Enhanced Nitric Oxide Production during Lead (Pb2+) Exposure Recovers Protein Expression but not Presynaptic Localization of Synaptic Proteins in Developing Hippocampal Neurons

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Abstract

We have previously reported that lead (Pb^{2+}) exposure results in both presynaptic and postsynaptic changes in developing neurons as a result of inhibition of the N-methyl-D-aspartate receptor (NMDAR). NMDAR inhibition by Pb^{2+} during synaptogenesis disrupts downstream trans-synaptic signaling of brain-derived neurotrophic factor (BDNF) and exogenous addition of BDNF can recover the effects of Pb^{2+} on both presynaptic protein expression and presynaptic vesicular release. NMDAR activity can modulate other trans-synaptic signaling pathways, such as nitric oxide (NO) signaling. Thus, it is possible that other trans-synaptic pathways in addition to BDNF signaling may be disrupted by Pb^{2+} exposure. The current study investigated whether exogenous addition of NO could recover the presynaptic vesicular proteins lost as a result of Pb^{2+} exposure during synaptogenesis, namely Synaptophysin (Syn) and Synaptobrevin (Syb). We observed that exogenous addition of NO during Pb^{2+} exposure results in complete recovery of whole-cell Syn levels and partial recovery of Syn and Syb synaptic targeting in Pb^{2+}-exposed neurons.

Keywords

Pb^{2+}; Nitric oxide; synaptophysin; synaptobrevin; synaptogenesis; hippocampal neurons

1. Introduction

Lead (Pb^{2+}) is a ubiquitous environmental neurotoxicant which causes cognitive and behavioral deficits in exposed children and disrupts spatial learning tasks in animal models (Jusko et al., 2008; Lanphear et al., 2005; Toscano and Guilarte, 2005). These neurological effects of Pb^{2+} exposure are believed to be mediated by its interaction with the N-methyl-D-

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aspartate receptor (NMDAR). Pb^{2+} is a potent inhibitor of the NMDAR (Alkondon et al., 1990; Guilarte and Miceli, 1992; Paoletti et al., 2000; Rachline et al., 2005), which is essential for spatial memory processes in the hippocampus (Bliss and Collingridge, 1993; Lynch, 2004).

The NMDAR is a heteromultimeric ion channel composed of an obligatory NR1 subunit and accessory NR2 or NR3 subunits. In the developing hippocampus, NR2A and NR2B are the predominant accessory NMDAR subunits and exhibit differential expression during development. Early in hippocampal development, NR2B subunits predominate while NR2A subunits become increasingly incorporated as development progresses (Monyer et al., 1994). However, in rodent models of chronic developmental Pb^{2+} exposure, there is decreased protein and mRNA levels of NR2A (Guilarte and McGlothan, 1998; Nihei et al., 2000; Nihei and Guilarte, 1999; Zhang et al., 2002) and increased levels of NR2B-containing NMDARs (NR2B-NMDARs) (Toscano et al., 2002). Furthermore, we have recently shown that exposure to Pb^{2+} during synaptogenesis in primary hippocampal neurons results in decreased levels of synaptic NR2A-containing NMDARs (NR2A-NMDARs) and an increase in NR2B-NMDARs (Neal et al., 2011; Neal and Guilarte, 2010). This change in NMDAR subunit ontogeny, detected in both animal and cell culture models of Pb^{2+} exposure, suggests that Pb^{2+} exposure arrests or delays the critical developmental switch from NR2B- to NR2A-NMDARs (Neal et al., 2011; Neal and Guilarte, 2010; Toscano and Guilarte, 2005)

Disruption of normal NMDAR ontogeny can have consequences on neuronal signaling. NMDARs exhibit distinct downstream signaling based on subunit composition. NR2A-NMDARs are responsible for activating pro-survival pathways including cyclic AMP response element binding protein (CREB)-mediated signaling and increasing expression of the neurotrophin brain-derived neurotrophic factor (BDNF). Alternately, NR2B-receptors are responsible for activating pro-death pathways and CREB shutoff (Hardingham et al., 2002; Ivanov et al., 2006; Soriano et al., 2008; Vanhoutte and Bading, 2003). Thus, altered NMDAR subunit composition due to chronic Pb^{2+} exposure may result in changes in intracellular signaling. This hypothesis is supported by evidence of altered MAPK signaling (Cordova et al., 2004), calcium/calmodulin kinase II (CamKII) activity (Toscano et al., 2005), and CREB phosphorylation status and binding affinity in the hippocampi of animals developmentally exposed to Pb^{2+} (Toscano et al., 2003; Toscano et al., 2002).

NMDAR activity has been linked to the production and release of BDNF (Hartmann et al., 2001; Jiang et al., 2005; Walz et al., 2006), a trans-synaptic signaling molecule implicated in presynaptic plasticity and development (Cohen-Cory et al., 2010). Postsynaptically-derived BDNF can stimulate glutamate release and stabilize nascent synapses (Magby et al., 2006; Walz et al., 2006), and in the absence of BDNF vesicular protein levels are reduced and vesicular release is impaired (Hu et al., 2005; Pozzo-Miller et al., 1999). We have previously shown that Pb^{2+} exposure during synaptogenesis in rat hippocampal neurons results in a selective decrease in the levels of Synaptobrevin (Syb) and Synaptophysin (Syn) (Neal et al., 2010), two proteins involved in vesicular release and recycling (Daly and Ziff, 2002; Deak et al., 2004; Pennuto et al., 2003; Schoch et al., 2001), which is consistent with interruption of NMDAR-dependent BDNF signaling (Hu et al., 2005; Pozzo-Miller et al., 1999). The loss of these proteins results in significant impairments in vesicular release (Neal et al., 2010), and may underlie the altered neurotransmission reported in animals chronically exposed to Pb^{2+} (Gilbert et al., 1999). Furthermore, exogenous addition of BDNF remediated presynaptic effects of Pb^{2+}, suggesting that disruption of NMDAR activity-dependent BDNF signaling is responsible for some of the effects of Pb^{2+} exposure on presynaptic plasticity (Neal et al., 2010).

