ENVIRONMENTAL LEAD EXPOSURE AND DOPAMINERGIC DYSFUNCTION

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Abstract

Parkinson Disease (PD) is a chronic and progressive neurodegenerative disorder that affects millions of Americans. Although several genes have been identified in the pathology of PD, approximately 90% of PD cases are of unknown origin. Epidemiological studies have recently shown the prevalence of PD in US states is positively associated with the quantity of acid rain where it is hypothesized that acid rain could mobilize metals, such as lead (Pb^{2+}), from the soil increasing their levels in drinking water. In addition, there is a significant positive correlation between lead municipal drinking water service lines still utilized in the US with the prevalence of PD. Exposure to Pb^{2+} has been implicated in neurotoxicity especially with regard to dopamine (DA) producing neurons. Dopaminergic (DAergic) neurons release DA which controls motor function, mood, reward and cognition. Extraneuronal DA levels are controlled spatially and temporally by DA transporter (DAT)-mediated uptake of released transmitter back into presynaptic neurons. The experiments proposed in this study are consistent with the priorities and goals of the DaCCoTA Translating Epidemiology to Experiments (TREE) pilot grant mechanism where we translate the epidemiologic findings implicating Pb^{2+} in drinking water with the risk of PD to potential altered DAT function and extraneuronal DA clearance in cellular and animal model systems. Preliminary results suggest that DA uptake in kidney epithelial cells expressing the DAT are not affected by acute exposure to Pb^{2+} (0-500 µM), however, ongoing studies in neuronal and animal models system may reveal effects of chronic exposure.

Methods

Cell Culture. Lilly Laboratory Cell Porcine Kidney ($LLC\text{-}PK_1$) cells or immortalized rat dopaminergic neurons (N27 cells) stably expressing WT rDAT were grown in Dulbecco's Modified Eagle Medium (DMEM) or RPMI medium containing 5% fetal bovine serum, 100 µg/mL penicillin/streptomycin, and supplemented with 200 µg/mL of Geneticin (G418) for maintenance of stable expression. Cells were maintained in a humidified incubator gassed with 5% CO_2 at 37˚C. DAT expression levels were verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting of the cellular lysates against human DAT specific antibody (Mab16).

Synaptosome Preparation. $LLC\text{-}PK_1$ cells expressing the indicated NETs were grown in 100 mm plates to 85% confluency. Cells were washed twice with 3 mL of ice-cold Buffer B (0.25 mM sucrose, 10 mM triethanolamine, pH 7.8), scraped, and collected in 500 µL of Buffer B containing a protease inhibitor cocktail of 1 µM phenylmethylsulphonyl fluoride (PMSF) and 5 µM Ethylenediaminetetraacetic acid (EDTA) at 4˚C and transferred to a 2 mL microcentrifuge tube. Cells were then pelleted *via* centrifugation at 3,000 *x g* for 5 min at 4˚C, the supernatant fraction was removed, and the cell pellet was suspended in 1 mL of ice-cold Buffer C (0.25 M sucrose, 10 mM triethanolamine, 1 mM EDTA, 1 µM PMSF, pH 7.8) and subsequently homogenized *via* 30 strokes of the pestle in a Dounce homogenizer. Homogenates were cleared of cellular debris and nuclei by centrifugation at 800 *x g* for 10 min. The post-nuclear supernatant fraction was collected and centrifuged at 18,000 *x g* for 12 min at 4°C to pellet cell membranes. The resulting membrane pellet was suspended in 1mL of sucrose phosphate (SP) buffer (10 mM sodium phosphate, 0.32 M sucrose, pH 7.4 with 1 µM PMSF and 5 µM EDTA) and assayed for protein concentration.

