JOURNAL OF
EMERGING INVESTIGATORS

Article

Chlorpyrifos exposure in mice leads to altered expression of key neurodevelopmental proteins

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SUMMARY

Exposure to organophosphates in humans has been linked to adverse neurodevelopmental effects. Chlorpyrifos (CPF) is an organophosphate insecticide used widely in a variety of settings. Although some countries have banned or restricted the use of CPF, particularly in agriculture, other applications still continue. In an effort to predict the potential neurodevelopmental impact of CPF on humans, we investigated the effects of CPF exposure in mice. Specifically, we measured changes in levels of four proteins found in the frontal cortex, tyrosine hydroxylase, vesicular monoamine transporter 2, N-methyl-D-aspartate receptor, and gamma-aminobutyric acid transporter 1 in the frontal cortex. We hypothesized that the levels of these proteins would change upon exposure to CPF. The results of the experiments indicated that significant increases and decreases occurred in all four proteins in both the male and female mice upon CPF exposure. The results were sex-dependent; for example, CPF exposure induced a significant decrease in NMDAR expression in males and a significant increase in females. Changes in these proteins are associated with several mental health conditions including schizophrenia, depression, cognitive and social deficits, and Parkinson's disease. Thus, our findings highlight the risk of neurodevelopmental concerns in neonates due to pre- and postnatal exposure to CPF.

INTRODUCTION

Over the last several decades, the use of insecticides has increased considerably, especially in agricultural processes (1). Typically, the human population is exposed to insecticides through food, water, and the air they breathe. In recent years, insecticides have been shown to be detrimental to human health, especially to the development and function of the nervous system (2). Chlorpyrifos (CPF), an organophosphate insecticide, works by inhibiting acetylcholinesterase, an enzyme essential for the proper functioning of the nervous system in insects (3). Acetylcholinesterase inhibits the function of the essential neurotransmitter acetylcholine by breaking it down into acetic acid and choline (4). Therefore, when acetylcholinesterase is inhibited, acetylcholine is not broken down and results in an excess in the neuronal synapse, leading to a suite of symptoms, ranging from gastrointestinal distress to paralysis and respiratory failure (3). Previous research also suggests that CPF-induced toxicity may alter glutamatergic signaling by increasing the expression of N-methyl-D-aspartate receptor (NMDAR) subunits, potentially leading to excitotoxic damage and disruptions in normal brain development (5).

CPF has been used as a pesticide for both agricultural and non-agricultural uses since 1965 (6). Due to the health concerns that have been raised through observational and laboratory studies, some countries and jurisdictions have banned or restricted the use of CPF in certain applications, particularly in agriculture (6). Pursuant to a ruling by the Environmental Protection Agency (EPA), all CPF tolerances expired in the United States on February 28, 2022, so CPF insecticides can no longer be used on food products (6). However, non-agricultural, non-food uses — such as in industrial settings and for commercial production (e.g., flowers and Christmas trees) — are unaffected by EPA's final tolerance rule. As a result, the risk of CPF exposure, while reduced significantly, persists. In addition, the ban on CPF is not worldwide and several countries continue its use in agriculture (3). Hence, in this study, an investigation was performed to discern the effects of CPF exposure during fetal and postnatal development in mice to estimate the potential impact on humans.

Previous studies have investigated the effects of various insecticides on the frontal cortex and found evidence of neurotoxic effects, such as the impairment of neurotransmitter circuits and changes in protein levels (7, 8). Endosulfan, when given to female mice during gestation and lactation, caused changes in GABAergic, glutamatergic, and dopaminergic neurotransmitter systems in male offspring (7). Deltamethrin causes nerve cell loss and neuronal impairment in layer III of the frontal cortex, which is responsible for sending signals to other cortical areas and the thalamus, affecting functions like working memory and higher cognitive processes (8). Diazinon, another organophosphate insecticide that works similarly to CPF, decreases fetal frontal cerebral cortex thickness, which inhibits brain development and leads to behavioral changes (9).