Nitric oxide (NO) production has also been linked to NMDAR activity. Nitric oxide synthase (nNOS) has been shown to be associated with both the NR2A and NR2B NMDAR subunits, and is activated by NMDAR activity-dependent phosphorylation (Al Hallaq et al., 2007; Christopherson et al., 1999; Rameau et al., 2007; Rameau et al., 2004). Increased nNOS activity increases the levels of NO, which can act locally in the postsynaptic density or diffuse to the presynaptic active zone to stimulate soluable guanylyl cyclase (sGC) and increase cyclic GMP production (Arancio et al., 1996). Cyclic GMP signaling has been associated with enhanced neurotransmitter release, increased release volume (Arancio et al., 1996; O'Dell et al., 1991; Steinert et al., 2008), and increased Syn expression at glutamatergic synapses *in vitro* (Wang et al., 2005) and *in vivo* (Ota et al, 2010). Thus, disruption of NMDAR-dependent NO signaling by Pb^{2+} may account for some of the presynaptic changes associated with chronic Pb^{2+} exposure. The current studies were undertaken to determine whether exogenous addition of NO could recover presynaptic protein levels lost as a result of Pb^{2+} exposure during synaptogenesis. We observed that exogenous addition of NO for the final 24 hours of Pb^{2+} exposure in primary hippocampal neurons fully recovered Syn whole-cell levels but did not remediate the effects of Pb^{2+} on the synaptic targeting of Syn and Syb.

2. Results

In the current study we used a primary hippocampal culture system as described previously (Neal et al, 2011; Neal et al, 2010). Briefly, hippocampi were removed from E18 rat embryos and grown in culture for seven days (DIV7), at which point they were exposed to either vehicle- or 1.0 μM Pb^{2+}-containing feeding media. Pb^{2+} exposure lasted for 5 days and cells were harvested on DIV12. The current work was originally undertaken at the same time as our previously published studies on the effect of exogenous addition of 25 ng/mL BDNF for the final 24 hours of Pb^{2+} exposure (Neal et al., 2010). The present work is focused on sister experiments on the effect of exogenous NO for the final 24 hours of Pb^{2+} exposure using the NO donor, DETA NONOate (DETA).

We first determined that exposure to neither 1.0 μM Pb^{2+} nor 10 μM DETA resulted in a loss of neuron viability (Figure 1A). Cultures treated with Pb^{2+} and/or DETA exhibited similar viability relative to control. We verified that DETA spontaneously released NO by assessing the levels of stable NO decomposition products with the Greiss reaction (Figure 1B), which is a colorimetric assay designed to detect the levels of nitrite in biological media (Green et al., 1982). 10 μM DETA significantly increased the levels of NO decomposition products in both control- and Pb^{2+}-treated cultures (p<0.01). We observed that control cultures treated with 10 μM DETA for 24 hours experienced a rise in nitrite levels from 1.7 ± 0.4 μM to 4.7 ± 0.7 μM and Pb^{2+}-exposed cultures experienced a rise from 1.1 ± 0.7 μM to 4.7 ± 0.4 μM. Thus, incubation with 10 μM DETA for the final 24 hours of Pb^{2+} exposure increased the levels of NO present by about 3-fold but did not cause a reduction in cell viability for either control or Pb^{2+}-treated cultures.

In our previous work we observed that Pb^{2+} reduced Syn whole-cell and presynaptic expression in a dose-dependent manner (Neal et al, 2010). Others have shown that Syn expression increases as a result of NO signaling at glutamatergic synapses (Ota et al, 2010; Wang et al, 2005). In the present study we investigated whether the decrease in Syn protein levels by Pb^{2+} could be remediated by incubation with 10 μM DETA for the final 24 hours of Pb^{2+} exposure. As shown in Figure 2, we observed a similar decrease in Syn levels during Pb^{2+} exposure as previously published (decrease to $85.5 \pm 3.0\%$ of control, p<0.05). This loss of Syn protein was completely recovered by exposure to DETA (recovery to $104.8 \pm 4.1\%$ of control, p<0.05). However, we also observed that exposure to DETA alone (without Pb^{2+} exposure) resulted in a significant elevation of Syn protein relative to control cells

(elevation to $113.5 \pm 6.9\%$, $p<0.05$). In contrast we did not observe any significant effect of Pb^{2+} or DETA on Syb whole-cell expression, although a non-significant decrease during Pb^{2+} exposure occurred. This would suggest that the whole-cell expression of Syn (but not Syb) is linked to NO signaling, in agreement with other work (Ota et al, 2010; Wang et al, 2005), and further shows that NO supplementation can fully recover whole-cell Syn expression in Pb^{2+}-exposed neurons.

Next we determined whether incubation with 10 μM DETA for the final 24 hours of Pb^{2+} exposure could recover the presynaptic levels of Syn and Syb, which decrease during Pb^{2+} exposure (Neal et al., 2010). For these studies we measured the distinctive punctate expression of presynaptic proteins (Syn and Syb) with immunofluorescence. Using the postsynaptic marker microtubule associated protein 2 (MAP2) to visualize neuronal dendrites, we measured the characteristics of the fluorescent presynaptic proteins juxtaposed to the dendrites in terms of puncta density (number of synapses per um dendrite), puncta intensity (average grey value), puncta area, and puncta total grey value (TGV; a semi-qualitative measurement of quantity). In Figure 3 we show that incubation with DETA for the final 24 h of Pb^{2+} exposure resulted in partial recovery of Syn expression in the synapses of Pb^{2+}-treated neurons. As previously published by us (Neal et al., 2010), Syn puncta density, area, intensity, and total grey value were significantly decreased after exposure to Pb^{2+}. Here we show that treatment of Pb^{2+}-exposed neurons with DETA resulted in partial recovery of Syn puncta density ($p<0.01$), area ($p<0.01$), and TGV ($p<0.01$). Syn puncta intensity was unchanged between Pb^{2+} and Pb^{2+} + DETA treatment groups. In contrast to other studies in hippocampal cultures (Wang et al., 2005), we did not observe an effect of DETA on Syn puncta density under control conditions. In our hands, control neurons treated with DETA for 24 hours exhibited a decrease in Syn puncta area, intensity, and TGV, but no difference in puncta density relative to control. Thus, supplementation with 10 μM DETA for the final 24 hours of Pb^{2+} exposure can partially recover the number of Syn-containing synapses in Pb^{2+}-exposed neurons, based on partial recovery of Syn puncta density, area, and TGV. However, in control cultures DETA reduced the levels of synaptic Syn expression based on decreased puncta intensity, area, and TGV relative to control.

