Neurological Effects of Chlorpyrifos on Prenatally-Exposed Rats

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Abstract

Though extensively documented, autism is still poorly understood. Possible factors include prenatal or neonatal chemical exposure that causes declined synaptic transmission. One chemical, the pesticide known as chlorpyrifos, has been shown to cause anatomical differences in the areas of rats’ brains that are linked to behavioral symptoms closely related to autism. With the knowledge of the increased susceptibility of males to prenatal drug exposure, it was hypothesized that male rats exposed to chlorpyrifos before birth would exhibit such symptoms. Two tests, an elevated plus maze and a Morris water maze, were used to test anxiety and cognition, respectively. Anxiety tests yielded no differences between rats prenatally exposed to the chemical, or between males and females. Similarly, cognition tests showed no such correlations. Though these results suggest no apparent connection between chlorpyrifos and autism-like behavior, it is still unclear whether the rats experienced internal anatomical change due to their exposure. Used as a pilot study, this experiment could be coupled with a cellular analysis to fully illustrate the effects of chlorpyrifos.
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Introduction

Autism Spectral Disorders (ASD) encompass an entire range of traits and behaviors, and are showing increasing prevalence in children (Bourgeron & Persico, 2006). While the exact causes of ASD are unclear, one widely accepted explanation is a change in normal neurodevelopment (Bourgeron & Persico, 2006). Furthermore, males are generally more at risk of having these disorders, possibly due to their increased susceptibility to prenatal drug exposure (Sakuma, 2004).

Though recently banned for residential purposes, agricultural use of the insecticide chlorpyrifos (CPF) is still prevalent throughout the world (Padilla et al., 2002; Aldridge et al., 2004). An organophosphate, CPF has been linked to developmental disorders in rats postnatally exposed to the pesticide (Padilla et al., 2002). However, late gestational CPF treatment has yielded only slight anatomical differences between neonates treated with both non-toxic dosages (Chen et al., 1998) or dosages that exceed toxicity levels for pregnant dams (Padilla et al., 2002). Such functional and structural inconsistencies included inhibition of fetal brain cholinesterase, growth impairment (Padilla et al., 2002), and altered serotonin development (Aldridge et al., 2005). This variation of serotonin levels sparked interest in using CPF to test for autism-like symptoms in rats, as the effects resulting from a sharp increase in serotonin levels closely resembled autistic symptoms in humans (Kanai et al., 2006).

Increased male susceptibility to autism coupled with the overall ambiguity of the disorder created a research objective aimed at exploring both the gender discrepancies within autism and the causes of the disease. In the present study, rats were initially comprised of six pregnant dams, half of which were given CPF treatment. After these
dams gave birth, 40 offspring were separated based on gender and treatment. It was hypothesized that, given our understanding of the effects of CPF and the propensity of males to exhibit increased rates of autism, male rats prenatally exposed to CPF would show differences in performance in memory and anxiety tests. Anxiety was tested using an elevated plus maze modified from Bhattacharya et al. (1997). Behavior of specific experimental animals in such a test would indicate autism-like symptoms in the rats. Because CPF has also been shown to affect cognitive abilities, a Morris water maze was used to judge memory skills, and was performed according to the procedure of Hager (2003) and Pletnikoff (2006).
Literature Review

Chlorpyrifos

Chlorpyrifos is one of the most widely used organophosphate pesticides, both indoors and outdoors (Ahmed & Davis, 1998). Under the name Lorsban, it is used agriculturally, while under another name, Dursban, it is intended for use around the home (Gibson et al., 1998; Liburd & Weihman, 2006). However, restrictions for home use have been created due to concern of its potential hazards to pregnant women and children (Qiao et al., 2001). As Ahmed and Davis (1998) have demonstrated, CPF has the ability to vaporize into the gas phase after it has been applied indoors as a liquid spray, and can become absorbed into several solid surfaces, including furniture and children’s toys. Although CPF has a half-life of 30 days, it has been shown to exist for up to eight years following indoor treatment (Ahmed & Davis, 1998).

Extensive use does not come without extensive study. By 1998, 250 studies had already been done investigating the possible health risks associated with CPF treatment, and enough empirical evidence had been found to permit its use (Gibson et al., 1998). Furthermore, Gibson et al. (1998) stated that studies performed according to EPA guidelines did not find CPF to be mutagenic, teratogenic, or carcinogenic. Qiao et al. (2001) argued otherwise, conducting a study that provided evidence of CPF as a neuroteratogen. In that study, neuron cells and glial cells were exposed in vitro to chlorpyrifos. It was discovered that CPF adversely affected DNA synthesis (and thus neural cell development) of both cell types, while the active metabolite of CPF, chlorpyrifos oxon, inhibited cholinesterase.
A previous study performed by Sherman (1996), described several birth defects of four children prenatally exposed to Dursban. Such defects included abnormalities of the eyes, brain, genitalia, heart, and teeth. All children experienced growth impairment, while three had severe mental retardation. Sherman (1996) proposed the presence of an "unrecognized syndrome" as the cause of these defects, along with prenatal exposure to Dursban.

