Oxidative Stress Accumulation in Parvalbumin Neurons after Early Life Stress

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Abstract

Early life adversity in humans is linked to cognitive deficits and increased risk of mental illnesses, including depression, bipolar disorder, and schizophrenia. Modeling early life adversity in rodents shows similar neuropsychological deficits that may partially be driven by sex-dependent dysfunction in parvalbumin (PV) interneurons in the prefrontal cortex (PFC). Research demonstrates that PV interneurons are particularly susceptible to oxidative stress; therefore, accumulation of oxidative damage may be driving PV dysfunction following early life adversity. The goal of this study was to quantify oxidative stress accumulation in PV neurons in the PFC of rats exposed to maternal separation (MS). To do this, pups were separated from their dam for 4 hours per day from postnatal day (P)2 to 20. Serial sections from the prelimbic (PL) and infralimbic (IL) PFC of juvenile (P21) and adolescent (P40) rats of both sexes were immunohistochemically stained with antibodies against PV, as well as 8-oxo-dG, a marker for oxidative DNA damage. Colocalization was measured in the PFC to determine the oxidative effect of MS and establish whether its progression varies between sexes. A significant increase in colocalization of PV and 8-oxo-dG was found in the PL of juvenile rats, indicating increased oxidative stress immediately following MS. Additionally, sex differences in PV cell count were found in both the PL and IL of adolescent rats. These data identify a potential timepoint at which antioxidant treatment may be administered in order to prevent further neurological dysfunction in individuals subject to early life adversity.
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Introduction

Exposure to trauma during early life is common and linked to mental disorders later in life. The National Survey of Children’s Exposure to Violence found that 38.7% of children in the United States had experienced multiple incidents of maltreatment – including physical, sexual, or emotional abuse or neglect – in the past year. Childhood neglect has been linked to a reduction in cognitive ability in adulthood, and early life trauma is associated with psychiatric disorders in adults, including depression, bipolar disorder, and schizophrenia.

The link between early life stress (ELS) and psychiatric disease has been connected to deficits in specific brain regions, particularly the prefrontal cortex (PFC). In adults diagnosed with schizophrenia, childhood neglect is associated with decreased gray matter in the PFC. Additionally, a meta-analysis of MRI data found that adults with a history of childhood trauma displayed reduced gray matter in the dorsolateral PFC. MRI data has also shown reduced gray matter volume in the ventromedial PFC of children diagnosed with post-traumatic stress disorder. These results have been reproduced in rodent models: rats subject to maternal deprivation on P3 showed reductions in PFC volume in adulthood, and rats subject to maternal deprivation on P9 displayed decreases in thickness and neuron density in the PFC as adults. Thus, it is important to monitor changes in the PFC in order to develop a better understanding of the effects of early life trauma.

The clinical symptoms associated with childhood trauma tend to manifest in adolescence, suggesting that the effects of ELS progress throughout development, and that it could be possible to identify a critical timepoint for intervention that occurs before the symptoms appear. The human PFC develops throughout childhood and adolescence, finally stabilizing in adulthood. Research shows that development follows the same timeline in rats. Early childhood is a
critical period for the PFC, making it particularly susceptible to ELS, which can cause severe consequences later in life.

The biological mechanism by which ELS modifies brain development is unclear. A potential source could be oxidative stress produced in neurons as a result of childhood trauma. There is evidence that reactive oxygen species, which are associated with cell malfunction and neurodegeneration, accumulate in the brain following other stressors, such as brain injury, immobilization, and social isolation. Furthermore, oxidative stress accumulation is linked to psychiatric diseases in humans, including schizophrenia, depression, and bipolar disorder.

A group of fast-spiking interneurons have been identified that seem particularly susceptible to oxidative stress. They express the calcium-binding protein PV and release GABA, modulating the firing rates of pyramidal neurons in the PFC. PV neurons drive gamma oscillations in the neocortex, which enhance signal transmission and information processing. These neurons modulate the neocortical balance of excitation and inhibition, which has important implications for cognitive and social functioning. Oxidative stress induced by genetic, pharmacological, psychosocial, and surgical manipulations have been linked to decreased PV immunoreactivity. MS has been shown to reduce PV expression in adolescence, but the mechanism driving this reduction has yet to be identified.