We next investigated whether DETA could recover the synaptic levels of Syb (Figure 4). Previously we have shown that the synaptic expression of Syb, a vesicular SNARE protein essential for fast neurotransmitter release, was significantly reduced by Pb^{2+} exposure. As previously published (Neal et al., 2010), we again observed a significant decrease in synaptic Syb levels after exposure to 1.0 μM Pb^{2+} (Figure 4E–H). However, in neurons exposed to Pb^{2+} + DETA, Syb puncta density ($p<0.01$) and intensity ($p<0.01$) exhibited a partial recovery of Syb expression while Syb area ($p<0.01$), and TGV ($p<0.01$) fully recovered to control levels. Neurons from control conditions did not exhibit any difference in Syb expression when treated with DETA for 24 hours. Thus, exposure to 10 μM DETA can partially recover Syb presynaptic expression in Pb^{2+}-exposed neurons with no adverse effect on presynaptic Syb expression under control conditions.

3. Discussion

Pb^{2+} exposure during synaptogenesis results in changes in both pre- and postsynaptic protein expression (Neal et al, 2011; Neal et al, 2010). In particular, Pb^{2+} exposure results in decreased levels of whole-cell and synaptic Syn expression and decreased synaptic Syb expression (Neal et al, 2010). The changes in presynaptic protein expression are a result of inhibition of NMDARs by Pb^{2+} and may be responsible for impaired vesicular release in Pb^{2+}-exposed neurons (Neal et al., 2010). These effects of Pb^{2+} on presynaptic structure and function can be fully remediated by supplementation with BDNF for the final 24 hours of Pb^{2+} exposure. Furthermore, BDNF expression and release are decreased during Pb^{2+}

exposure, indicating that Pb^{2+} can disrupt trans-synaptic BDNF signaling. However, NMDARs mediate multiple trans-synaptic pathways besides BDNF-mediated signaling and these alternate pathways may also be implicated in the effects of Pb^{2+}. One of these alternate pathways is transsynaptic NO signaling and the present study determined whether supplementation with the NO donor DETA during Pb^{2+} exposure was sufficient to rescue neurons from the effects of Pb^{2+}. The results support the following conclusions. First, the decreased whole-cell expression of Syn caused by Pb^{2+} exposure during synaptogenesis can be fully remediated by supplementation with DETA for the final 24 hours of Pb^{2+} exposure. Second, the decreased synaptic expression of Syn and Syb caused by Pb^{2+} exposure cannot be fully remediated by DETA, but does exhibit partial recovery after NO supplementation.

We interpret the full recovery of whole-cell, but not synaptic, Syn expression in Pb^{2+}-exposed neurons to indicate that NO signaling can stimulate the expression but not the targeting of Syn in Pb^{2+}-exposed neurons. DETA exposure in absence of Pb^{2+} elevated Syn expression above control levels, an effect which was not observed with Syb. This may indicate that NO is specifically linked to Syn expression. The increased cellular expression of Syn is supported by the increase in somatic staining of Syn in Figure 3; this diffuse staining pattern, which may be indicative of Syn protein that has not yet been transported to presynaptic active zones, appears to be increased in neurons exposed to DETA with or without Pb^{2+} exposure (particularly evident in Figure 3D vs 3B). This hypothesis is supported by evidence *in vitro* that glutamatergic stimulation activates the NOS-NO-cGMP-cGK-RhoA pathway which ultimately results in increased Syn expression in hippocampal neurons (Wang et al, 2005). Inhibition of this pathway by NOS or cGMP dependent protein kinase (cGK) inhibitors prevents the glutamate-stimulated increase of Syn (Wang et al, 2005). While this study observed that stimulation of NOS resulted in increased synaptic targeting of Syn, which was not observed in our study, the increased targeting of Syn was attributed to cytoskeletal restructuring by vasodilator-stimulated phosphoprotein (VASP) and/or Ras homolog gene family member A (RhoA) activation. Both VASP and RhoA are downstream to activation of sGC by NO (Wang et al, 2005). Thus, disruption of NO signaling may affect presynaptic Syn localization; however, our work indicates that supplementation with NO is not sufficient to reverse or recover the decreased presynaptic Syn levels in Pb^{2+}-exposed neurons.

In contrast to Syn targeting, the transcription and/or translation of Syn may involve pre- or postsynaptic activation of CaMKII and subsequent phosphorylation events, based on the results of an *in vivo* study which observed increased Syn levels in the lateral amygdala after fear conditioning (Ota et al, 2010). In this study, inhibition of the NMDAR or CaMKII blocked the increase in Syn expression associated with fear conditioning. CaMKIIα can be activated by protein kinase G, which in turn is stimulated by cGMP. Thus, NO is implicated in both the CamKII- and RhoA-mediated effects on Syn expression and targeting since increased cGMP levels can activate both pathways (Ota et al, 2010; Wang et al, 2005). In Pb^{2+}-exposed cultures supplementation with NO for 24 hours was not sufficient to fully recover the deficits in Syn targeting caused by Pb^{2+}, but could fully recover whole-cell levels of Syn.

We have previously determined that decreases in Syn and Syb synaptic expression was already evident in sister cultures after a shorter (4 days) exposure to Pb^{2+} (Neal et al., 2010). In those studies we observed that the number of presynaptic sites that contain Syn were reduced by roughly 25%, although no significant effect was observed on Syn puncta area, intensity or TGV at this time point. Thus, it appears that exogenous addition of NO halts the further loss of Syn from synapses during the final 24 hours of Pb^{2+} exposure, but does not affect the Syn puncta area, intensity, or TGV. Again, this may indicate that DETA or NO is not sufficient to recover deficits in Syn targeting. In contrast, Syb puncta area, intensity, and

TGV, but not density, were significantly reduced after 4 days' exposure to Pb^{2+} (Neal et al., 2010). Syb puncta area was reduced to $61 \pm 7\%$, Syb puncta intensity decreased to $91.6 \pm 2.8\%$, and Syb puncta TGV decreased to $59 \pm 9\%$ of control (Neal et al., 2010). These values are lower than what is observed in neurons exposed to Pb^{2+} for 5 days but supplemented with DETA (Figure 4). Thus, exogenous addition of DETA for the final 24 hours of Pb^{2+} exposure may recover Syb protein levels in regards to puncta area, intensity, and TGV, and may prevent the loss of Syb puncta density that occurs in neurons exposed to Pb^{2+} for longer than 4 days.

