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Lead Exposure: Expression and Activity Levels of Oct-2 in the Developing Rat Brain

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Lead is a highly neurotoxic metal, and the developing central nervous system is particularly vulnerable to the effects of lead. In this study, transcription factors (TFs) that are altered due to lead exposure were identified using macroarray analysis. Rat pups were lactationally exposed to 0.2% lead acetate from birth through weaning. Changes in the developmental profiles of 30 TFs were screened in hippocampal tissue on postnatal day (PND) 5, 15, and 30. The temporal patterns of some TFs were transiently upregulated or repressed following lead exposure in a stage-specific manner; however, Oct-2, which is involved in the regulation of key developmental processes, exhibited sustained elevations during the entire period of study. Lead-induced elevation of Oct-2 was validated by reverse transcriptase–polymerase chain reaction analysis; however, significant elevation of Oct-2 mRNA expression was detected only on PND 5. The DNA-binding activity and protein levels of Oct-2 were further evaluated and found to be consistently induced on PND 5. The elevations observed in Oct-2 mRNA and protein levels as well as DNA-binding activity on PND 5 suggest that developmental maintenance of Oct-2 DNA binding could be impacted through *de novo* synthesis. These findings identify Oct-2 as a potential molecular target for Pb and suggest that Oct-2 may be associated with lead-induced disturbances in gene expression.

Key Words: **Oct-2; lead; development; brain; macroarray.**

Lead exposure and toxicity continue to be a major public health problem in urban areas in the United States and around the world. Cognitive deficits associated with lead exposure at blood lead concentrations lower than 5 μg/dl have also been documented (Lanphear *et al.*, 2000). Lead exposure affects the nervous system including cellular processes such as apoptosis, second messengers, and neurotransmission. Lead exposure can also affect several cell types in the brain, such as cerebrovascular endothelial cells, astroglia, and oligodendroglia (Faustman *et al.*, 2000). Although all of lead's toxic effects cannot be tied together by a single unifying mechanism, lead's ability to substitute for calcium (Ca) and zinc (Zn) is a feature common to many of its toxic actions (Bressler and Goldstein, 1991; Lidsky and Schneider, 2003; Zawia, 2003). The ability of lead to interfere with several signal transduction pathways and transcription factors (TFs) also results in alterations in gene expression and thereby interferes with multiple cellular events in the developing brain (Zawia and Harry, 1996). Lead has proven to be a potent toxicant capable of disrupting the activity of a number of important proteins as well as altering the expression patterns of numerous genes (Basha *et al.*, 2003; Bouton and Pevsner, 2000; Schmitt *et al.*, 1996; Zawia and Harry, 1996; Zawia *et al.*, 1994, 1998).

The study of gene expression and transcriptional regulation is an important aspect of understanding the mechanisms associated with lead neurotoxicity. A possible mechanism through which lead may cause neuronal damage is by perturbation of brain gene expression through alterations in numerous TFs and signal transduction intermediates involved in development and differentiation. Although the expression of a number of target genes and the involvement of some TFs has been shown to occur following exposure to lead (Basha *et al.*, 2003; Harry *et al.*, 1996; Hossain *et al.*, 2000; Kim *et al.*, 1997; Pennypaker *et al.*, 1997; Zawia and Harry, 1996), most of these characterizations were conducted *in vitro*. It is still unknown how many genes and which families of TFs are associated with the response to lead exposure.

The development of new approaches such as DNA and protein array techniques has provided a new tool to delineate the scope of TFs that might be involved in lead neurotoxicity and identify unknown TFs that respond to lead exposure. This study aims to utilize macroarray screening methods to determine and identify TFs that are specifically modulated by lead in an ontological manner. Unlike screens that are conducted at a single time point, this study examined changes in prototypical TFs at multiple times during postnatal development.