There is some evidence to suggest that the effects of ELS are different between sexes. Reduction in PV immunoreactivity following MS has been shown to occur earlier in females than in males. Female mice that experienced ELS induced by limited bedding displayed depressive-like behaviors in adolescence and adulthood, while their male counterparts did not. Sex differences are also seen in humans that experience ELS: In adolescent girls, ELS was associated with internalizing problems as well as increased activation of the ventromedial PFC.
during emotion regulation tasks, while adolescent boys that experienced ELS showed no association in these areas.29 The mechanisms driving these sex differences is not clear, so more research is needed utilizing both male and female subjects.

The main objective of the present study was to measure oxidative stress in PV neurons in the PFC of juvenile and adolescent rats of both sexes exposed to ELS. A secondary objective was to examine the effects of MS on PV cell count at these two timepoints, which may signify sensitive periods in development. This was accomplished through immunohistochemical assays measuring PV and 8-oxo-dG, a marker of DNA oxidation,21 in brain sections collected from rats subject to MS during the pre-weaning period. These data will provide insight about the mechanism by which ELS reduces PV expression in the brain, with implications for the study of development and psychiatric disease.

Methods

Subjects

Pregnant Sprague-Dawley rats at gestational day 15 were ordered from Charles River Laboratories (Wilmington, MA). The day of birth was designated P0. On P1, litters were culled to 10 pups with as close to 5 males and 5 females as possible. Animals were housed in a temperature (22-23°C)- and humidity-controlled environment on a 12-hour light/dark cycle (the light period occurred from 0700-1900, at approximately 332 lux) in standard wire-top cages with pine shaving bedding. Standard chow (ProLab5P00) and water were available ad libitum throughout gestation and development. All experiments were performed in accordance with the NIH 1996 Guide for the Care and Use of Laboratory Animals with approval from the Institutional Animal Care and Use Committee at Northeastern University.
Maternal Separation

Each litter was randomly assigned to either the ELS or CON condition on P0 ($n = 7$-8/group). To avoid litter effects, no more than 2 pups per litter were assigned to each group. The MS protocol began on P2. Pups in the ELS group were isolated for 4 hours each day from P2 to 20 in a thermo-regulated environment at 37°C. From P2-14, pups were placed in individual cups in a warm water bath. At P15, once the pups were able to adequately thermoregulate, they were separated in small cages. Apart from weighing and normal husbandry procedures, pups in the CON group were left undisturbed with their mothers during this time. All pups were weaned and pair-housed with a cage-mate matched for age, sex, and experimental group, on P21. The experimental timeline is visually represented in Figure 1A.

Immunohistochemistry

For immunohistochemical analyses, rats were euthanized with carbon dioxide on either P21 or 40 and intracardially perfused with 0.9% saline followed by ice-cold 4% paraformaldehyde. Their brains were extracted and stored in PFA for 3 days at 4°C, then cryoprotected in 30% sucrose solution. Brains were sectioned on a freezing microtome (Leica) at 40 μm and stored in freezing solution at -20°C until immunohistochemical staining. Sections were washed in phosphate-buffered saline (PBS) and then incubated in RNase solution (5 mg/mL RNase A in 5 mM Tris-HCl, 7.5 mM NaCl) for 1h at 37°C to prevent anti-8-oxo-dG binding to RNA. Sections were then washed again in PBS and blocked in PBS with normal goat serum (NGS, NC9660079, Jackson) and 0.2% Triton-X. Sections were subsequently incubated in primary antibodies mouse anti-8-oxo-dG (1:300, 4354-MC-050, Trevigen) and rabbit anti-PV (1:10,000, PA1-933, Thermofisher) overnight at 4°C. On day 2, sections were washed in PBS with 0.2% Triton-X and incubated in goat secondary antibodies anti-mouse conjugated with
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Alexa Fluor 568 nm (1:500, A11031, Invitrogen) to visualize 8-oxo-dG and anti-rabbit conjugated with Alexa Fluor 488 nm (1:500, A11034, Invitrogen) to visualize PV. Following this, sections were mounted on slides and coverslipped with ProLong Gold antifade mounting medium.