Analysis of Palmitoylation by Acyl-Biotinyl Exchange. Palmitoylation of DAT will be assessed by the acyl-biotinyl exchange assay (ABE) using a method modified from Wan et al (Fig 1). Protein (400 - 800 µg) from synaptosome samples prepared as described above are solubilized in lysis buffer (50 mM HEPES, pH 7.0, 2% SDS, 1 mM EDTA) containing 25 mM NEM to block free thiols. Lysates were incubated for 20 minutes at 37˚ C followed by end-over-end mixing at ambient temperature for 1 hour followed by acetone precipitation, three times consecutively, and re-suspension in lysis buffer containing NEM and incubation at ambient temperature overnight with end-over-end mixing. Excess NEM was removed by two sequential acetone precipitations followed by re-suspension of the precipitated proteins in 200 µL of a buffer containing 4% (w/v) SDS (4SB: 4% SDS, 50 mM Tris, 5 mM EDTA, pH 7.4). Each sample was divided into two equal portions (100 µL) that were treated for 2 hours at ambient temperature with 50 mM Tris, pH 7.4, as control or 0.7 M hydroxylamine (NH_2OH, a reducing agent), pH 7.4, to cleave thioester bonds, removing palmitate from cysteine sulfhydryls. NH_2OH was removed by three sequential acetone precipitations followed by re-suspension of the precipitated proteins in 150 µL of 4SB buffer followed by dilution with 1000 µL (1 ml) of 50 mM Tris, pH 7.4, containing 4 mM sulfhydryl-reactive (N-(6-(biotinamido)hexyl)-3'(2'-pyridyldithio)-propionamide (HPDP-Biotin) and incubation overnight at 4 ˚ C with end-over-end mixing. Unreacted (N-(6-(biotinamido)hexyl)-3'-(2'-pyridyldithio)-propionamide was removed by three sequential acetone precipitations followed by resuspension of the final pellet in 75 µL of lysis buffer without NEM. Samples were diluted (1:20) with 50 mM Tris, pH 7.4, to contain 0.1% SDS, and the biotinylated proteins in samples were affinity-purified using NeutrAvidin resin. Proteins are eluted with sample buffer (60 mM Tris, pH 6.8, 2% SDS, 10% glycerol) containing 100 mM dithiothreitol and 3% 2-mercaptoethanol and subjected to SDS-PAGE and immunoblotting using anti-rat DAT monoclonal antibody (MAb16).

DA Uptake analysis. $LLC\text{-}PK_1$ cells expressing DAT were grown in 24-well plates until 80% confluent. The cells were washed twice with 0.5 mL 37°C Krebs Ringers HEPES (KRH: 120 mM NaCl, 5mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM $NaHCO_3$, 5.5 mM HEPES, 1mM D-glucose) and DA uptake was immediately conducted for 8 min at 37°C with 0.32, 0.6, 1, 3, 10, and 20 µM total DA containing 10 nM [^3H]DA; nonspecific uptake was determined in the presence of 100 µM cocaine. Cells were rapidly washed twice with 500 µL ice-cold KRH buffer and lysed with 1% Tx-100 for at least 20 min with rocking at ambient temperature. Lysates were collected and analyzed for [^3H] content by liquid scintillation counting. Uptake values were normalized to total cellular protein (pmol/min/mg).

Acyl-Biotinyl Exchange (ABE)