Due to its physiochemical properties, CPF can easily cross the placenta, leading to exposure to the developing fetus (10). Moreover, CPF has been found to collect in breast milk, providing an additional route of exposure to the newborn infant (11). Exposure to CPF during critical periods of neurodevelopment have been found to lead to decreased IQ and increased symptoms of Attention Deficit/Hyperactivity Disorder (ADHD) in offspring of women exposed to CPF during pregnancy (10, 12). Since exposure to CPF can lead to detrimental health effects not only for the current population, but also future generations, it is a critical problem to investigate and address as early as possible.

Previous research has also explored the effects of CPF exposure in animal models. For instance, one study

https://doi.org/10.59720/24-145

found that early or late postnatal exposure of CPF showed evidence of working- and reference-memory impairment in rats (13). The study also found evidence of the neurochemical changes being distinctly gender-selective, which has also been supported by other studies (14). Additionally, it found that even when CPF was administered to neonatal rats at levels that do not elicit any obvious signs of toxicity, CPF can cause behavioral abnormalities (14). The effects tended to be acute in males but delayed in females. A study where CPF was administered to neonatal rats on postnatal days 1–4 found that that neonatal CPF exposure produces a wide range of issues with cholinergic synaptic function that can appear long after exposure and can continue into adulthood (15). In another study, CPF was administered to pregnant rats on gestational days 17–20 at low enough levels to not elicit any obvious signs of toxicity, and the authors showed that there were adverse effects on cholinergic systems in both the prenatal and postnatal periods (16). Another study that evaluated the developmental effects by observing the effects on serotonin with CPF exposures during different stages found persistent changes of varying impacts in each of these exposure windows (17). Several of these studies employed behavioral tests such as motor function assessments and memory related tasks, while others involved brain sample analyses.

While previous studies have highlighted the neurodevelopmental effects of CPF exposure, the specific regions of the brain and neurotransmitter circuits which are affected remain unknown. In this study, we evaluate pre- and postsynaptic proteins that are involved in regulating neurotransmitter signaling known to be altered in neurodevelopment disorders. We specifically investigate how the levels of the proteins glyceraldehyde-3-phosphate dehydrogenase (GAPDH), NMDAR, gamma-aminobutyric acid (GABA) transporter 1 (GAT1), vesicular monoamine transporter 2 (VMAT2), and tyrosine hydroxylase (TH) in the frontal cortex are affected by exposure to CPF during fetal development. These proteins were chosen as levels of these proteins have been associated with certain neural and neurodegenerative diseases (4, 7, 18–23). We hypothesized that the levels of TH, VMAT2, NMDAR, and GAT1 in the frontal cortex of the brain would change as a result of CPF exposure. Our results supported this hypothesis, showing statistically significant changes in all of these proteins with exposure to CPF during development. Our results lay the groundwork for future studies to further understand and mitigate the effects of CPF.

RESULTS

In this study, we evaluated frontal cortex samples from the brains of mice after they had been exposed to a vehicle control or CPF during gestation and lactation. Protein expression was quantified using western blot (**Figure 1**). The expression of cortical TH in male mice exposed to CPF showed a 183% increase compared with controls (Student's t-test, treatment group: 5.1 ± 0.93, control group: 1.8 ± 0.895, $p = 0.0004$). In contrast, exposure to CPF did not appear to have a significant effect on TH expression levels in female mice (Student's t-test, treatment group: 3.8 ± 1.3, control group: 3.0 ± 0.94, $p = 0.27$). (**Figure 2**). Assessment of cortical VMAT2 identified an opposite effect to that observed with cortical TH. While VMAT2 expression in male mice exposed to CPF did not significantly differ from control (Student's t-test, treatment group: 4.9 ± 1.2, control group: 4.4 ± 1.0, $p = 0.46$), CPF exposed female mice showed a 46% increase in VMAT2 expression compared with control mice (Student's t-test, treatment group: 3.8 ± 0.6, control group: 2.6 ± 0.43, $p = 0.009$) (**Figure 3**).