There have also been extensive studies involving CPF exposure in rats. Chen et al. (1998) noted several publications, as well as their own, in which rats prenatally exposed to CPF show no unique sensitivity when compared to the dams. Therefore, CPF was not considered to be a selective developmental neurotoxin. Methods included the administration of various CPF dosages by force-feeding the dams from gestation day six until day ten of nursing. None of the dosages caused alterations in cognitive functions. Only the highest dosage, which caused obvious maternal toxicity, elicited adverse effects on the progeny, which were then attributed to maternal neglect. Chen et al. (1998) concluded that CPF was not considered to be a selective developmental neurotoxin. More recent studies provide contradictory results.

Padilla et al. (2002) subcutaneously injected pregnant dams with varied dosages of CPF on different days of gestation. At comparable dosages used in the Chen et al. (1998) study, it was found that neonates experienced changes in cholinergic synaptic markers and cholinesterase inhibition. While testing only fetal exposure, Padilla et al. (2002) concluded that neonates were not as susceptible to CPF as fetuses. Seidler et al. (1995) suggested that dissolving CPF in dimethyl sulfoxide would allow for rapid and
complete absorption, meaning that force-feeding of CPF may not be the best method of introduction.

Aldridge et al. (2005) conducted a similar experiment and demonstrated that even at levels below inhibition of brain cholinesterase, normal neural cell development was disrupted. Studies such as these provide alarming evidence of the potential effects CPF could have on humans.

**Autism Spectral Disorders**

Autism is defined as an intricate neurodevelopmental disorder, causing repetitive behavior habits, social isolation, and inhibited communication skills (Daniels, 2006). The cause of ASD is still very much a mystery, but many believe it to have both genetic and environmental roots (Croen et al., 2006). Studies of family genetics suggest this disorder is inherited, while the increase in diagnosed cases points to environmental origins (Croen et al., 2006). However, Daniels (2006) mentioned that ASD rates may not be increasing – rather, our methods of diagnosing patients have improved, and there are few data collected for a single population over time. Whether or not rates are increasing, a gene-environment interaction is still a widely accepted cause (Daniels, 2006).

Bourgeron and Persico (2006) compiled a table with all proteins known to be associated in some way to ASD. Functions are varied, but all proteins are involved with synaptic abilities and neurodevelopment (Bourgeron & Persico, 2006). In studying the characteristics of these proteins, it was possible to construct three pathways of ASD pathogenesis: one that alters cell migration, another that affects the glutamate-gamma-aminobutyric acid equilibrium, and a third that includes proper synapse development.
(Bourgeron & Persico, 2006). These data, in conjunction with environmental clues, are making progress towards understanding the exact causes of ASD (Bourgeron & Persico, 2006).

Thalidomide (THAL) was a drug used in the 1950s and 1960s to treat anxiety and insomnia (Kanai et al., 2006). Usage halted when THAL, taken by pregnant women, was linked to a plethora of birth defects, including heart problems, absence of limbs, and autism (Kanai et al., 2006). When studied in rats, it was found that THAL caused a dramatic increase in the amount of serotonin in the brains of the progeny sired by treatment females (Kanai et al., 2006). Alteration of serotonin levels is also caused by CPF, according to a study performed by Aldridge et al. (2005). Thus, a direct link between CPF exposure and ASD can be proposed.

**Memory and Learning**

Long-term memory is formulated chiefly by the hippocampus and nearby cortex (Miezin et al., 1992). Together, this brain system is responsible for the acquisition, storage, and retrieval of memory associated with events (Miezin et al., 1992). This type of memory, known as declarative memory, is considered to be conscious (Squire & Zola, 1996). Declarative memory differs from nondeclarative memory in that it deals with specific facts and events, rather than skills and habits (Squire & Zola, 1996).

Declarative memory can be adversely affected by damage to the hippocampus (Squire & Zola, 1996). Some forms of encephalitis can damage the hippocampus and cause amnesic syndromes. Human patients show a loss in this type of memory, and are unable to recall names, dates or facts (Rozin, 1976).
Damstra et al. (1975) stated that biological changes associated with learning do not occur in localized regions of the brain, but rather involve entire regions of brain tissue. Spatial learning, however, has been attributed to the hippocampus, and more specifically, the dorsal hippocampus (Andersen et al., 1995).