MATERIALS AND METHODS

Animal exposure. Timed-pregnant Long-Evans hooded rats were obtained from Charles River Laboratories (Wilmington, MA). Twenty-four hours following birth on postnatal day 1 (PND 1), all the pups were pooled and new litters consisting of eight to nine males were randomly selected and placed with

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each dam. Exposure to lead was initiated on PND 1 with the addition of 0.2% lead acetate (Sigma, St Louis, MO) to the deionized drinking water of the dam and continued until PND 20 (Atkins *et al.*, 2003; Basha *et al.*, 2003, 2005; Zawia *et al.*, 1998). Control dams received deionized drinking water. Food and water were freely available throughout the study, and the fluid consumption of dams was regularly monitored until weaning. The animals were housed at a constant temperature (21°C ± 2°C) and relative humidity (50 ± 10%) with a 12-h light/dark cycle (0700 h–1900 h). On selected time points (PND 5, 15, and 30), randomly selected pups were removed from each litter and replaced with filler pups to maintain a constant litter size. Animals were decapitated following exposure to CO_2 and hypothermia, and various brain regions were isolated and stored at − 80°C. The animal exposure protocol and the maintenance of the animal facilities were regularly monitored by the Institutional Animal Care and Use Committee of the University of Rhode Island.

Macroarray analysis. The *Message Hunter TM* macroarray screening system was used to study the simultaneous expression of multiple genes (Genotech, St Louis, MO). Blots contain the fragments of 15 genes from various families of TFs in variable concentrations (2 ng and 20 ng) and prearranged on nylon membranes, along with actin, reduced form of guanosine adenine dinucleotide phosphate (GAPDH), and pUC19 DNA as positive or negative controls. Samples of brain mRNA from control and Pb-exposed animals were isolated and reverse transcribed in the presence of [α^{32}P]-dCTP and hybridized to the gene array organized on the nylon membrane. Each gene on the membrane was 200 bp long and contained equimolar amount of DNA. Sequences corresponding to about 30 genes of representative TF families were immobilized on several filters. The filters were then stringently washed and exposed to x-ray films. The resultant bands were scanned and quantitated using UVP Epi chemi II Darkroom software. The data in each filter were normalized to the housekeeping genes on the same filter and then expressed as a percentage of the values derived from filters probed with RNA obtained from Pb-exposed animals. The array data were collected from two sets of experiments, and each set consisted of tissue pooled from three animals (Bakheet and Zawia, 2004).

Total RNA isolation and estimation. RNA was isolated according to the TRIzol method (Invitrogen, Carlsbad, CA). One milliliter of TRIzol Reagent was added to each sample. Then 0.1 ml of chloroform was added to the reaction mixture followed by centrifugation at 12,000 × g for 15 min. The RNA was recovered from the aqueous phase by precipitation with an equal volume of isopropanol and chilling at − 70°C for 30 min. The precipitated RNA was collected by centrifugation at 12,000 × g. The pellet was washed twice with 75% ethanol and twice with 100% ethanol, then air-dried and resuspended in 50 µl of RNAse-free water. Total RNA was estimated using a spectrophotometer (Beckman, Fullerton, CA). Readings at 260 nm were used to determine the concentration of the RNA samples. A 5-µg aliquot of the total RNA was fractionated on 1.0% agarose/formaldehyde gels to examine the quality of the RNA preparation.

Synthesis of cDNA. RNA was reverse transcribed to obtain cDNAs. The first-strand cDNA synthesis kit was obtained from Invitrogen. The reaction was catalyzed by SuperScript II RNase H reverse transcriptase (RT). The RNA/primer mixture containing 50 ng of total RNA, 1 µl of 10mM dNTP mix, and 1 µl oligo (dT) was incubated at 65°C for 5 min. A reaction mixture containing 10× RT buffer, 25mM $MgCl_2$, 0.1M dithiothreitol (DTT), and RNaseOUT recombinant RNase inhibitor was added and incubated at 42°C for 2 min. One microliter of SuperScript II RT was added and incubated at 42°C for 50 min. The reaction was terminated at 70°C for 15 min. One microliter of RNase H was then added and the reaction was incubated for 20 min at 37°C. The final product was stored at − 20°C and used in the polymerase chain reaction (PCR) step.