Our finding that exogenous addition of the NO-releasing chemical DETA can reduce synaptic Syn levels and yet boost whole-cell expression under control conditions is interesting and contrasts with work from other groups (Wang et al., 2005). As more is learned about NO signaling in hippocampal neurons, it becomes increasingly clear that NO-dependent signaling pathways are complex, often with contradictory cellular effects. For example, NO generation linked to NR2B-containing receptors has been implicated in cell-death pathways in response to excitotoxic conditions (Hardingham et al., 2002; Soriano et al., 2008). However, NO-dependent stimulation of sGC has been shown to be important for vesicular release and activity-dependent regulation of synaptic proteins (Arancio et al., 1996; Wang et al., 2005). Perhaps the key issue is the concentration of released NO: although we did not detect any difference in total decomposition products we would not be able to detect differences in NO levels at individual synapses. It is possible that normal synapses release tightly regulated amounts of NO, and additional NO from DETA initiates a negative feed-back loop for synaptic targeting. We have recently shown that the exogenous addition of the neurotrophic factor BDNF using the same experimental design as in the present study results in complete recovery of the Pb^{2+}-induced decrease in synaptic Syn and Syb as well as remediate deficits in vesicular release (Neal et al., 2010). Combined, these studies suggest that Pb^{2+} exposure during synaptogenesis of hippocampal neurons may disrupt more than one NMDAR-dependent trans-synaptic retrograde signaling pathway.

4. Experimental Procedure

4.1 Animal Care and Use Statement

All animal studies were reviewed and approved by the Johns Hopkins University Animal Care and Use Committee and have been carried out in accordance with the Guide for Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health.

4.2 Cell culture

Primary hippocampal cultures were obtained from E18 Sprague-Dawley rat pups (Harlan, Frederick, MD). Low density cultures (14,000 cells/cm^2) were seeded and maintained as described elsewhere (Neal et al., 2010). Feeding medium was composed of fetal bovine serum (FBS, 1% v/v, Hyclone-Thermo Scientific, Waltham, MA), 2 mM glutamax (Invitrogen, Carlsbad, CA), and penicillin/streptomycin (100 units each, Invitrogen) in neurobasal medium (Invitrogen). Pb-acetate (95% pure, Sigma Aldrich, St. Louis, MO) was added to the feeding medium on DIV7 while feeding media without Pb-acetate was added to control cultures. Neurons were harvested 5 days after dosing (DIV12), without media exchange between DIV7 and DIV12. DETA NONOate (Sigma) was added to cultures on Pb^{2+} exposure day 4 diluted in neurobasal medium to achieve a final concentration of 10 μM DETA. Sister plate controls received vehicle (neurobasal medium).

4.3 Pb^{2+} analysis

Samples of the stock solutions of Pb^{2+} (100 μM and 10 μM) used to dose cells were sent periodically to ESA Laboratories, Inc. (Magellan Biosciences, Chelmsford, MA) for atomic absorption spectroscopy to verify accuracy. Based on the reports from ESA labs, our 100 μM and 10 μM Pb^{2+} stock solutions were within the intended range (91.4 ± 4.9, n=9 samples; and 9.6 ± 0.4 μM, n=4 samples, respectively).

4.4 Immunocytochemistry

On DIV12, immunocytochemistry was performed as described elsewhere (Neal et al., 2010). Briefly neurons grown on glass coverslips were initially fixed in 4% paraformaldehyde (v/v), 4% sucrose (w/v) in phosphate buffered saline (PBS) followed by secondary fixation in ice cold methanol. Cells were permeablized in 0.2% Triton in PBS (v/v) and blocked in 10% normal goat serum in PBS (v/v). Samples were incubated in primary antibodies diluted in blocking solution overnight at 4° C using the following dilutions: 1:1000 MAP2 (Santa Cruz sc74421, Santa Cruz, CA); 1:500 Synaptophysin (Sigma S5768); 1:500 Synaptobrevin (Synaptic Systems 104202, Goettingen, German). After incubation in primary antibodies the neurons were washed in PBS and incubated in the appropriate secondary antibodies (10 μg/mL Alexafluor488 or Alexafluor594; Invitrogen-Molecular Probes, Carlsbad, CA) diluted in blocking solution (10% v/v normal goat serum in PBS) at room temperature. Following another series of washes in PBS the coverslips were mounted onto slides in ProLong Gold mounting media (Molecular Probes). Slides were coded to ensure that imaging and analyses were conducted in a blinded fashion.

4.5 Imaging and Image Analysis of Fixed Coverslips

Immunofluorescently-labeled neurons were imaged at 63× magnification using a single-point, laser scanning confocal microscope (LSM510-Meta, Zeiss, Thornwood, NY) utilizing LSM image software at the Johns Hopkins University School of Medicine Microscope Facility. All coverslips stained under the same conditions were imaged using the same scanning parameters on the same day. Four to 7 confocal stacks of single neurons were obtained for each experimental condition. Confocal stacks were projected into single images using the maximum fluorescence. Images were analyzed using Metamorph Offline (Molecular Devices, Downingtown, PA). Images obtained from the same experiment were thresholded at the same level for analysis. Several parameters of synaptic protein expression were measured using integrated morphometry analysis. They include immunofluorescent puncta density (number of puncta per μm dendrite), area (average area of puncta), intensity (average grey value of puncta), and total grey value (integration of puncta intensity relative to area). Puncta intensity thus measures the average intensity of the fluorescent puncta while puncta total grey value gives a semi-quantitative measurement of protein quantity. For colocalization (or juxtaposition) analysis, gray scale images at each wavelength for the same neuron were used to select 3 - 6 dendritic regions. Selected dendritic regions were at least 10 μm from the cell body, were clearly identifiable as single processes, and could be traced back to the imaged neuron. All dendrites which fit these criteria in a single image were sampled.

