment. In the electrophysiological studies of midbrain DA neurons, both anatomical regions (SN and VTA) were examined in each rat exposed to lead- or sodium-acetate. The mean values for number of cells per track, discharge rate, and percentage of cells with burst discharges calculated for each animal were analyzed as dependent variables for treatment effects using a three-way analysis of variance (ANOVA) with repeated measures on two variables, cell type (SN or VTA), and amphetamine challenge (before or after). Subsequent planned comparison of means was conducted using contrast analysis or the Fisher’s LSD test. In the iontophoretic study, a difference between the mean basal firing rates of the cells sampled from the two treatment groups was observed. The iontophoresis results were therefore analyzed using repeated measures analysis of covariance (ANCOVA) with ejection current as a repeated measures factor, treatment as a between-subjects factor, discharge rate as the dependent variable, and basal discharge rate as the covariate.

In the immunohistochemical study, two mean values (one for SN and one for VTA) were analyzed for treatment effects using ANCOVA with repeated measures (i.e., cell type). The two factors in this analysis were treatment (sodium/lead) and cell type (SN/VTA).

The covariate was duration of sodium/lead exposure. In a similar fashion, a multivariate analysis of covariance was used to simultaneously analyze the two dependent variables body weight and brain weight using duration of exposure as a covariate. Blood lead levels were analyzed using ANCOVA with duration of exposure as a covariate. The use of ANCOVA in the analysis of electrophysiological data has been previously described by Pitts et al. (1990).

**Drugs and Chemicals.** Apomorphine hydrochloride, haloperidol, (-)-SKF-38393 hydrochloride, normal goat serum, and goat anti-mouse IgG were purchased from Sigma-Aldrich (St. Louis, MO). Apomorphine hydrochloride was dissolved in 0.9% saline for i.v. administration. Haloperidol was dissolved in dimethyl sulfoxide (0.5 mg/200 μl) for i.p. administration (Zhanga et al., 1996). The monoclonal primary antibody (LNC1) for TH was a gift from Dr. Greg Kapatos, Wayne State University.

## Results

**Effects on Growth and Blood Lead Level.** Table 1 illustrates the mean values for growth parameters and blood lead levels. The mean body weights (gram) at the beginning of the study were 60.1 ± 1.1 (n = 12) and 59.8 ± 1.2 (n = 12) for the sodium and lead groups, respectively. There was no significant difference between the initial body weights. No significant difference in body weight or brain weight (P > 0.60, Wilks’ lambda) was found between lead- and sodium-treated animals after 3 to 6 weeks of treatment. There was a significant positive relationship (P < 0.001, Wilks’ lambda) between the covariate, duration of exposure, and the weight measurements indicating that growth was still occurring during the exposure period. Although the mean values of the body and brain weights for lead-treated animals were slightly smaller than control values, both the body and brain weight of the lead-treated animals were still approximately 98% of control values. If lead affected growth in these studies, there was not enough power to detect an effect of such small magnitude (~2%). The blood lead level of controls was found to be below detection (<1.4 μg/dl, n = 7) or at a very low level of 2.1 ± 0.4 μg/dl (n = 5) relative to lead-treated animals. ANCOVA indicated that lead treatment significantly (P < 0.001) elevated blood lead levels to a mean value of 30.8 ± 1.2. There was not a significant relationship between the covariate, duration of exposure, and the blood lead level (P > 0.30). A one-way ANOVA examining only the lead-treated group did not reveal any significant main effect of treatment duration (3 to 6 weeks) on blood lead levels (P > 0.20). This analysis of the blood lead levels indicates that blood levels were relatively stable between the 3rd and 6th week of exposure.

**Electrophysiological Study of Midbrain DA Neurons.** Electrophysiological studies were conducted to determine the number of spontaneously active midbrain dopamine DA-containing neurons (Fig. 1A) per electrode track in sodium/lead-treated animals before and after an acute apomorphine challenge.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Sodium Acetate (n = 12)</th>
<th>Lead Acetate (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>259 ± 12</td>
<td>250 ± 12</td>
</tr>
<tr>
<td>Brain weight (g)</td>
<td>1.76 ± 0.03</td>
<td>1.74 ± 0.03</td>
</tr>
<tr>
<td>Blood Pb (μg/dl)</td>
<td>2.1 ± 0.4 (n = 5)</td>
<td>30.8 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>&lt;1.4, B.D. (n = 7)</td>
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B.D., below detection limits of instrument.
(APO) challenge dose (50 µg/kg i.v.). Three different post-weaning treatment groups examined are sodium treated (250 ppm, n = 15), lead treated (250 ppm, n = 15), and haloperidol treated (500 µg/kg i.p., n = 14). Figure 2 illustrates the effect of apomorphine treatment on the number of spontaneously active DA neurons in the SN (panel A) and VTA (panel B), respectively. Three-way ANOVA indicated that there was a significant APO main effect (P < 0.05) and a significant interaction (sodium/lead/HAL) x APO interaction (P < 0.001). Fisher’s LSD test indicated that haloperidol-treated animals before APO challenge, significantly decreased the number of spontaneously active DA neurons relative to their within-subject pre-APO control.