Polymerase chain reaction. Primers were selected from the Oct-2 gene sequences so that their amplified PCR products would include all spliced forms of the messages (sense: GGCCCTCAACCTGAGCTTCAAG; antisense: GATCAGCAGGATCTCCTCT). Control primer for housekeeping gene (GAPDH) was obtained commercially. The PCR step consisted of the reaction of 1 µl of the primer (20µM), 2 µl of the cDNA strand, and 46 µl Platinum PCR SuperMix obtained from Invitrogen. Using a MiniCycler (MJ Research, Inc., Waltham, MA), the reaction was then denatured at 94°C for 2 min and subsequently underwent 35 temperature cycles (95°C denaturing for 45 sec, 55°C annealing for 1 min, and 72°C elongation for 2 min). The reaction was then held at 72°C for 5 min for a final elongation period. The final PCR product was held at 4°C and then was run on a 1% agarose/ethidium bromide gel. The samples contained 10 µl of the final PCR product and 2 µl gel-loading dye (Sigma). The gel was run at 90 V in 0.5× TBE buffer for approximately 90 min and was analyzed in an imaging system.

Preparation of nuclear extracts. Nuclear proteins were extracted from hippocampal tissue according to the method given by Dignam *et al.* (1983), with slight modifications. Tissue samples were homogenized with 1 ml phosphate buffer saline (PBS, pH 7.4) and centrifuged at 2500 × g for 10 min. The pellets obtained were suspended in 5 volumes of buffer A (10mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid [HEPES] at pH 7.9, 1.5mM $MgCl_2$, 0.5mM DTT, 0.5mM ethylenediaminetetraacetic acid (EDTA), and 0.2mM phenylmethylsulfonyl fluoride [PMSF]) and centrifuged at 6000 × g for 2 min at 4°C. The pellets were resuspended in 3 volumes of buffer A and centrifuged at 6000 × g for 2 min at 4°C. The resulting pellets were then resuspended in 5 volumes of buffer C (20mM HEPES at pH 7.9, 1.5mM $MgCl_2$, 0.5mM DTT, 0.5mM EDTA, 420mM NaCl, 20% glycerol, 0.2mM PMSF, 2 µg/ml aprotinin, and 0.5 µg/ml leupeptin) and homogenized with a Polytron homogenizer. The final suspensions were centrifuged at 12,000 × g for 10 min at 4°C. The supernatants were transferred to 1.5-ml tubes, snap frozen in an ethanol dry-ice bath, and then stored at − 80°C (Basha *et al.*, 2003; Zawia *et al.*, 1998).

Gel mobility shift and supershift assay. Changes in the profiles of the Oct-2 TF were studied through electrophoretic mobility shift assay. Nuclear extracts were derived from the hippocampal tissues as described above. The consensus oligonucleotide sequence for Oct-2 was obtained from commercial sources (Promega, Madison, WI) and radiolabeled by 5′ labeling procedures. The oligo (1.75 pmol) was incubated with 1 µl [γ-^{32}P]adenosine triphosphate (3000 Ci/mmol, Perkin-Elmer, Boston, MA) and 10 units T4 polynucleotide kinase (Promega) in a final volume of 10 µl for 15 min at 37°C. The reaction was terminated and the DNA probe was isolated through purification with a Sephadex G-25 column. A binding reaction was set up containing 5000–20,000 cpm of the DNA probe (0.1–0.5 ng), 0.5–2 µg of poly–dI-dC, 5–20 µg of the nuclear extract and binding buffer (2mM HEPES, 2.5mM $MgCl_2$, 2.5mM KCl, 0.5mM DTT, 2.5% glycerol, 0.001% NP40, 50 µg/ml bovine serum albumin, 0.2% protease inhibitor cocktail [Sigma], 0.2% ficoll, and 20 ng/ml poly dI-dC). For supershift assay, 4 µl of Oct-2 rabbit polyclonal antibody (200 µg/0.1 ml) was added to the reaction mixture in order to identify the band corresponding to Oct-2 DNA binding. The binding reaction was carried out at room temperature for 15 min. The reaction mix was separated by 6% polyacrylamide gel electrophoresis (PAGE). The gel was dried and exposed to a phosphor screen for 2 h and scanned in the Typhoon Variable Mode Imager 9410. The resulting scanned gels were analyzed for shifted bands and quantitated using the Image Quant 5.0 software (Basha *et al.*, 2003; Zawia *et al.*, 1998).