Cortical NMDAR expression was significantly affected by CPF in both male and female mice. While male mice exposed to CPF demonstrated a 61% reduction in NMDAR expression

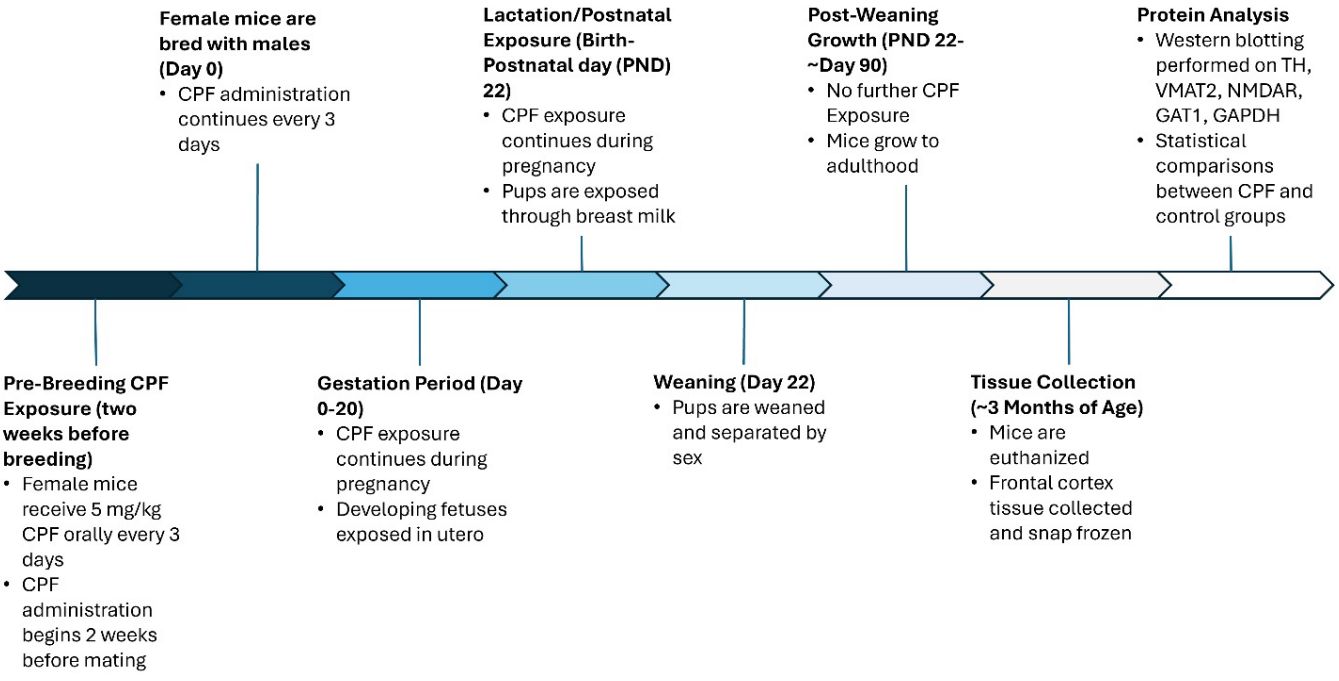

Figure 1: Timeline of experimental analysis procedures. The above figure shows the methodological steps in the sequence they were performed and provides the duration for the steps involved.