The cells per track analysis indicates that both lead and HAL treatment significantly decreased the number of spontaneously active VTA DA neurons, but APO only “reversed” the effects on the VTA DA neurons in the HAL group. Although the number of spontaneously active SN DA neurons before APO challenge was significantly decreased by lead, the number of active neurons was not significantly reduced by HAL (P > 0.20 relative to sodium-treated, Fisher’s LSD). APO did not significantly affect either lead- or HAL-treated neurons relative to their within-subject pre-APO challenge control in the SN.

The effect of apomorphine challenge on the discharge rate of spontaneously active DA neurons in lead/sodium/HAL-treated animals before and after APO challenge in SN and VTA is shown in Fig. 2, panel C and D, respectively. A three-way analysis of variance indicated that there was a significant treatment main effect (P < 0.05) but no other significant main effects or interactions. When ignoring cell type and APO challenge as factors, contrast analysis indicated that HAL treatment significantly increased discharge rate relative to lead treatment (P < 0.05) but not sodium treatment (P > 0.05). Contrast analysis applied in a similar manner did not detect any significant differences between lead and sodium treatment (P > 0.30). There was a trend for a treatment (sodium/lead/HAL) x APO x cell type interaction (P < 0.08).

The effect of apomorphine challenge on DA neuron burst discharges in all three groups (HAL/sodium/lead) in the VTA and SN can be seen in panel E and F, respectively, of Fig. 2. There was a significant treatment effect (P < 0.005) and a significant treatment (sodium/lead/HAL) x APO x cell type interaction (P < 0.005) and a

Fig. 2. Panel A and B depict the effect of APO treatment on the number of spontaneously active DA neurons in sodium- (n = 15; 250 ppm), lead- (n = 15; 250 ppm), and haloperidol-treated animals (n = 14; 500 µg/kg i.p.) for the SN and VTA, respectively. A three-way ANOVA indicated that there was a significant treatment (sodium/lead/HAL) by APO interaction (P < 0.001). Fisher’s LSD test was used to examine differences between groups before apomorphine and to do a within-subjects comparison of the effect of apomorphine (i.e., before versus after). Significant differences from sodium-treated controls before apomorphine are indicated by an asterisk (*, P < 0.05 when compared with sodium-treated control). Significant effects of apomorphine are indicated by the carrot symbol (ˆ, P < 0.05 when compared with before condition). N.S., no significant difference. Panel E and F show the effect of apomorphine challenge on the number of spontaneously active DA neurons in the SN and VTA, respectively. A three-way analysis of variance indicated that there was a significant treatment main effect (P < 0.05). See Results for a more detailed statistical analysis. Panel E and F show the effect of apomorphine challenge on the number of DA neurons with burst discharges in the SN and VTA, respectively. A three-way analysis of variance indicated that there was a significant treatment effect (P < 0.005) and treatment x cell type interaction (P < 0.01). See Results for a more detailed description of the statistical analysis.
treated group being significantly lower (P < 0.001) indicating a higher number of TH (+) cells in the VTA.

Even when the duration of exposure for the 250-ppm exposure is increased relative to that used in the electrophysiological studies (increased to 7 to 13 weeks), these immunohistochemical results indicate that there are no significant changes in the number of TH (+) neurons. Although there is a trend for lead to reduce histological cell count, the calculated percent of control values for SN and VTA are high at 94% and 95%, respectively. For the purpose of comparison, the calculated percent of control values for spontaneously active DA neurons following lead treatment in the electrophysiology study were approximately 60% for both the SN and VTA.

**Effects on Nucleus Accumbens D1 Receptor Sensitivity.** The effect of the DA D1 receptor selective agonist SKF-38393 on the firing of type I nucleus accumbens (Nacb) neurons (Fig. 1B) was investigated in lead- and sodium-treated animals. The SKF-38393 ejection currents exerted a current-dependent inhibitory effect. Figure 3 shows examples of ratemeter histograms of type I Nacb neurons from a sodium- (panel A) and a lead-treated (panel B) animal. Note the difference between these neurons from treated animals in basal discharge rate and the difference in the slope of the inhibitory response in relation to iontophoretic drug application.