Western blot analysis. Polyclonal antibodies for rabbit Oct-2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used to probe Western blots. Western blotting and quantitation procedures were carried out as described previously (Atwood *et al.*, 1998; Nunomura *et al.*, 1999) with slight modifications. The protein samples (80 µg) were subjected to sodium dodecyl sulfate–PAGE, transferred to nitrocellulose membranes, and probed with (Oct-2) polyclonal rabbit antibody. The blots were incubated with antirabbit horseradish peroxidase (Pierce, Rockford, IL) and the chemiluminescent signal was captured using Kodak Image Station, and the images were analyzed and quantified.

Lead determination. On PND 20, blood and hippocampal tissue were obtained from both the control and Pb-exposed animals. The samples were prepared for the estimation of tissue lead levels, as described in previous studies (Basha *et al.*, 2003, 2005) following the method of Smith *et al.* (1998). Then the

samples were analyzed at the RI analytical laboratory, Warwick, Rhode Island using Environmental Protection Agency–approved methodology (method: 200.9).

Statistical analysis. Data were expressed as mean ± SEM, and statistical significance was assessed using a two-way ANOVA followed by a Student-Newman-Keuls (SNK) *post hoc* test to compare the relationship among various groups. Differences were considered significant at $p < 0.05$.

RESULTS

Exposure to lead is known to alter a number of TFs in the rodent brain; however, the scope of changes is unknown. Macroarray screening analysis was conducted to identify the changes in the levels of certain TFs that were not previously implicated in mediating the effects of Pb exposure on the central nervous system. We also intended to examine the ontological patterns of the expression of a key TF across a dynamic range of time, that is, during postnatal development. The animals used in this study did not experience any gross nutritional disturbances such as body weight or overt behavioral changes. Postnatal exposure of pups to 0.2% lead acetate from birth to weaning resulted in significantly greater blood lead levels (46.43 ± 1.95 μg/dl) than that of the control group (< 2.0 μg/dl) in PND 20 animals (Basha *et al.*, 2003, 2005). Furthermore, tissue lead levels in the hippocampus were significantly higher in exposed animals (0.34 ± 0.012 μg/g) than those in the control group (< 0.2 μg/g).

Macroarray Screening Analysis

The mRNA expression profile of a number of TFs was altered following exposure to lead and 10 days after exposure to lead had ceased. While various TFs belonging to different families of TFs were upregulated, TFs belonging to the steroid receptor nuclear family of receptors were downregulated (Fig. 1). Ranking the changes of expression showed that different groups of TFs were highly altered depending on the period they were examined, except for Oct-2 which sustained the highest elevations across all the time points studied (Fig. 1).

Validation of Macroarray Analysis for Oct-2 by RT-PCR

The identity of Oct-2 and Sp1 TFs discovered by macroarray screening analysis was confirmed using RT-PCR techniques (Fig. 2). Sp1 was used to validate our screening process. Sp1 data were consistent with our previous published findings and thus are not presented (Basha *et al.*, 2003, 2005). The level of mRNA expression of Oct-2 was significantly elevated only on PND 5 in lead-exposed animals compared with controls (Fig. 2a), while the expression of the housekeeping gene GAPDH was not altered by lead (Fig. 2b). Oct-2 mRNA profiling by macroarray and RT-PCR techniques display similar expression profiles on PND 5 only.