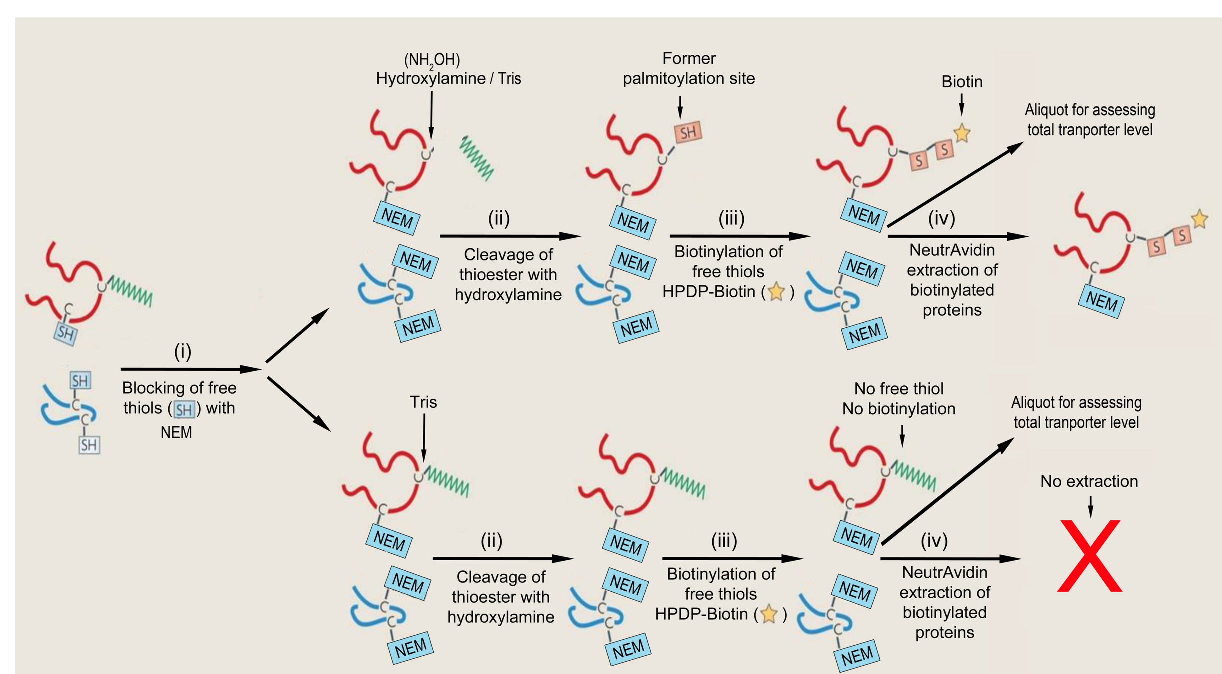

Figure 1. Schematic diagram of Acyl Biotinyl Exchange (ABE). The blocking of free thiols with NEM is followed by the sample being spilt into two equal portions. The experimental portion is subsequently treated with hydroxylamine (NH_2OH) to hydrolyze thioester bonds and remove the palmitate group while the other is treated with Tris as a negative control. After treatment, both samples are incubated with HPDP-biotin which conjugates with the free thiols generated by NH_2OH treatment. The biotinylated proteins are then affinity purified using NeutrAvidin resin and the protein of interest is identified by immunoblotting.

Purpose

- The goal of this study is to translate epidemiologic data implicating (Pb^{2+}) in water with the risk of Parkinson Disease (PD) to an experimental setting. Thus, this research is directly responsive to the TREE (Translating Research in Epidemiology to Experiment) mechanism in the DaCCoTA NIH grant.
  - Epidemiological studies have recently shown the prevalence of PD in US states is positively associated with the quantity of acid rain where it is hypothesized that acid rain could mobilize metals, such as lead (Pb^{2+}), from the soil increasing their levels in drinking water (Fig. 2).
  - It was also shown that there is a significant positive correlation between the presence of lead water service lines, which are common private lines still in use in the US that deliver water from the municipal water main to the home, with the prevalence of PD.
- The cause of PD in most individuals is unknown but involves the degeneration of dopaminergic (DAergic) neurons in the brain resulting in motor deficits and cognitive changes.
  - DAergic neurons release dopamine (DA), which controls motor function, mood, reward and cognition.
  - Extraneuronal DA levels are controlled spatially and temporally by DA transporter (DAT)-mediated uptake of released transmitter back into presynaptic neurons (Fig. 3).
  - DAT activity or transport capacity is regulated by the posttranslational modifications, phosphorylation and palmitoylation (Fig. 4), which are modulated by protein kinase C (PKC) activation.
  - Pb^{2+} alters calcium (Ca^{2+})-mediated cellular processes, is stored in bone and mimics Ca^{2+} in binding and activating protein kinase C (PKC).
  - Studies with C. elegans suggest that Pb^{2+}-induced neurotoxicity involves the DAT and that PKC-deficient worms are resistant to Pb^{2+} toxicity.
  - Together, these studies suggest that chronic PKC activation, which results in DAT phosphorylation and decreased DAT palmitoylation, may be influenced by Pb^{2+} and result in altered dopamine homeostasis and neuronal degradation.

Figure 2. Potential role of Pb^{2+} in drinking water with dopaminergic dysfunction via DAT dysregulation and the development of Parkinson Disease.