Figure 4A shows the mean responses of type I Nacb neurons to the microiontophoretically applied SKF-38393 in lead- and sodium-treated rats. The basal discharge rate of the nucleus accumbens neurons was found to differ between the sodium- and lead-treated groups, with that of the lead-treated group being significantly lower (P < 0.005, t test). The current-response relationship was analyzed using a two-way ANCOVA with basal discharge rate as the covariate.

There was a significant interaction between treatment group and current (P < 0.005). This can be observed as a shallow slope for the lead-treated group and a steeper slope for the control group. The difference in slopes can also be seen in the inset of Fig. 4A, which shows the iontophoretic data redrawn as mean change scores. This indicates that the nucleus accumbens neurons from the lead-treated group are less sensitive to iontophoretically applied SKF-38393 than those from the sodium-treated group. The D1 receptors on Nacb neurons from the lead-treated group appear to be "down-regulated". Figure 4B shows a plot of the adjusted least square means derived from ANCOVA. Again, note that the relative difference in the slopes of the two current-response curves remains after adjusting for the covariate basal discharge rate.

**Discussion**

The results of this study confirm and extend the findings of the previous report by Tavakoli-Nezhad et al. (2001) showing a significant lead-induced decrease in the number of spontaneously active midbrain DA neurons in the SN and VTA. Based on TH immunopositive (TH+) cell counts, Tavakoli-Nezhad et al. (2001) suggested that lead exposure may have "silenced" the activity of some DA neurons without causing cell death. However, some of the physiologically "silent" neu-
rons might have been compromised by lead exposure and were silent because of ongoing pathological responses to the insult (e.g., necrosis or apoptosis). It remained possible that a significant decline in TH (+) neurons might be detected if a later period were evaluated after the initiation of lead exposure.

When the lead exposure was extended in duration in the present study, TH (+) cell counts were not found to be significantly different between sodium- and lead-treated groups. Therefore, it seems most probable that the significant lead-induced decrease in spontaneously active DA neurons observed in the electrophysiological studies was not due to cell death. These findings suggest that the predominant effect of the longer lead exposure regimen is an alteration in the midbrain DA neurons without lead-induced cell loss.

At least two different pathophysiological mechanisms could be proposed to explain the silenced DA neurons: 1) excess hyperpolarization of DA neurons or 2) excess depolarization of DA neurons (i.e., depolarization inactivation). Haloperidol, a selective DA D2-like receptor antagonist, has been shown to reduce the number of active DA neurons below control in both the SN and the VTA following a 3-week treatment (Bunney and Grace, 1978; Chiodo and Bunney, 1983; White and Wang, 1983). DA neurons in chronic haloperidol-treated animals have been shown to be in a tonic depolarized state (Bunney and Grace, 1978; Chiodo and Bunney, 1983). Therefore, if lead caused depolarization inactivation, systemic administration of the hyperpolarizing DA D2-like agonist apomorphine should restore DA neuron activity (e.g., see Chiodo and Bunney, 1983; White and Wang, 1983; Grace and Bunney, 1986).

Haloperidol was found to significantly decrease the number of spontaneously active VTA DA neurons in the present study. There was also a nonsignificant decrease in the number of spontaneously active SN DA neurons. Based on previous reports, hyperpolarization induced by systemic apomorphine should relieve DA neurons from depolarization inactivation by repolarizing the membrane and thereby increase the number of spontaneous active DA neurons in the haloperidol-treated rats relative to preapomorphine controls (e.g., see Bunney and Grace, 1978; Chiodo and Bunney, 1983; White and Wang, 1983). Following apomorphine challenge, the number of spontaneously active VTA DA neurons found in the haloperidol-treated group significantly increased relative to the preapomorphine control condition in this study. This result indicates that apomorphine was capable of reversing the effects of haloperidol on the VTA DA neurons. The lack of effect of apomorphine on the SN DA neurons of the haloperidol group is most likely due to the smaller non-significant reduction in the number of spontaneously active neurons in the preapomorphine challenge condition. Based on the utility of the haloperidol and apomorphine doses used, the best test of the hypothesis that lead exposure caused significant depolarization inactivation of midbrain DA neurons resides in the evaluation of the effects of apomorphine on VTA neurons from lead-treated animals.

The apomorphine challenge in the lead-treated group did not produce any significant effects. Given the expected significant decrease in the cells per track in the sodium-treated group and the significant apomorphine-induced reversal of the effects of haloperidol on the VTA neurons, it can be concluded that this otherwise active dose of apomorphine (50 μg/kg i.v.) did not affect the lead-treated group. The results of the present study indicate that the significant decrease in the number of spontaneously active VTA DA neurons following 250 ppm lead exposure cannot be readily explained by a depolarization inactivation mechanism. It does, however, remain possible that lead treatment interfered with the function of the somatodendritic autoreceptors, which mediate the hyperpolarizing effects of apomorphine. To the best of our knowledge, the effect of lead on somatodendritic autoreceptors has not been evaluated. It is also possible that this normally strongly hyperpolarizing apomorphine dose was not optimal for repolarizing DA neurons in the lead-treated group that may have been inactivated by a depolarizing mechanism.