Oct-2 DNA-Binding and Protein Levels

The DNA-binding activity of specific TFs is elevated by lead-induced increases in their level. Alterations in the DNA binding of TFs are brought about mainly by a buildup in their levels. Electrophoretic mobility shift assay and supershift assay was conducted using nuclear extracts from control and lead-exposed animals to compare whether lead-elevated Oct-2 expression corresponds to enhanced DNA-binding activity (Fig. 3a). The results showed that Oct-2 DNA-binding activity was significantly elevated following developmental exposure to lead on PND 5 and returned back to basal levels on subsequent time points (Fig. 3b). To test whether alterations observed in Oct-2 DNA binding are reflected in the Oct-2 protein, Oct-2 protein levels were evaluated by Western blot in control and Pb-exposed animals during PND 5, 15, and 30. Consistent with the results of Oct-2 DNA binding, the protein levels also exhibited a significant increase on PND 5 and gradually returned to basal levels on PND 15–30 (Fig. 3c).

DISCUSSION

Brain development is a very complex process executed by complicated and time-directed programs. TFs play an important role in ensuring that growth and differentiation occur in a coordinated manner that enables neurons to establish precise networks. Disturbances in these master regulators may have a long-term impact and affect the ultimate makeup of the brain.

Lead-induced learning impairments have been investigated following postnatal exposure in several areas of the brain, including the hippocampus (Brown, 1975; Cory-Slechta and Thompson, 1979). The hippocampus has been specifically implicated in lead toxicity because of its suggested role in learning and the high concentrations of zinc and exogenous heavy metals that are commonly found in this region. The major growth and development of the hippocampus occurs postnatally, and thus, this region serves as a good model to identify key TFs that play a major role during hippocampal development and the impact of environmental agents on developmental events in the brain (Brown, 1975; Cory-Slechta and Thompson, 1979).

The early and marked induction in Oct-2 mRNA expression on PND 5 revealed by macroarray analysis suggests that this TF plays a key role in the early development of the hippocampus. This was validated by RT-PCR analysis; however, this method did not detect changes beyond PND 5. This discrepancy between macroarry analysis and RT-PCR profiling maybe due to inherent problems with macroarray screening methods as experienced by others (El Yahyaoui *et al.*, 2004; Sgarlato *et al.*, 2005; Weber and Jung, 2002). Nevertheless, the macroarray screening can help in identifying potential changes; it requires further confirmation and validation.

In addition to TFs associated with growth and development, such as Oct-2, numerous other TFs were modulated by lead exposure; however, the changes in the expression of these TFs were less marked (Fig. 1). The macroarray screening analysis