Multichannel images of PFC PV and 8-oxo-dG immunofluorescence in 4 serial sections were taken at 20x magnification on a Zeiss AxioImager M2 microscope. Regions of interest were outlined (Figure 1B) using pre-defined boundaries and landmarks in accordance with a rat brain atlas. In each section, 2 images were captured for the PL and 1 in the IL, bilaterally, resulting in 24 images per subject. Acquired images were analyzed in ImageJ. Cell count was quantified in each image for all of the PV-positive cells and the cells expressing both PV and 8-oxo-dG.

Statistics

Differences in PV cell count and colocalization of 8-oxo-dG and PV were calculated using a two-way (Sex x Rearing Group) analysis of variance (ANOVA), with Bonferroni correction for multiple comparisons. Colocalization of 8-oxo-dG and PV was quantified by dividing the number of 8-oxo-dG-positive PV-positive cells over the total number of PV-positive cells.

Results

Effects of MS in PL at P21

We first measured PV and 8-oxo-dG immunofluorescence in the PL of P21 animals subjected to MS. Representative images can be seen in Figure 2A. While there was no two-way interaction of Sex x Rearing Group ($F_{1,28} = 0.181, p = 0.6737$) and no significant effect of Sex
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\((F_{1,28} = 0.03447, p = 0.8541)\), there was a significant effect of Rearing Group on colocalization of 8-oxo-dG and PV \((F_{1,28} = 5.658, p = 0.0244)\). ELS increased colocalization (Figure 3A). The Bonferroni multiple comparisons test did not reveal any significant differences between groups. PV cell count was not affected by Sex \((F_{1,28} = 0.4548, p = 0.5056; \text{Figure } 3B)\), Rearing Group \((F_{1,28} = 2.055, p = 0.1628)\), or an interaction between the two \((F_{1,28} = 0.07536, p = 0.7857)\).

Effects of MS in IL at P21

We measured PV and 8-oxo-dG immunofluorescence in the IL of P21 animals subjected to MS. Representative images can be seen in Figure 2B. Colocalization of 8-oxo-dG and PV was not affected by Sex \((F_{1,28} = 0.9139, p = 0.3473; \text{Figure } 4A)\), Rearing Group \((F_{1,28} = 0.468, p = 0.4995)\), or an interaction between the two \((F_{1,28} = 1.217, p = 0.2793)\). There was also no two-way interaction of Sex x Rearing Group on PV cell count \((F_{1,28} = 0.6203, p = 0.4375; \text{Figure } 4B)\), nor was there a significant effect of Sex \((F_{1,28} = 0.171, p = 0.6824)\) or Rearing Group \((F_{1,28} = 0.003489, p = 0.9533)\).

Effects of MS in PL at P40

We measured PV and 8-oxo-dG immunofluorescence in the PL of P40 animals subjected to MS. Representative images can be seen in Figure 2C. While there was no two-way interaction of Sex x Rearing Group \((F_{1,27} = 4.192, p = 0.0504)\) and no significant effect of Stress \((F_{1,27} = 1.139, p = 0.2953)\), there was a significant effect of Sex on colocalization of 8-oxo-dG and PV \((F_{1,27} = 13.8, p = 0.0009)\). The Bonferroni multiple comparisons test revealed that Female CON animals displayed increased colocalization of 8-oxo-dG and PV in the PL compared to their male counterparts \((p = 0.0018; \text{Figure } 5A)\). Relatedly, there was a significant effect of Sex on PV cell count \((F_{1,27} = 20.77, p < 0.0001)\). The Bonferroni multiple comparisons test revealed that there
were significant differences between the Male and Female CON animals ($p = 0.0203$) and between the Male and Female ELS animals ($p = 0.0194$), indicating that males had a higher number of PV+ cells in the PL than their female counterparts (Figure 5B). There was no two-way interaction of Sex x Rearing Group on PV cell count ($F_{1,27} = 0.00839, p = 0.9277$); nor was there a significant effect of Rearing Group alone ($F_{1,27} = 0.01361, p = 0.9080$).