4.6 MTS Cell Viability Assay

3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assays were performed per the manufacturer's instructions (CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay, Promega). Briefly, hippocampal neurons were plated in 96-well plates at 14,000 cells/cm^2. Cells were exposed to 1.0 μM Pb^{2+} for 5 days or to 0–10 μM DETA for 24 h. Exposure to 100 μM glutamate for 24 hours was used as a positive cytotoxic control. After the exposure period, 20 μL of the

MTS reagent was added to the wells for 2 hours at 37°C. Absorbencies were read at 490 nm using a multi-channel plate reader.

4.7 Determination of Nitric Oxide Levels

Nitric oxide levels were indirectly estimated using the nitrate/nitrite Greiss reaction (Green et al., 1982). Culture media samples were taken on the 4th day of Pb^{2+} exposure, prior to the addition of DETA. Another set of samples were obtained 24 hours later, prior to harvesting the cells for immunocytochemistry. Standards were made from $NaNO_2$ (0 to 50 μM) dissolved in neuron feeding media (NM1). Neither NM1 nor Pb^{2+} interfered with the Greiss reaction. The Greiss reagent was added in a 1:1 ratio with standards or samples in triplicate. After 15 minutes, the absorbance at 540 nm was recorded with a multiplate reader.

4.8 Whole Cell Immunoblotting

For whole cell protein levels, cells were harvested on DIV12 according to the method of Brewer et al. (2007). Western blot membranes were incubated in the appropriate primary antibodies: 1:1000 Synaptophysin (Cell Signaling 4329), 1:1000 Synaptobrevin (Synaptic Systems 104 202), 1:1000 Actin (Santa Cruz sc-1616) diluted in blocking solution overnight at 4°C. The membranes were visualized using the Odyssey imaging system (LiCor). Integrated intensity of the protein of interest was normalized to b-actin levels from the same blot.

Acknowledgments

Reference List

Al Hallaq RA, Conrads TP, Veenstra TD, Wenthold RJ. NMDA di-heteromeric receptor populations and associated proteins in rat hippocampus. J Neurosci. 2007; 27:8334–8343. [PubMed: 17670980]

Alkondon M, Alberto CS, Radhakrishnan V, Aronstam RS, Albuquerque EX. Selective blockade of NMDA-activated channel currents may be implicated in learning deficits caused by lead. FEBS Lett. 1990; 261:124–30. [PubMed: 1689669]

Arancio O, Kiebler M, Lee CJ, Lev-Ram V, Tsien RY, Kandel ER, Hawkins RD. Nitric oxide acts directly in the presynaptic neuron to produce long-term potentiation in cultured hippocampal neurons. Cell. 1996; 87:1025–35. [PubMed: 8978607]

Bliss TV, Collingridge GL. A synaptic model of memory: long-term potentiation in the hippocampus. Nature. 1993; 361:31–39. [PubMed: 8421494]

Brewer LD, Thibault O, Staton J, Thibault V, Rogers JT, et al. Increased vulnerability of hippocampal neurons with age in culture: temporal association with increases in NMDA receptor current, NR2A subunit expression and recruitment of L-type calcium channels. Brain Res. 2007; 1151:20–31. [PubMed: 17433272]

Christopherson KS, Hillier BJ, Lim WA, Bredt DS. PSD-95 assembles a ternary complex with the N-methyl-D-aspartic acid receptor and a bivalent neuronal NO synthase PDZ domain. J Biol Chem. 1999; 274:27467–27473. [PubMed: 10488080]

Cohen-Cory S, Kidane AH, Shirkey NJ, Marshak S. Brain-derived neurotrophic factor and the development of structural neuronal connectivity. Dev Neurobiol. 2010; 70:271–288. [PubMed: 20186709]

Cordova FM, Rodrigues LS, Giocomelli MBO, Oliveira CS, Posser T, Dunkley PR, Leal RB. Lead stimulates ERK1/2 and p38MAPK phosphorylation in the hippocampus of immature rats. Brain Res. 2004; 998:65–72. [PubMed: 14725969]

Daly C, Ziff EB. Ca^{2+}-dependent formation of a dynamin-synaptophysin complex: Potential role in synaptic vesicle endocytosis. J Biol Chem. 2002; 277:9010–9015. [PubMed: 11779869]

Deak F, Schoch S, Liu X, Sudhof TC, Kavalali ET. Synaptobrevin is essential for fast synaptic-vesicle endocytosis. Nat Cell Biol. 2004; 6:1102. [PubMed: 15475946]

Gilbert ME, Mack CM, Lasley SM. Chronic developmental lead exposure and hippocampal long-term potentiation: biphasic dose-response relationship. NeuroToxicology. 1999; 20:71–82. [PubMed: 10091860]

Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of Nitrate, Nitrite and 15N Nitrate in Biological Fluids. Anal Biochem. 1982; 126:131–138. [PubMed: 7181105]

Guilarte TR, McGlothan JL. Hippocampal NMDA receptor mRNA undergoes subunit specific changes during developmental lead exposure. Brain Res. 1998; 790:98–107. [PubMed: 9593842]

Guilarte TR, Miceli RC. Age-dependent effects of lead on [3H]MK-801 binding to the NMDA receptor-gated ionophore: in vitro and in vivo studies. Neurosci Lett. 1992; 148:27–30. [PubMed: 1300499]

Hardingham GE, Fukunaga Y, Bading H. Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways. Nat Neurosci. 2002; 5:405–414. [PubMed: 11953750]

Hartmann M, Heumann R, Lessmann V. Synaptic secretion of BDNF after high-frequency stimulation of glutamatergic synapses. EMBO J. 2001; 20:5887–5897. [PubMed: 11689429]

Hu B, Nikolakopoulou AM, Cohen-Cory S. BDNF stabilizes synapses and maintains the structural complexity of optic axons in vivo. Development. 2005; 132:4285–4298. [PubMed: 16141221]