Rats and humans possess similar developmental patterns of the central nervous system, including deficiencies in long-term memory (Campbell & Coulter, 1976). Thus, studies performed with rats would be appropriate in inferring similar conclusions in humans (Campbell & Coulter, 1976).

Several studies have shown that CPF alters the development of proper serotonin function (Aldridge et al., 2005). According to Flood and Jarvik (1976), serotonin may have an effect on the acquisition of memory, although such effects have not been well documented in humans.
Materials and Methods

Animals

Six pregnant female Wistar rats were acquired on their 11th day of pregnancy from Simonson Labs in California. Rats were housed in separate cages in a room kept at a constant 80°F. They were given free access to Mazuri Rat Chow and water, and initially weighed between 230 and 300 grams.

Treatment

Beginning on their 12th day of gestation and ending on the day before birth, all dams received daily subcutaneous injections. Three of the dams were given 0.1 mL vehicle injections of dimethyl sulfoxide (DMSO), while the other three were given 0.1 mL of 3 mg/kg CPF dissolved in DMSO. The CPF dosage was determined using body mass ratios and recommended amounts outlined by Padilla et al. (2002). Such a dosage was below the amount that elicited maternal toxicity (5 mg/kg) and above the threshold for inhibition of fetal brain cholinesterase (2 mg/kg).

Offspring

Born on either gestation day 20 or 21, offspring were separated after 21 days of weaning based on gender and treatment. A total of 40 rats were kept, evenly distributed into four groups: Male DMSO, Female DMSO, Male CPF, and Female CPF. Like their mothers, the offspring were given free access to food and water, and stored three or four per cage in the same secured room.
Anxiety Testing

Based on the protocol of Bhattacharya et al. (1997), an elevated plus maze was constructed to test anxiety. This was made out of wood and cardboard, with a height of 112 centimeters. Two wooden beams, each 112 centimeters long, crossed each other to form a "plus" sign. One of these beams contained cardboard corridors that encompassed each open end to a height of 30.5 centimeters. The other beam was left open. Along this open beam, colored lines were drawn at two, four, and six inches outwards from the intersection. The dimensions were adapted from the Bonhoeffer et al. (2003).

Rats were 25 days old when testing began. Each one was set into the maze directly on the intersection, facing an open beam of the maze. They were left in the maze for five minutes and recorded on video. After the five minutes, the rat was removed and placed back in his or her cage. Rats were evaluated on the following criteria: time spent in open arms, number of entries into open arms, corridor changes, furthest exploration, and open arms visited.

Cognition Testing

A Morris water maze was used to test cognition. A plastic pool 2.44 meters in diameter was filled with water to a depth of 16.5 centimeters, and then dyed using soluble, non-toxic purple paint that made the water opaque. Approximately 15 centimeters from the pool wall, a platform was set that was 1.5 centimeters below the surface of the water, and thus impossible to be seen from the perspective of the rats. The platform was an upside-down 1-liter beaker with wire mesh attached to the upper surface for grip. Set up around the pool were conspicuous visual markers to provide orientation about the location of the platform to the rats. Subjects were placed in the pool roughly 15
cm from the wall opposite the platform. The researcher would then hide and monitor the latency. Each rat was given a maximum time of two minutes in the pool before being manually placed on the platform for 25 seconds. If the rat found the platform in less than 15 seconds, he or she was immediately removed from the pool. Times were recorded for each rat for 21 consecutive days until all were considered to have learned the location of the platform. “Learning” was defined as reaching this location in less than 15 seconds for four consecutive days.

**Statistical Analysis**

Data from both tests were analyzed using the MANOVA and ANOVA analyses. Graphs and pertinent figures were created using Microsoft Excel.
Results

Anxiety Testing

The anxiety levels of the rats were measured first. Using an elevated plus maze, I focused on the apprehension of the rats to explore their surroundings. Figures 1-5 present results of the following: time spent on open arms, furthest exploration, corridor changes, open arm entries, and number of arms visited. Table 1 outlines the results of a MANOVA test, which shows no significant differences across any of the four groups for any variable (P>0.05).

Time Spent on Open Arms

No significant differences were found in the elevated plus maze in regard to average time spent on the open arms of the maze (Figure 1). Large error values come from small sample sizes and great variation between individuals.