**Effects of MS in IL at P40**

Finally, we measured PV and 8-oxo-dG immunofluorescence in the IL of P40 animals subjected to MS. While there was no two-way interaction of Sex x Rearing Group ($F_{1,27} = 0.5233, p = 0.4757$) and no significant effect of Rearing Group ($F_{1,27} = 0.2627, p = 0.6125$), there was a significant effect of Sex on colocalization of 8-oxo-dG and PV ($F_{1,27} = 4.601, p = 0.0411$). Females had increased colocalization compared to Males, irrespective of Rearing Group. The Bonferroni multiple comparisons test revealed no significant differences between groups. PV cell count was also affected by Sex ($F_{1,27} = 22.06, p < 0.0001$), but not Rearing Group ($F_{1,27} = 0.1038, p = 0.7498$) or an interaction between the two ($F_{1,27} = 0.03447, p = 0.8541$). The Bonferroni multiple comparisons test revealed that there were significant differences between the Male and Female CON animals ($p = 0.0187$) and between the Male and Female ELS animals ($p = 0.0128$), indicating that Male animals had higher PV+ cell counts than their Female counterparts.

**Discussion**

We investigated the effects of MS on the accumulation of 8-oxo-dG in PV neurons in the PL and IL of the PFC. A significant increase in colocalization of 8-oxo-dG and PV was found in the PL of juvenile animals subjected to ELS. There were no significant effects of ELS in the PL.
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in adolescence or in the IL at either time point. There was an overall reduction in PV expression in females in both the PL and IL in adolescent animals, corresponding with an increase in colocalization of 8-oxo-dG and PV in female CON animals. Here, we provide evidence that adversity in early life leads to oxidative damage in PV neurons, which may underlie the subsequent neurobehavioral dysfunction seen later in development.

We demonstrated an increase in the percentage of 8-oxo-dG+ PV+ neurons in the PL of animals that underwent MS. This increase existed only in juveniles and normalized by adolescence. Other studies have reported neurological differences that arise in juvenility and disappear later. Rats exposed to a similar MS protocol demonstrated decreased basal spine density in the PFC at juvenility, but there were no differences in spine density 8 months later. We have also shown previously that female rats exposed to MS displayed decreased PFC PV protein expression at juvenility, which normalized by adolescence. This suggests that certain biological indicators of stress may peak in juvenility, immediately after MS exposure, but disappear over time. However, the lack of certain biological markers in adolescence does not indicate that the effects of ELS are no longer present.

We have previously demonstrated a link between ELS and later deficits in PV neurons and behavior. Rats that underwent MS displayed reduced PV expression in the medial PFC and cognitive deficits on the win-shift maze in adolescence. Oxidative stress has also been shown to cause neurobehavioral deficits over development. Rats exposed to a glutathione inhibitor in juvenility displayed impaired performance in water and radial maze tasks as adults. Furthermore, perinatal phencyclidine administration resulted in impaired prepulse inhibition in juvenility, which was prevented by treatment with M40403, a superoxide dismutase mimetic.
Our findings suggest that increased oxidative stress in PV neurons may underlie the behavioral dysfunction caused by ELS.

We also found reduced PV cell count in both the PL and IL of adolescent females, regardless of stress condition. Although few studies compare PV levels in control animals, there is evidence to suggest that sex differences exist. In a study comparing outbred lines of extreme high and low trait anxiety rats, decreased PV immunoreactivity was found in the PFC of adult females from both lines. This supports our data, indicating that, irrespective of stress and anxiety, females display reductions in PV compared to males.