The capacity of DA neurons to discharge in the burst mode provides a mechanism for phasic increases in DA levels within the synapse, which is spatially restricted to the synapse as a result of extremely efficient reuptake mechanisms that limit overflow into the extrasynaptic space (Floresco et al., 2003). A model has been described (Grace, 1991, 2000; Floresco et al., 2003), where the “background” or “tonic” level
of extrasynaptic DA present in the striatum is regulated by the total number of spontaneously active DA neurons present and by modulatory afferent activity at the level of the forebrain DA terminal. If there is no change in midbrain DA neuron discharge rate and pattern and afferent regulation of terminal release in the forebrain remains unchanged, a decrease in the population of spontaneously active spike-generating DA neurons would be expected to result in a reduction in the tonic release of DA and a reduction in extrasynaptic DA at terminal regions (Floresco et al., 2003).

In both the present and the previous study by Tavakoli-Nezhad et al. (2001), a significant lead-induced reduction in the number of spontaneously active DA neurons was detected, but significant alterations in discharge rate or percentage of bursting cells were not detected. These findings suggest that a lead-induced decrease in forebrain DA release would likely be observed after such an exposure. Although forebrain DA release was not examined in the present study, Kala and Jadhav (1995a) have reported a decrease in basal and K+-induced DA release in the nucleus accumbens induced by 50 ppm lead exposure for 90 days. The time course for the alteration in DA release following low-level lead exposure is apparently not known. Long-term alterations in the tonic level of extrasynaptic DA have been postulated to regulate post-synaptic DA receptor sensitivity through up-and-down-regulation (Grace, 1991). Since DA release was not measured in the present study, it is not known if the 250 ppm lead exposure altered DA release in the forebrain. The basal discharge rate of type I nucleus accumbens neurons was found to be significantly lower in the lead-treated (250 ppm) animals relative to controls. The mechanism for this effect is not known but could be mediated by direct effects on the type I nucleus accumbens neurons or indirectly through altered afferent activity. Our results indicate that lead exposure reduces the sensitivity of type I nucleus accumbens neurons to the inhibitory effects of the selective DA D1 receptor agonist SKF-38393 relative to controls and strongly suggests that lead exposure alters the sensitivity of DA D1 receptors in the nucleus accumbens. Therefore, at the 250 ppm-exposure level, lead depressed the discharge rate of type I nucleus accumbens neurons and decreased their sensitivity to DA D1 receptor stimulation. Similarly, Lewis and Pitts (2004) have reported that 250 ppm lead exposure results in a greatly attenuated ability of an acute amphetamine challenge to induce cFOS immunoreactivity in the striatum. Since it is known that DA D1 receptor stimulation is a necessary requirement for amphetamine-induced increase in cFOS immunoreactivity (Robertson et al., 1991), it is possible that the suppression of amphetamine-induced striatal cFOS expression by lead may involve down-regulation of striatal DA D1 receptors.

More than one cellular site could mediate the DA D1 receptor responses to iontophoretically applied SKF-38393 (Nicola et al., 2000). For example, it is possible that lead affected D1 receptors on glutamatergic afferent terminals (Nicola et al., 2000). Although the exact mechanism for the effect of lead on spontaneously active DA neurons has yet to be determined, the observation of decreased DA D1 receptor sensitivity in the nucleus accumbens would be consistent with at least two different scenarios: 1) a lead-induced increase in forebrain DA neurotransmission resulting in DA D1 receptor down-regulation and/or 2) a direct effect on postsynaptic targets of the midbrain DA system that results in an apparent down-regulation of DA D1 receptors. It is particularly noteworthy that prenatal or perinatal cocaine exposure (Minabe et al., 1992; Wang and Pitts, 1994), prenatal haloperidol exposure (Zhang et al., 1996), and prenatal ethanol exposure (Shen et al., 1999) have also been found to reduce the number of spontaneously active midbrain dopamine neurons in rats. These results and those of the present study suggest that developmental exposure to specific agents known to affect the midbrain dopaminergic system can result in relatively similar changes in the population activity of midbrain DA neurons. As also suggested by Shen et al. (1999), perturbation of DA neurotransmission during critical periods of development by certain agents/mechanisms may have relatively similar cellular outcomes that are potentially permanent in nature.

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