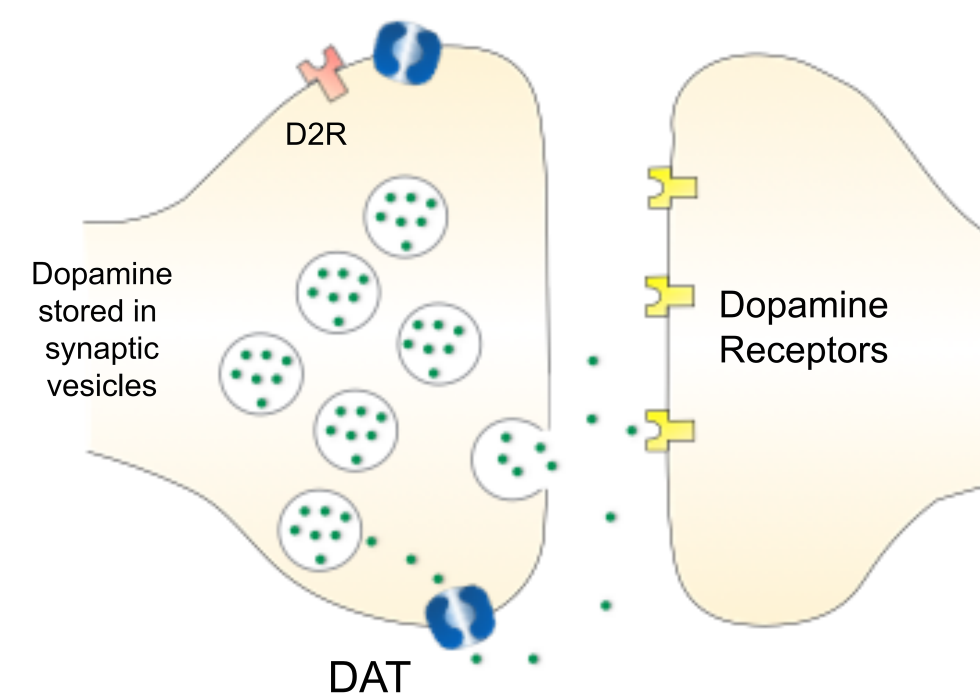

Figure 3. Dopaminergic synaptic neurotransmission. Dopamine is released from the presynaptic neuron into the synapse where it can bind and activate receptors on the postsynaptic neuron. The dopamine transporter (DAT) translocates dopamine back into the presynaptic neuron where it is repackaged into synaptic vesicles thereby terminating synaptic transmission. This is a tightly regulated process where it is essential that dopamine is sequestered and not allowed to be oxidized which can cause neuronal degradation.

Preliminary Results

DA uptake in $LLCPK_1$ cells expressing WT rDAT in the absence and presence of Pb^{2+} for 2 hours

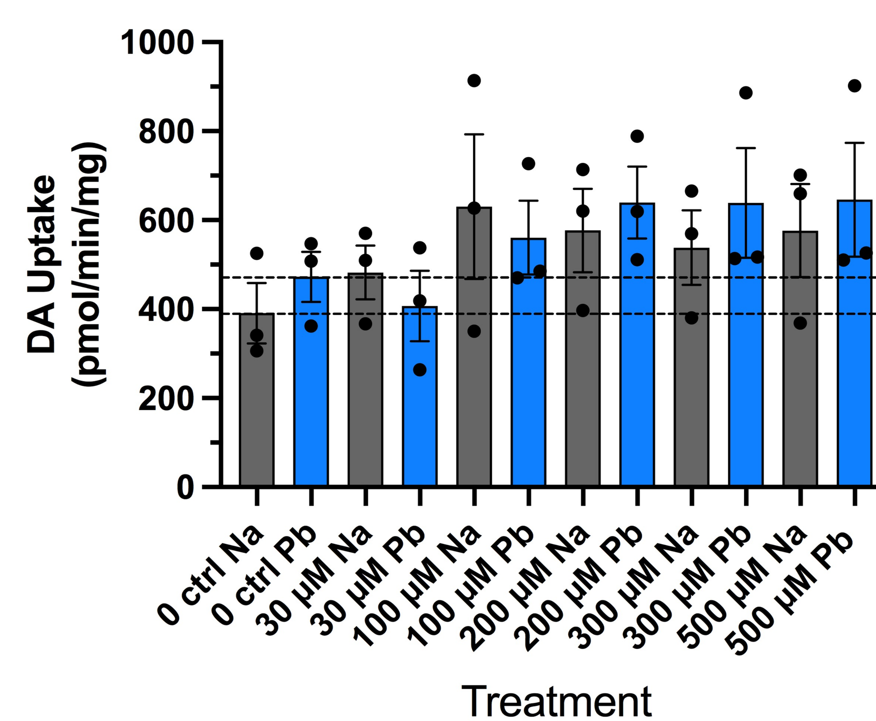

Figure 5. Lead Acetate had no affect on DA uptake in LLCPK cells expressing the rat DAT. Cell were incubated with the indicated concentrations (0-500 µM) of either sodium acetate ( ) or lead acetate ( ) in KRH buffer, pH 7.4, for 2 hours at 37°C followed by DA uptake assay with 3 µM total DA. Data are expressed as mean pmol/min/mg protein ± SD ; n=3.