Ivanov A, Pellegrino C, Rama S, Dumalska I, Salyha Y, Ben Ari Y, Medina I. Opposing role of synaptic and extrasynaptic NMDA receptors in regulation of the extracellular signal-regulated kinases (ERK) activity in cultured rat hippocampal neurons. J Physiol. 2006; 572.3:789–798. [PubMed: 16513670]

Jiang X, Tian F, Mearow K, Okagaki P, Lipsky RH, Marini AM. The excitoprotective effect of N-methyl-D-aspartate receptors is mediated by a brain-derived neurotrophic factor autocrine loop in cultured hippocampal neurons. J Neurochem. 2005; 94:713–722. [PubMed: 16000165]

Jusko TA, Henderson CR, Lanphear BP, Cory-Slechta DA, Parsons PJ, Canfield RL. Blood lead concentrations < 10 microg/dL and child intelligence at 6 years of age. Environ Health Perspect. 2008; 116:243–248. [PubMed: 18288325]

Lanphear BP, Hornung R, Khoury J, Yolton K, Baghurst P, Bellinger DC, Canfield RL, Dietrich KN, Bornschein R, Greene T, Rothenberg SJ, Needleman HL, Schnaas L, Wasserman G, Graziano J, Roberts R. Low-level environmental lead exposure and children's intellectual function: An international pooled analysis. Environ Health Perspect. 2005; 113:894–899. [PubMed: 16002379]

Lynch MA. Long-term potentiation and memory. Physiol Rev. 2004; 84:87–136. [PubMed: 14715912]

Magby JP, Bi C, Chen ZY, Lee FS, Plummer MR. Single-cell characterization of retrograde signaling by brain-derived neurotrophic factor. J Neurosci. 2006; 26:13531–13536. [PubMed: 17192436]

Monyer H, Burnashev N, Laurie DJ, Sakmann B, Seeburg PH. Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. Neuron. 1994; 12:529–540. [PubMed: 7512349]

Neal AP, Guilarte TR. Molecular Neurobiology of lead (Pb^{2+}): Effects on synaptic function. Mol Neurobiol. 2010; 42:151–160. [PubMed: 21042954]

Neal AP, Stansfield KH, Worley PF, Thompson RE, Guilarte TR. Lead exposure during synaptogenesis alters vesicular proteins and impairs vesicular release: Potential role of NMDA receptor-dependent BDNF signaling. Toxicol Sci. 2010; 116:249–263. [PubMed: 20375082]

Neal AP, Worley PF, Guilarte T. Lead exposure during synaptogenesis alters NMDA receptor targeting via NMDA receptor inhibition. NeuroToxicology. 2011; 32:281–289. [PubMed: 21192972]

Nihei MK, Desmond NL, McGlothan JL, Kuhlmann AC, Guilarte TR. N-methyl-D-aspartate receptor subunit changes are associated with lead-induced deficits of long-term potentiation and spatial learning. Neuroscience. 2000; 99:233–242. [PubMed: 10938429]

Nihei MK, Guilarte TR. NMDAR-2A subunit protein expression is reduced in the hippocampus of rats exposed to Pb^{2+} during development. Mol Brain Res. 1999; 66:42–49. [PubMed: 10095076]

O'Dell TJ, Hawkins RD, Kandel ER, Arancio O. Tests of the roles of two diffusible substances in long-term potentiation: evidence for nitric oxide as a possible early retrograde messenger. Proc Nat Acad Sci USA. 1991; 88:11285–11289. [PubMed: 1684863]

Ota KT, Monsey MS, Wu MS, Schafe GE. Synaptic Plasticity and NO-CGMP-PKG Signaling Regulate Pre- and Postsynaptic Alterations at Rat Lateral Amygdala Synapses Following Fear Conditioning. PLoS One. 2010; 5:e11236. [PubMed: 20574537]

Paoletti P, Perin-Dureau F, Fayyazuddin A, Le Goff A, Callebaut I, Neyton J. Molecular organization of a zinc binding n-terminal modulatory domain in a NMDA receptor subunit. Neuron. 2000; 28:911–925. [PubMed: 11163276]

Pennuto M, Bonanomi D, Benfenati F, Valtorta F. Synaptophysin I Controls the Targeting of VAMP2/ Synaptobrevin II to Synaptic Vesicles. Mol Biol Cell. 2003; 14:4909–4919. [PubMed: 14528015]

Pozzo-Miller LD, Gottschalk W, Zhang L, McDermott K, Du J, Gopalakrishnan R, Oho C, Sheng ZH, Lu B. Impairments in high-frequency transmission, synaptic vesicle docking, and synaptic protein distribution in the hippocampus of BDNF knockout mice. J Neurosci. 1999; 19:4972–4983. [PubMed: 10366630]

Rachline J, Perin-Dureau F, Le Goff A, Neyton J, Paoletti P. The micromolar zinc-binding domain on the NMDA receptor subunit NR2B. J Neurosci. 2005; 25:308–317. [PubMed: 15647474]

Rameau GA, Chiu LY, Ziff EB. Bidirectional regulation of neuronal nitric-oxide synthase phosphorylation at serine 847 by the N-methyl-D-aspartate receptor. J Biol Chem. 2004; 279:14307–14314. [PubMed: 14722119]

Rameau GA, Tukey DS, Garcin-Hosfield ED, Titcombe RF, Misra C, Khatri L, Getzoff ED, Ziff EB. Biphasic coupling of neuronal nitric oxide synthase phosphorylation to the NMDA receptor regulates AMPA receptor trafficking and neuronal cell death. J Neurosci. 2007; 27:3445–3455. [PubMed: 17392461]

Schoch S, Deak F, Konigstorfer A, Mozhayeva M, Sara Y, Sudhof TC, Kavalali ET. SNARE function analyzed in synaptobrevin/VAMP knockout mice. Science. 2001; 294:1117–1122. [PubMed: 11691998]

Soriano FX, Martel MA, Papadia S, Vaslin A, Baxter P, Rickman C, Forder J, Tymianski M, Duncan R, Aarts M, Clarke PGH, Wyllie DJA, Hardingham GE. Specific targeting of pro-Death NMDA receptor signals with differing reliance on the NR2B PDZ ligand. J Neurosci. 2008; 28:10696–10710. [PubMed: 18923045]