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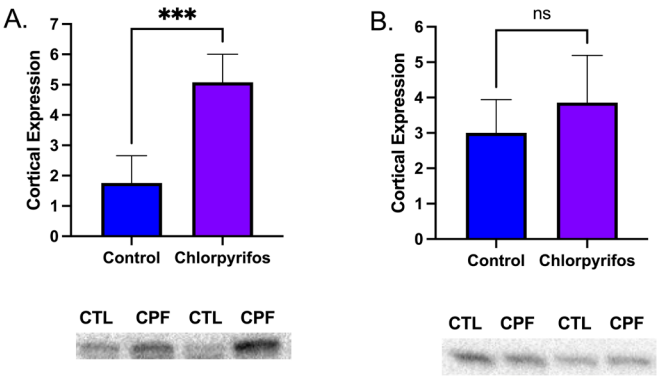

Figure 2: Cortical tyrosine hydroxylase (TH) protein expression with chlorpyrifos exposure in males (A) and females (B) and western blot bands for two biological replicates of males (C) and females (D). Protein expression was measured by western blot. Unpaired Student's t-tests were conducted. Bar graphs with whiskers showing mean ± SD for cortical TH protein expression. n=4-6 for each graph. The p-value for A is 0.0004. The p-value for B is 0.27, so it is nonsignificant. ***p < 0.0005.

compared with control (Student's t-test, treatment group: 0.18 ± 0.11, control group: 0.46 ± 0.09, p = 0.002), we observed a 190% increase in NMDAR expression in female mice exposed to CPF (Student's t-test, treatment group: 0.58 ± 0.22, control group: 0.2 ± 0.14, p = 0.01) (**Figure 4**). Exposure of male offspring to CPF did not have a profound impact on GAT1 expression (Student's t-test, treatment group: 4.9 ± 1.6, control group: 4.6 ± 1.4, p = 0.75). However, CPF exposure led to a 44% reduction in GAT1 levels in female mice (Student's t-test, treatment group: 1.8 ± 0.37, control group: 3.2 ± 0.62, p = 0.002) (**Figure 5**). Finally, assessment of cortical GAPDH found no change in expression based on treatment or sex. Exposure of male mice to CPF found GAPDH expression to be 2.3 ± 0.47, while expression in control mice was 2.3 ± 0.38. Similarly, cortical GAPDH in treated female mice was 1.12 ± 0.13 and in control female mice was 1.08 ± 0.13 (**Figure 6**).

DISCUSSION

We hypothesized that the levels of TH, VMAT2, NMDAR, and GAT1 in the frontal cortex of the brain would change as a result of CPF exposure. Our results supported this hypothesis showing statistically significant changes in several of these proteins with exposure to CPF during development.

A significant increase of TH expression was observed in the male mice with CPF exposure, but not in the female mice (**Figure 2**). TH is an enzyme that helps with the synthesis of dopamine and norepinephrine (24). An increase in TH expression could mean that more dopamine is produced, or that not enough dopamine is being produced so the neurons are being stimulated to create more (25). Increased TH expression has been found to be associated with an increase in chronic stress which decreases the working memory (9). Traumatic brain injury has also been found to cause an increase in TH (25). A decrease in TH has been shown to affect behavior, social memory, fear learning, response inhibition, but not motor, sensory, or instrumental learning (26). It also regulates aggressive behavior (27). There has been evidence showing that a reduction in TH expression can be related to Parkinson's disease and schizophrenia since they result from a decrease or imbalance in dopamine (18, 19). These results therefore indicate that increased TH levels in the offspring resulting from CPF exposure during gestation period in the mother can lead to several health risks, and male offspring may be more susceptible to changes in TH levels as a result of CPF exposure.

Increased expression of VMAT2 was observed for both male and female mice offspring with CPF exposure (**Figure 3**). The VMAT2 protein is located on synaptic vesicles and functions to transport dopamine, norepinephrine, or serotonin into synaptic vesicles (28). An increase in VMAT2 may enhance the function of the monoaminergic neurons, as dopamine and norepinephrine that are not packaged into synaptic vesicles can be oxidized in the cytosol of the neuron and damage other parts of the neuron (28, 29). VMAT2 has been shown to protect against the neurotoxicant 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which results in Parkinsonism (29, 30). MPTP causes damage to the nigrostriatal dopamine (28). A decrease in VMAT2 expression has been linked to Parkinson's disease (20, 28). Cytosolic dopamine (i.e., dopamine that has not been packaged by VMAT2) can oxidize and form reactive species and other compounds that react and modify proteins, lipids, and DNA, and an elevated level of these modified compounds have been observed within the brains of patients with Parkinson's disease (31). However, since our results indicate an increased expression of VMAT2 for both male and female mice, rather than a decrease, such risks are potentially minimized, as increased VMAT2 has been shown to protect dopamine neurons from methamphetamine and MPTP (29, 30). Increased VMAT2 can protect dopamine terminals by keeping dopamine in vesicles, which supports dopamine release even after exposure to toxins, which indicates a compensatory response (29, 30).

