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Research Report

Enhanced nitric oxide production during lead (Pb^{2+}) exposure recovers protein expression but not presynaptic localization of synaptic proteins in developing hippocampal neurons

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ARTICLE INFO

Article history:
Accepted 17 December 2011
Available online 29 December 2011

Keywords:
Pb^{2+}
Nitric oxide
Synaptophysin
Synaptobrevin
Synaptogenesis
Hippocampal neuron

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.

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-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

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doi:10.1016/j.brainres.2011.12.037

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 and Guilarte, 1999; Nihei et al., 2000; 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 and Guilarte, 2010; Neal et al., 2011). 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 and Guilarte, 2010; Neal et al., 2011; 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., 2002, 2003).

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 l., 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., 2004, 2007). 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 h 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., 2010, 2011). 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 h 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 h 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 (Fig. 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 (Fig. 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 h 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 h 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