Steinert JR, Kopp-Scheinpflug C, Baker C, Challiss RAJ, Mistry R, Haustein MD, Griffin SJ, Tong H, Graham BP, Forsythe ID. Nitric oxide is a volume transmitter regulating postsynaptic excitability at a glutamatergic synapse. Neuron. 2008; 60:642–656. [PubMed: 19038221]

Toscano CD, Guilarte TR. Lead neurotoxicity: From exposure to molecular effects. Brain Res Rev. 2005; 49:529–555. [PubMed: 16269318]

Toscano CD, Hashemzadeh-Gargari H, McGlothan JL, Guilarte TR. Developmental Pb^{2+} exposure alters NMDAR subtypes and reduces CREB phosphorylation in the rat brain. Dev Brain Res. 2002; 139:217–226. [PubMed: 12480136]

Toscano CD, McGlothan JL, Guilarte TR. Lead exposure alters cyclic-AMP response element binding protein phosphorylation and binding activity in the developing rat brain. Dev Brain Res. 2003; 145:219–228. [PubMed: 14604762]

Toscano CD, O'Callaghan JP, Guilarte TR. Calcium/calmodulin-dependent protein kinase II activity and expression are altered in the hippocampus of Pb^{2+}-exposed rats. Brain Res. 2005; 1044:51–58. [PubMed: 15862789]

Vanhoutte P, Bading H. Opposing roles of synaptic and extrasynaptic NMDA receptors in neuronal calcium signaling and BDNF gene regulation. Curr Op Neurobiol. 2003; 13:366–371. [PubMed: 12850222]

Walz C, Jungling KL, Gottmann K. Presynaptic plasticity in an immature neocortical network requires NMDA receptor activation and BDNF release. J Neurophysiol. 2006; 96:3512–3516. [PubMed: 17110740]

Wang HG, Lu FM, Jin I, Udo H, Kandel ER, de Vente J, Walter U, Lohmann SM, Hawkins RD, Antonova I. Presynaptic and postsynaptic roles of NO, cGK, and RhoA in long-lasting potentiation and aggregation of synaptic proteins. Neuron. 2005; 45:389. [PubMed: 15694326]

Zhang XY, Liu AP, Ruan DY, Liu J. Effect of developmental lead exposure on the expression of specific NMDA receptor subunit mRNAs in the hippocampus of neonatal rats by digoxigenin-labeled in situ hybridization histochemistry. Neurotoxicol Teratol. 2002; 24:149–160. [PubMed: 11943503]

Highlights

- Pb^{2+} reduces cellular synaptophysin levels and synaptic targeting of presynaptic proteins

- Previous work showed that Pb^{2+} can disrupt NMDAR dependent trans-synaptic signaling

- Supplementation with NO during Pb^{2+} exposure fully rescued Syn whole cell expression

- NO supplementation did not recover Pb^{2+} effects on synaptic protein localization

- Thus NO recovers deficits in cellular but not synaptic protein expression due to Pb^{2+}
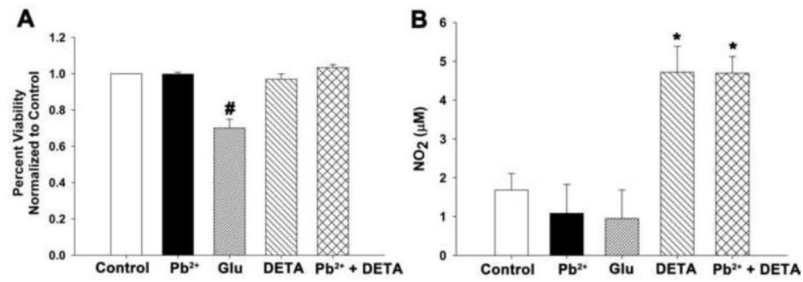
Figure 1. DETA NONOate added to neuronal culture media for the final 24 hours of Pb^{2+} exposure spontaneously releases NO and does not affect cell viability

(**A**) DETA (10 μM) did not cause a loss in cell viability as assessed by the MTS assay. Cell death was only observed with the excitotoxic concentration (100 μM) of glutamate (Glu). Data are the mean ± SEM and are the result of 4 assays. (**B**) During the 24 hr treatment period DETA supplementation resulted in significantly elevated levels of NO_2 confirming increased NO release. Data are the mean ± SEM and are the result of 3 assays. # = significance from all other treatments, * = significance from treatments without DETA.
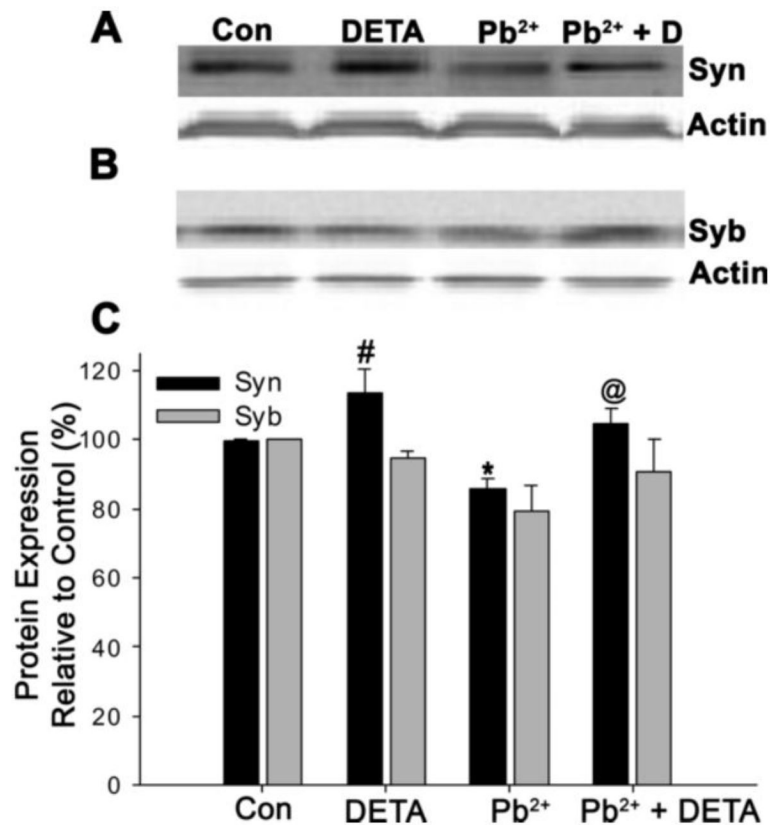
Figure 2. Exogenous addition of NO for the final 24 hours of Pb^{2+} exposure completely recovers whole-cell Syn levels