We observed a dramatic change in expression of NMDAR in the frontal cortex and the changes differed by sex (**Figure 4**). NMDAR is a receptor found throughout the brain that is activated by the neurotransmitter glutamate and is essential for synaptic refinement during cortical development (22). Glutamate is typically regarded as an excitatory neurotransmitter because when it interacts with NMDAR, it leads to activation of the neuron (32). Glutamate

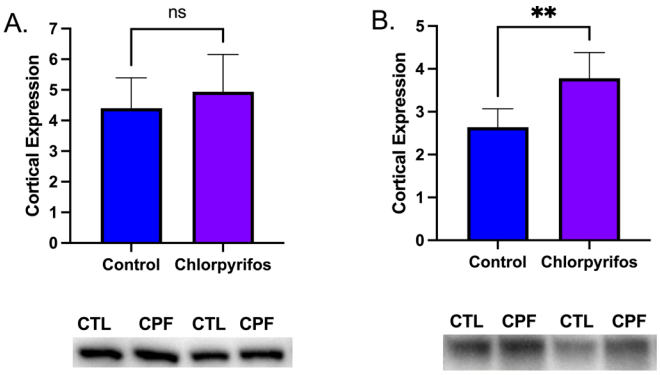

Figure 3: Cortical vesicular monoamine transporter 2 (VMAT 2) protein expression with chlorpyrifos exposure in males (A) and females (B) and western blot bands for two biological replicates of males (C) and females (D). Protein expression was measured by western blot. Unpaired Student's t-tests were conducted. Bar graphs with whiskers showing mean ± SD for cortical VMAT2 protein expression. n=4-6 for each graph. The p-value for A is 0.46, so it is nonsignificant. The p-value for B is 0.009. **p < 0.005.

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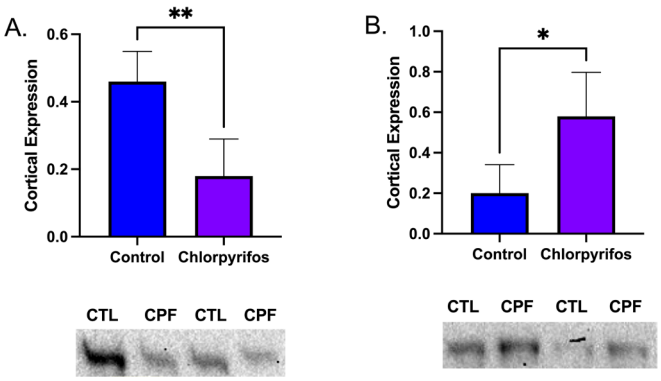

Figure 4: Cortical N-methyl-D-aspartate receptor (NMDAR) protein expression with chlorpyrifos exposure in males (A) and females (B) and western blot bands for two biological replicates of males (C) and females (D). Protein expression was measured by western blot. Unpaired Student's t-tests were conducted. Bar graphs with whiskers showing mean ± SD for cortical NMDAR protein expression. n=4-6 for each graph. The p-value for A is 0.002, and the p-value for B is 0.01. **p < 0.005 and *p < 0.05.