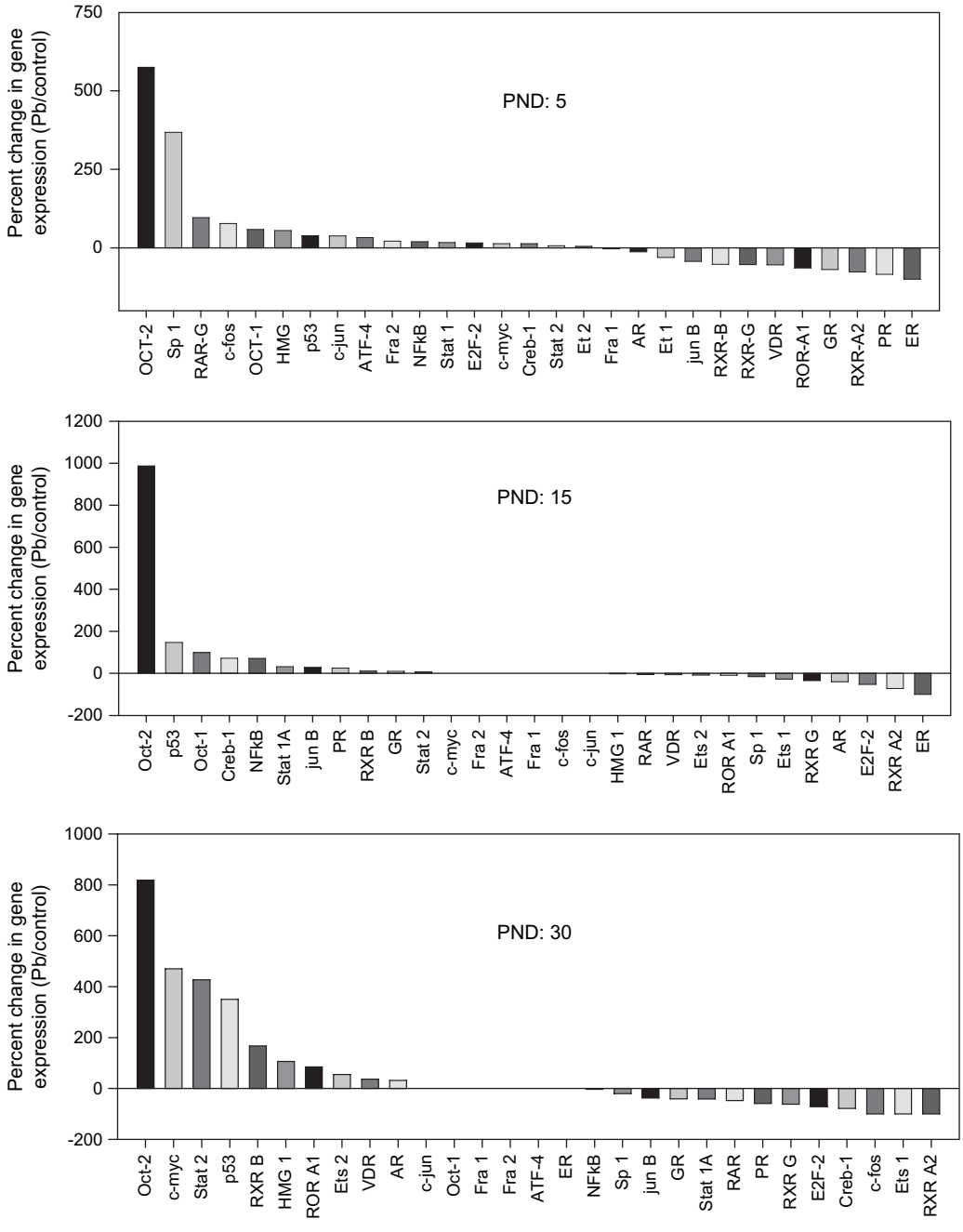

FIG. 1. Macroarray screening profiles of a series of TFs following developmental exposure to lead in the hippocampus of control and lead-exposed animals. Total RNA was isolated from pooled hippocampi of control and lead-exposed animals on PND 5, 15, and 30 and was reverse transcribed to cDNA while simultaneously incorporating radiolabeled dCTP. The labeled cDNA was then hybridized with Genotech nylon filters, washed, exposed to x-ray film, and the resulting bands were then digitized. Data were normalized for each filter and are expressed above as a percent change in lead group versus the control group. The array data shown were from two sets of experiments, and each set consisted of tissue pooled from 3 animals. AP-1, activator protein-1 (dimers of: Fos, Jun, Fra products); AR, androgen receptor; c-myc, oncogene; CREB, cAMP responsive element–binding protein; ER, estrogen receptor; Et, erythroblastosis virus oncogene homology; GR, glucocorticoid receptor; HMG, high-mobility group protein; NFκB, nuclear factor kappa B; Oct, octamer transcription factor; p53, tumor suppressor protein; PR, progesterone receptor; RAR, retinoic acid receptor (related factors: ROR and RXR); Sp, specificity protein; STAT, signal transducer and activator of transcription; VDR, vitamin D3 receptor.

also showed the upregulation of genes, which are activated later on during the period of myelination and glial maturation, such as Stat-2 and c-myc, as well as TFs associated with DNA damage, inflammation, and stress (Brivanlou and Darnell, 2002; Harry *et al.*, 1996; Zawia, 2003). The inductions in these TFs were sustained long after lead exposure had ended (Fig. 1). Moreover, the screening analysis revealed an overall pattern in which the steroid receptor superfamily was