S-Palmitoylation

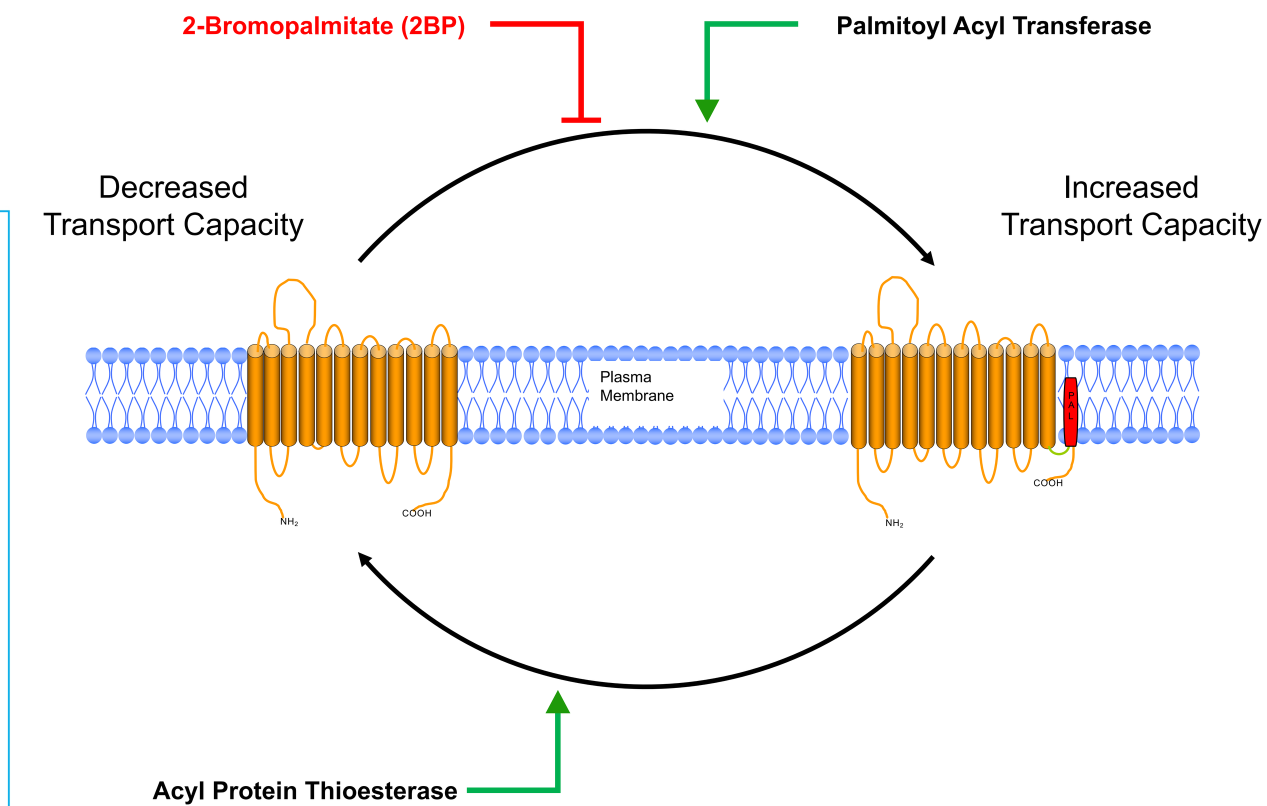

Figure 4. S-Palmitoylation is the reversible addition of palmitate to proteins through a thioester linkage. Palmitoylation status of a protein is modulated by palmitoyl acyl transferases (PATs) and acyl protein thioesterases (APTs). PATs catalyze the addition of palmitate to the protein at cysteine residues while APTs remove palmitate from the protein. 2-Bromopalmite is an irreversible inhibitor of PATs that inhibits palmitate incorporation into proteins. Palmitoylation is unique in that it is reversible, and no universal sequence motif exists for this lipid modification other than the presence of a cysteine residue.

Conclusions

1. Two hours of exposure with up to 500 µM Pb^{2+} had no effect on DA uptake in LLCPK kidney epithelial cells expressing the DAT suggesting that this model cell system may be resistant to the deleterious effects of Pb^{2+} or greater times of exposure are necessary for effects on uptake to be seen.
2. Follow up studies in a neuronal model are underway and this model system may be more sensitive to the effects of Pb^{2+}.
3. Long-term (30 day) exposure studies are underway where animals will be exposed to to lower and higher concentrations (300 ppm and 1000 ppm) of Pb^{2+} in their drinking water. We anticipate this chronic exposure will lead to altered DA uptake and DAT palmitoylation and phosphorylation status in striatal synaptosomes isolated from these animals.

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