works with GABA to create a balance to maintain proper function of the prefrontal circuit (22). NMDAR hypofunction during development is associated with local GABA deficits and circuitry dysfunction in the prefrontal cortex (21). This impaired connectivity in the prefrontal cortex has been linked to cognitive and social deficits (22). NMDA receptor hypofunction has also been associated with schizophrenia (33). Reduced expression of the NMDA receptor subunits NMDA receptor subunit 2A and NMDA receptor subunit 2B has been found in the prefrontal cortex of depressed subjects (32). The literature indicates an association between reduced protein expression of NMDAR and diseases such as depression, schizophrenia, and other cognitive deficits. Our results therefore indicate a higher risk in the female mice for such diseases in the context of CPF exposure. Conversely, increased protein expression of NMDAR has been associated with Huntington's disease, so our results therefore indicate a possible higher risk for this disease in males as a result of the CPF exposure (34).

A reduction in expression of GAT1 is seen in female offspring with exposure to CPF (**Figure 5**). The impaired functioning of the dorsolateral prefrontal cortex, which usually characterizes schizophrenia, is usually attributed to issues in GABA neurotransmission. A previous study found that such GABA neurotransmission issues in postmortem tissue of the dorsolateral prefrontal cortex from diagnosed schizophrenic patients could be related to changes of GAT1 protein in chandelier neurons (23). GAT1 has the same function as DAT; it recycles GABA from the neuron synapse back into the neuron from which it was released (23). When this happens, the activation of GABA with its receptors is halted. As a result, GABA is considered an inhibitory neurotransmitter. When GAT1 interacts with GABA receptors, it causes inactivation of the neuron. In this way, it works in an opposite way to glutamate (23). A reduction in expression of GAT1 could indicate that less GABA is being recycled, meaning more of it is able to interact with GABA receptors. The fact that the reduction is seen in female mice is interesting, given the increase in NMDAR seen in these same mice. This concurrence could indicate that the increase in NMDAR is leading to an increase in neuron excitation; the reduction in GAT1 may represent a compensatory mechanism intended to reduce this hyperexcitation.

The results of this study show that exposure to CPF during critical periods of neurodevelopment leads to statistically significant changes to protein expression in the frontal cortex of male and female mice. Changes in these protein levels have been found in previous research to be associated with several health conditions including schizophrenia, depression, cognitive and social deficits, and Parkinson's disease (9, 18–23, 25–34). Consequently, it is evident that the risk of development of these health conditions in neonates due to exposure to CPF during critical periods of neurodevelopment warrants further consideration by regulatory agencies. Therefore, it is critical to revisit the legislation and policies associated with the use of CPF to evaluate if further restrictions are required in a global context to reduce the health risks associated with developmental CPF exposure.

A limitation of this study is that the health risks discussed are based on associations rather than clinical outcomes in human populations. The changes in protein expression indicate that CPF can alter signaling pathways, but these alterations are markers of potential risk. Since this was not a clinical trial, we cannot confirm that the changes in protein expression would lead to the specific neurological disorders discussed or which symptoms would be most affected. Rather, the results suggest possible risks based on known links between these proteins and various health conditions. The findings should be viewed as a hypothesis about how exposure to CPF during development can cause different mental conditions that future studies can test.

Further studies in this area are needed to understand the mechanism leading to the changes in protein expression. For example, studies using RNA-sequencing or qPCR can be performed to determine whether the RNA transcripts for the proteins investigated in this study are also impacted.