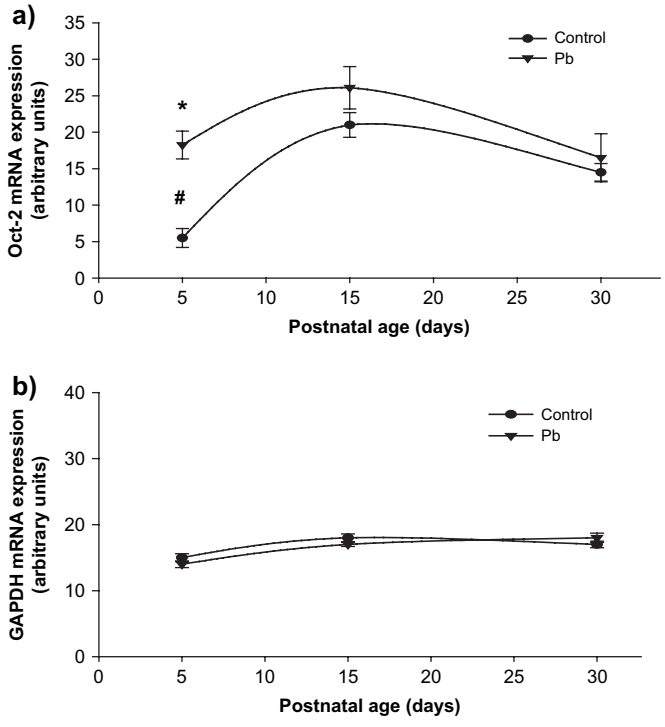

FIG. 2. Developmental profiles of (a) Oct-2 and (b) GAPDH mRNA expression in the hippocampus of control and lead-exposed animals. mRNA levels of both Oct-2 and GAPDH were analyzed using the RT-PCR technique, and its corresponding bands were quantitated using image acquisition and analysis software (UVP Lab. Products, CA). Values in the graphs indicate mean ± SEM of three to four independent determinations. The values marked with "*" are significantly different over corresponding controls, and the values marked with "#" are significantly different among various time points within the same group, as evaluated by ANOVA followed by SNK's *post hoc* test ($p < 0.05$).