Neuronal cultures were harvested and immunoblotted for Syn (**A**) or Syb (**B**) and Actin. Pb^{2+} exposure significantly reduced Syn expression relative to control but exposure to the NO donor DETA during Pb^{2+} exposure resulted in complete recovery of Syn levels. Exposure to DETA in absence of Pb^{2+} resulted in a significant elevation of Syn protein above control levels. No significant trends were observed with Syb, although a non-significant decrease in Syb expression occurred in Pb^{2+}-treated neurons. Quantification of protein expression relative to control is shown in **C**. Data are mean ± SEM and are the result of 4 independent trials. For Syn expression only, * = significance from Syn control, # = significance from all other Syn treatment groups, and @ = significance from Syn treatment with Pb^{2+} (Fisher's Protected LSD).
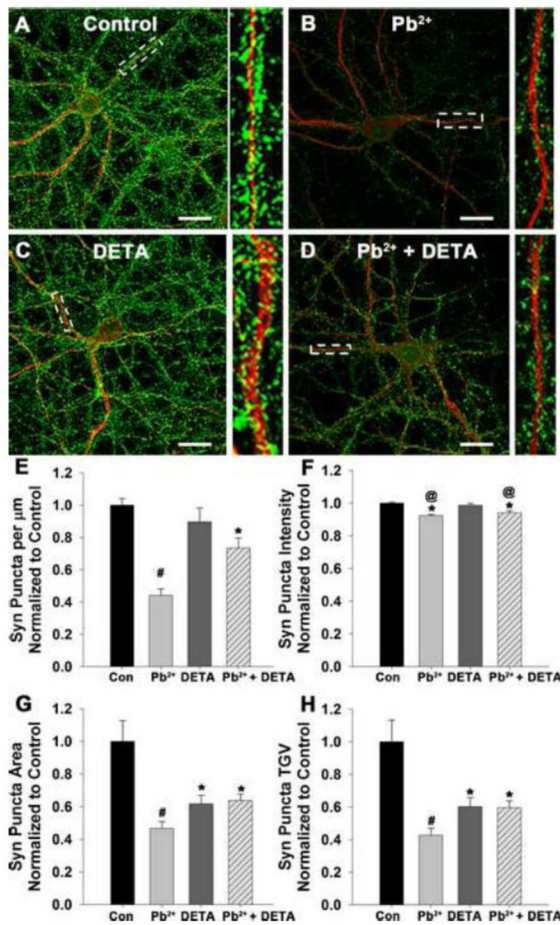
Figure 3. Exogenous addition of NO for the final 24 hours of Pb^{2+} exposure partially recovers Syn levels in Pb^{2+}-treated neurons but reduces Syn expression under control conditions
Representative images of neurons exposed to control medium (**A**), 1 μM Pb^{2+} (**B**) 10 μM DETA (**C**), or Pb^{2+} + DETA (**D**) stained for Syn (green) and MAP2 (red). Scale bar=20 μm. Quantification of Syn puncta density (**E**), intensity (**F**), area (**G**), and TGV (**H**) revealed that some effects of Pb^{2+} may be refractory to NO-mediated recovery. Data are mean ± SEM and are the result of 4 independent trials with 18–20 neurons per condition. * = significance from control, # = significance from all other treatment groups, and @ = significance from treatment with DETA alone (Fisher's Protected LSD).
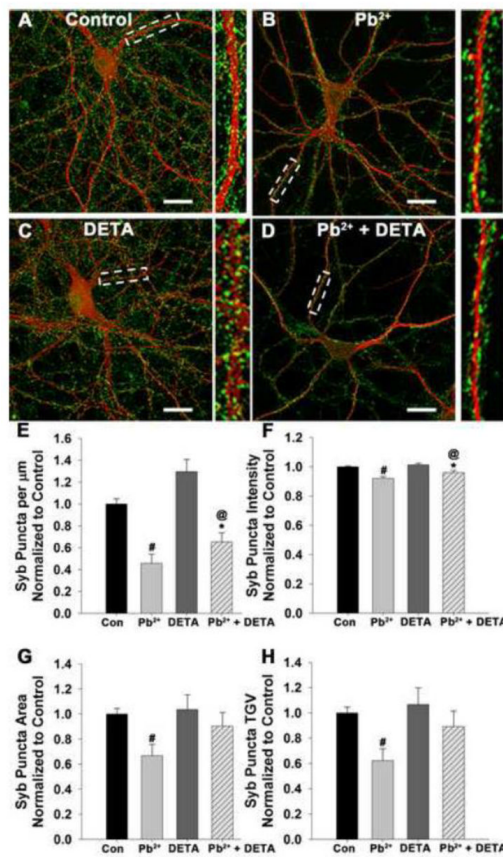
Figure 4. Exogenous addition of NO for the final 24 hours of Pb²⁺ exposure partially recovers Syb levels in Pb²⁺-treated neurons with no effect on Syb levels under control conditions
Representative images of neurons exposed to control medium (**A**), 1 μM Pb²⁺ (**B**) 10 μM DETA (**C**), or Pb²⁺ + DETA (**D**) stained for Syb (green) and MAP2 (red). Scale bar=20 μm. Quantification of Syb puncta density (**E**), intensity (**F**), area (**G**), and TGV (**H**) revealed that puncta area and TGV in Pb²⁺-exposed neurons were completely recovered by DETA treatment while puncta density and intensity exhibited partial recovery. Data are the mean ± SEM and are the result of 4 independent trials with 18–20 neurons per condition. * = significance from control, # = significance from all other treatment groups, and @ = significance from treatment with DETA alone.