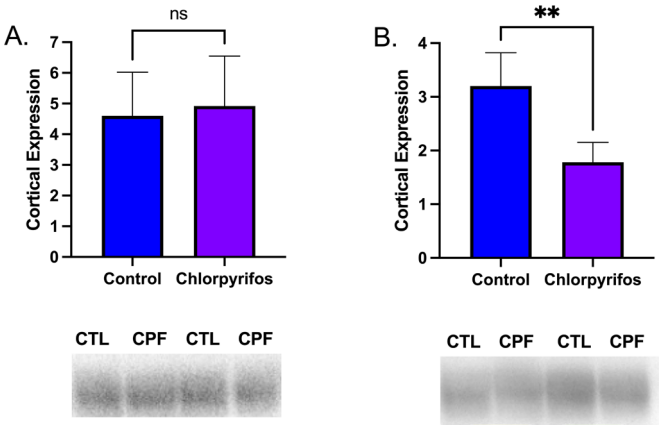

Figure 5: Cortical gamma-aminobutyric acid (GABA) transporter 1 (GAT 1) protein expression with chlorpyrifos exposure in males (A) and females (B) and western blot bands for two biological replicates of males (C) and females (D). Protein expression was measured by western blot. Unpaired Student's t-tests were conducted. Bar graphs with whiskers showing mean ± SD for cortical GAT1 protein expression. n=4-6 for each graph. The p-value for A is 0.75, so it is nonsignificant. The p-value for B is 0.002. **p < 0.005.

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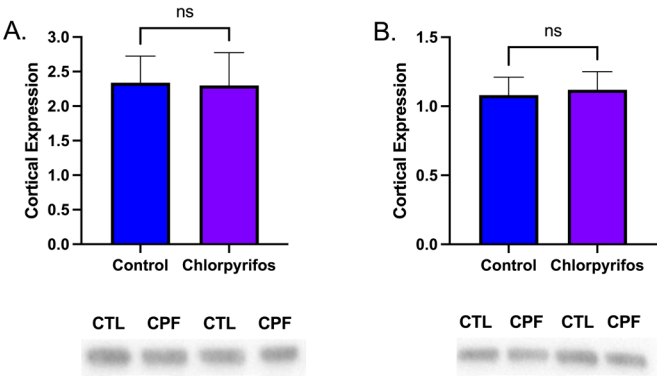

Figure 6: Cortical glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein expression with chlorpyrifos exposure in males (A) and females (B) and western blot bands for two biological replicates of males (C) and females (D). Protein expression was measured by western blot. Unpaired Student's t-tests were conducted. Bar graphs with whiskers showing mean ± SD for cortical GAPDH protein expression. n=4-6 for each graph. The p-value for A is 0.9 and for B is 0.64, so they are both nonsignificant.

This could lead to a better understanding of whether the observed changes in protein levels are due to transcriptional regulation or post-transcription modifications. Investigating upstream regulatory pathways and epigenetic changes could provide an explanation for how CPF exposure during critical developmental windows alters gene expression patterns and contributes to long-term neurodevelopmental effects. Overall, more research, including long-term behavioral studies and clinical investigations, is needed to fully understand the impact of CPF exposure on neurodevelopment. However, identifying these specific protein changes provides a foundation for future studies that could explore therapeutic strategies to help mitigate the effects of CPF.

MATERIALS AND METHODS

Animal Care and Treatment Administration

Eight-week-old female and male C57BL/6J mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA). Mice were maintained on a 12:12 light-dark cycle in polycarbonate cages at 23°C and 40% humidity with food (Purina Rodent Chow #5001; Research Diets, New Brunswick, NJ, USA) and water available ad libitum. All procedures were conducted in accordance with the U.S. National Institutes of Health (NIH) Guide for Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at Emory University (35). Female mice were randomly assigned to an exposure group. Chlorpyrifos (CPF) mice received orally administered 5 mg/kg of chlorpyrifos (ChemService, West Chester, PA) dissolved in corn oil vehicle every 3 days beginning 2 weeks prior to breeding continuing throughout gestation and lactation while the control group received only the corn oil in the same increments. This dose was chosen based on the reference dose (RfD) for chlorpyrifos (0.005 mg/kg/day), which is an estimate of the quantity of chemical that a person could be exposed to over their lifetime with no appreciable risk of adverse health effects (36). Based on this value, it is estimated that the average adult female is exposed to 0.387 mg/day chlorpyrifos. This value is three times less than the amount of chlorpyrifos our female mice received with each exposure. Animals were weaned on postnatal day (PND) 22, separated by sex, and euthanized at 3 months of age. The dosing paradigm is based off documented human exposures, including exposures within pregnant women (37–39). The experiments reported here represent 4–6 litters per treatment group. Each litter was treated as an individual experimental unit. One male and one female mouse per litter were utilized for each experiment to reduce confounding due to litter effects. All tissue samples were snap frozen and stored at 80ºC for analysis.