There were no significant effects of stress on either 8-oxo-dG or PV levels in the IL at either time point, suggesting a unique sensitivity of the PL to ELS. In fear learning studies, the PL has long been understood to control fear conditioning, while the IL drives fear extinction. The present study suggests a similar dichotomy relating to prolonged stress. Groups studying other stress paradigms have also found effects specific to the PL, relating to the deficits in inhibitory neurons as well as increased excitatory neurotransmission in this subdivision of the PFC. Rats subjected to peripubertal stress showed decreased levels of neuroligin-2, a synaptic cell adhesion molecule involved in stabilizing GABAergic synapses, only in the PL, which corresponded to attention deficits. Others have found an increase in the excitability of D1-expressing pyramidal neurons of the PL, but not the IL, following chronic unpredictable stress. Similarly, exposure to synthetic fox odor resulted in enhanced excitatory transmission in the PL and not the IL. These studies, along with our own, suggest that various forms of stress can drive deficits in GABAergic neurons in the PL, which may then result in enhanced excitatory signaling, and that this disruption in cortical excitation and inhibition is specific to the PL.
Overall, our data suggests a link between ELS and oxidative damage in the PFC. Other lines of evidence support this link. Rats subjected to maternal deprivation showed increases in lipid peroxidation in the PFC, which was reversed by treatment with antioxidants such as ω-3, folic acid, and N-acetylcysteine. Similarly, rats that experienced social isolation as juveniles had increased activity of antioxidant enzymes such as superoxide dismutase that lasted into adulthood. Mitochondrial oxidative phosphorylation is responsible for the vast majority of oxidative damage, and the mitochondria has also been shown to be affected by ELS. For example, rats subjected to maternal deprivation displayed decreased expression of mitochondrial proteins involved in energy metabolism in adulthood. Thus, the ability of ELS to drive oxidative stress in the brain is an important area of research and future studies should examine similar measures in other brain regions, as well.

There are a few methodological limitations in the present study. Immunofluorescent cells were counted by hand, not via stereological analysis, which may have introduced error. An additional limitation is the fact that the dams were purchased and shipped to the colony while pregnant. An interaction between ELS and the stress of shipment may have factored into the findings reported. It is also important to note that our study focused on 8-oxo-dG as our sole marker of oxidative stress. However, this is restricted to DNA oxidation; future studies should include measures of protein and lipid oxidation in order to fully understand the oxidative damage induced by ELS.

In summary, these findings indicate that ELS drives oxidative stress accumulation in PV neurons. Specifically, this occurs in the PL of juveniles subjected to MS, irrespective of sex. This highlights an important distinction between effects of ELS on different subdivisions within the
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PFC. These data provide a mechanism for the deficits seen in PV neurons following ELS, and introduce a potential target for interventions.
References


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Figure 1

(A) Graphical timeline of experiments. (B) Illustration of regions of interest analyzed for PFC immunohistochemistry
Representative images showing PV in red and 8-oxo-dG in green. (A) PL of P21 animals. (B) IL of P21 animals. (C) PL of P40 animals. (D) IL of P40 animals. Scale bar = 100 µm.
Effects of MS in PL at P21. (A) ELS resulted in a significant increase in colocalization of 8-oxo-dG and PV. (B) There was no effect of Sex or Rearing Group on PV cell count. n = 8/group. *p < 0.05.
Figure 4

Effects of MS in IL at P21. (A) There was no effect of Sex or Rearing Group on colocalization of 8-oxo-dG and PV. (B) There was no effect of Sex or Rearing Group on PV cell count. n = 8/group.
Effects of MS in PL at P40. (A) There was a significant main effect of Sex on colocalization of 8-oxo-dG and PV. A Bonferroni multiple comparison test showed a significant increase in Female CON animals compared to Male CON animals. (B) Males had a significant increase in PV cell count. $n = 7-8$/group. *$p < 0.05$. **$p < 0.01$. ***$p < 0.001$. 

**Figure 5**
Effects of MS in IL at P40. (A) There was no effect of Sex or Rearing Group on colocalization of 8-oxo-dG and PV. (B) There was a significant main effect of Sex on PV cell count. A Bonferroni multiple comparison test showed a significant increase in Male CON animals compared to Female CON animals, and in Male ELS animals compared to Female ELS animals. 

\( n = 7-8/\text{group.} \) \(*p < 0.05, \quad ****p < 0.0001.\)