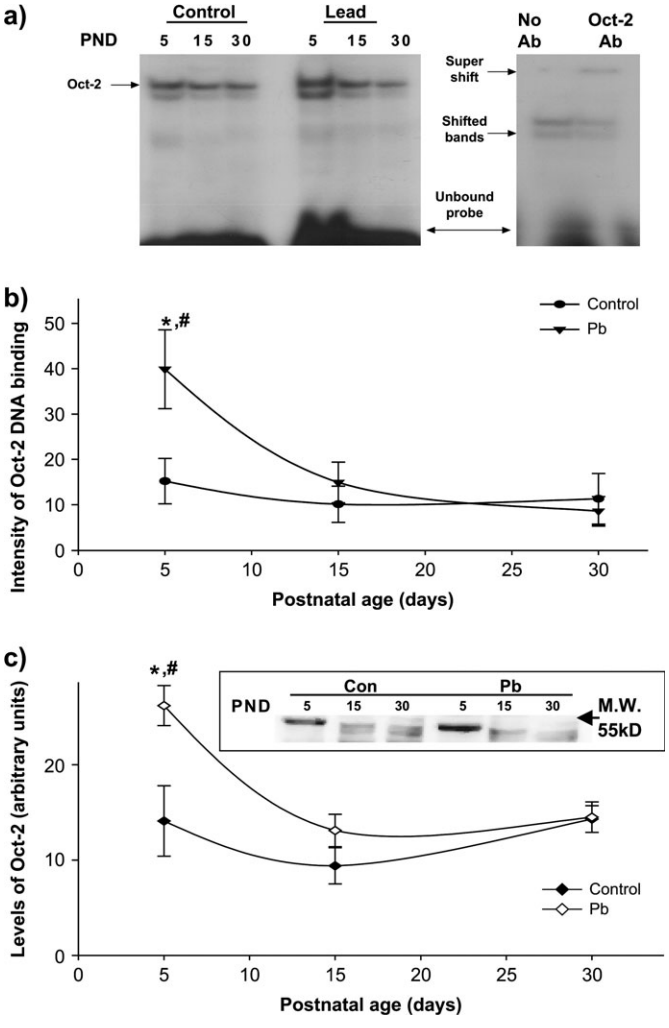

FIG. 3. Changes in the developmental profile of Oct-2 DNA binding and Oct-2 protein levels in the rat hippocampus. Oct-2 DNA binding was monitored in the nuclear extracts prepared from the control and Pb-exposed rats using the gel mobility shift assay. (a) A representative autoradiogram shows the shifted and supershifted bands. (b) Shifted bands were scanned and quantitated using image acquisition and analysis software (UVP Lab. Products, Upland, CA) and the data were shown in the graph for PND 5, 15, and 30. (c) Hippocampal nuclear protein extracts were prepared from the control and Pb-exposed hippocampal tissue and the Oct-2 levels were examined by Western blot analysis. All the values are mean ± SEM of four individual observations. The values marked with "*" are significantly different over corresponding controls, and the values marked with "#" are significantly different among various time points within the same group as evaluated by the ANOVA followed by SNK's *post hoc* test ($p < 0.05$).

consistently downregulated. The significance of all these alterations is not clear and should not be viewed as conclusive until future confirmation and validation is undertaken.

The discovery that Oct-2 mRNA expression was markedly increased by lead exposure required further validation and characterization. It was important to monitor whether changes in mRNA expression influenced the functionality of the Oct-2 TF. The induction of Oct-2 expression by lead exposure on PND 5 was accompanied by a rise in Oct-2 DNA binding as well as protein levels (Figs. 3b and c). The correspondence between Oct-2 mRNA and protein levels as well as DNA binding on PND 5 suggests that Oct-2 activity during early development is maintained through rapid *de novo* synthesis. Similar mechanism has been previously proposed for the TFs associated with developmental process (Brivanlou and Darnell, 2002).

In addition to the current study and our preliminary report (Bakheet and Zawia, 2002), there is just about a single report that has examined the effect of lead exposure on the expression and activity of Oct-2 (Chen *et al.*, 2004). The results of Chen *et al.*, which were conducted *in vitro*, demonstrated that exposure of cultured hippocampal cells to lead induce Oct-2 expression. The agreement between these studies suggests that Oct-2 is an important mediator of the effects of lead on growth and development. The association of Pit-Oct-Unc (POU) family of TFs, originally named for its founder members Pit-1, Oct-1 and -2, and *unc*-86, in the neurotoxicity of lead has been further reinforced by the work of Chang *et al.* (2006), who showed that lead causes alterations in the expression of Brn-3a,

a member of the POU family of TF predominantly expressed in the neuronal cells.

These experiments have identified Oct-2 as a key TF, which mediates lead-induced changes in developmental gene expression. Oct-2 is a member of the POU domain factors. The POU family as well as a large number of different TFs are present in both vertebrates and invertebrates. The POU domain common to these factors constitutes the DNA-binding domain of the protein and consists of two highly conserved regions: a POU-specific domain (approximately 75 amino acid N-terminal region), which is unique to these factors, and a POU homeodomain (60 amino acid C-terminal region), which is related to that found in the homeobox proteins. Homeodomain proteins comprise a superfamily of highly conserved DNA-binding factors that are involved in the transcriptional regulation of key developmental processes such as embryogenesis and histone gene expression (Latchman, 1999a,b; Phillips and Luisi, 2000).

In summary, we have employed macroarray screening analysis to simultaneously detect the expression levels of several TFs in the hippocampus of lead exposed animals during various periods of postnatal development. The findings of this study identify Oct-2 TF as an early, novel *in vivo* molecular target of lead. We suggest that the POU homeodomain TFs may play a role in lead neurotoxicity during brain development and their analysis in other brain regions should be pursued.

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