Sample Treatment

Male and female mice were euthanized by live decapitation, as approved by the Institutional Animal Care and Use Committee at Emory University. Whole brains were removed and transferred to a cold block for dissection. Brain regions of interest were isolated bilaterally and snap frozen in liquid nitrogen, then stored at -80ºC until analysis. Western blots were performed to quantify the amount of NMDAR, GAT1, TH, and VMAT2 **(Table 1)**. Briefly, frontal cortex samples were homogenized in a sucrose buffer (120 mM sucrose, 25 mM Tris-HCl, pH 7.4) containing protease inhibitor cocktail. Samples were centrifuged at 3,000xg for 5 minutes and the supernatant was transferred to a new tube. The supernatant was further centrifuged at 14,000 x*g* for 45 minutes and the pellet was resuspended in 100 µl of sucrose buffer containing protease inhibitors cocktail. Protein concentration of each sample was assessed by Bicinchoninic Acid (BCA) Kit (Sigma-Aldrich, St. Louis, MO). Samples (5–10 µg protein)

Antibody	Concentration	Incubation Time (hours)	Temperature	Supplier	Catalogue Number
Rabbit anti-NMDAR	1:2,500	18	4ºC	Sigma-Aldrich, St. Louis, MO	AB9864
Rabbit anti-GAT1	1:2,500	18	4ºC	Sigma-Aldrich, St. Louis, MO	AB1570W
Rabbit anti-TH	1:2,500	18	4ºC	Sigma-Aldrich, St. Louis, MO	AB152
Rabbit anti-VMAT2	1:2,500	18	4ºC	Covance Custom Immunology Services; Madison, WI	
Rabbit anti-GAPDH	1:2,500	18	4ºC	Abcam, Waltham, MA	AB245355

Table 1: Polyclonal antibodies for immunoblotting. This table shows the concentration, incubation time, incubation temperature, supplier, and catalogue number of the polyclonal antibody.

https://doi.org/10.59720/24-145

were subjected to polyacrylamide gel electrophoresis on 10% precast NuPage gels (Invitrogen, Carlsbad, CA). Samples were electrophoretically transferred to a polyvinylidene difluoride membrane, and nonspecific sites were blocked in 7.5% nonfat dry milk in Tris-buffered saline (135 mM NaCl, 2.5 mM KCl, 50 mM Tris, and 0.1% Tween 20, pH 7.4). Membranes were then incubated overnight in a polyclonal antibody to the NMDA receptor. NMDAR antibody binding was detected using a goat anti-rabbit horseradish peroxidase secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, 1:5,000) and enhanced chemiluminescence. The luminescence signal was captured on a BioRad Fluorochem imaging system (Temecula, CA, USA) and stored as a digital image for densitometric analysis. Membranes were stripped for 30 minutes at 37°C with a stripping buffer made of glycine (1.5g), SDS (0.1g), Tween-20 (1mL), and up to 100mL of dH2O, and then sequentially re-probed with additional antibodies VMAT2, TH, and GAT1. GAPDH blots were used to ensure equal protein loading across samples. Protein expression for each marker is represented as arbitrary units of intensity.

Statistical Analysis

GraphPad Prism 10.4.2 was used to perform unpaired Student's *t*-tests for comparisons involving only two groups. A litter was considered the smallest unit of analysis. For our analysis, an individual litter was considered the smallest unit of analysis, with each litter representing an independent replication (n = 4–6 litters/treatment group). Statistical significance is reported at $p < 0.05$.

Received: June 5, 2024
Accepted: August 22, 2025
Published: August 31, 2026

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