Furthest Exploration

No significant differences were found with respect to the average furthest distance explored on the open arms (Figure 2). Much like the time spent on the open arms, it seems that perhaps the female DMSO group was less adventurous, as it averaged merely 4.5 inches of exploration. However, great error values erase any significance.

Average Number of Corridor Changes

No significant differences were found in the number of corridor changes (Figure 3). Males, regardless of their treatment, appeared to be significantly less anxious than the females, as observed by more corridor changes. Statistical analyses, however, prove otherwise, displaying a P-value greater than 0.05 (Table 1).
Open Arm Entries

No significant differences were found in regard to the number of open arm entries (Figure 4). It appears that the CPF male group was surprisingly, though not significantly, less anxious than the others (Table 1).

Open Arms Visited

No significant differences were observed for the average number of open arms visited (Figure 5). Unlike the number of entries on the open arms, which counted total number of entries and repeated entries to the same arm, this category only had a maximum of two distinct arms available for exploration. Once an arm was visited, it was not counted again.

Cognition Testing

A Morris water maze was used to evaluate the cognition skills of the rats. Rats were tested on how quickly they could find the submerged platform for successive days (Figure 6). Figure 7 shows the average day each group learned the maze. Table 2 displays the results of an ANOVA test. Again, no significant differences appeared among the four groups (P>0.05).
Figure 1. Average times spent on either of the open arms of elevated plus maze. No significant differences were observed. Error bars represent standard error.
Figure 2. Average distance of furthest exploration onto either of the open arms of the elevated plus maze. Each arm was 19 inches long. No significant differences were seen. Error bars represent standard error.
Figure 3. Average number of corridor changes on the elevated plus maze. Corridors dictated the two “closed” arms of the maze, which were separated by the open arms intersection. Though males appear to have made more corridor changes than the females, there is no significant statistical differences between gender (Tables 1 and 2). Error bars represent standard error.
Figure 4. Average number of total entries into the two open arms of the elevated plus maze. So significant differences were observed. Error bars represent standard error.
Figure 5. Average number of open arms visited for each group on the elevated plus maze. Rats had a maximum of two arms to visit. No significant differences were seen among the groups. Error bars represent standard error.
Figure 6. Average times for each of the four test groups for 21 consecutive trials. A significant P-value was observed among trials (P<0.05), but not between treatment or gender (P>0.05). Error bars represent standard error.
Figure 7. Average day each group learned the Morris water maze. Learning the maze meant finding the platform in less than 15 seconds for four consecutive days. No significant differences were seen. Error bars represent standard error.
Table 1. MANOVA analysis of gender and treatment effects on all response variables for both mazes. Significant values (a P-value <0.05) do not exist.

<table>
<thead>
<tr>
<th>Effect</th>
<th>Wilks' Lambda</th>
<th>Rao’s R</th>
<th>df 1</th>
<th>df 2</th>
<th>P-value</th>
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<tbody>
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<td>5.000</td>
<td>32.00</td>
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<tr>
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<td>5.000</td>
<td>32.00</td>
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Table 2. ANOVA analysis of trial, gender, and treatment effects in all response variables for the Morris water maze. Unlike the MANOVA analysis, this analysis includes the effect of trial on the results. The only significant P-value (italicized) comes from the sole effect of trial on the results. This is to be expected, as every group, regardless of gender or treatment, experienced significant results in the Morris water maze: trial times decreased as trial number increased.

<table>
<thead>
<tr>
<th>Effect</th>
<th>df Effect</th>
<th>MS Effect</th>
<th>df Error</th>
<th>MS Error</th>
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<td>906.3</td>
<td>34.56</td>
<td>&lt;0.001</td>
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<td>906.3</td>
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<tr>
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Discussion

The results of this experiment suggest no link between prenatal exposure to CPF and autism-like symptoms or cognitive deficiencies in rats. Both tests yielded no significant differences across gender or treatment. My original hypothesis, that the male rats exposed to CPF would exhibit differences in performance in the elevated plus maze and the Morris water maze, was therefore not supported. All rats, regardless of gender or treatment, performed similarly in both tests. The only outstanding difference was observed between genders in the number of corridor changes on the elevated plus maze (Figure 3). Most likely due to a small sample size, these results show no significance (P>0.05).

These findings are not in agreement with those suggested by Aldridge et al. (2005). That particular experiment supported the idea that CPF is toxic to the developing rat brain, stating that there is a functional increase in serotonin levels. Such effects even occurred at CPF levels below those that would cause maternal or fetal toxicity. However, it is still uncertain if these anatomical differences would elicit behavioral changes, as was the aim of my study.

Tested rats may have experienced internal anatomical differences that could not be detected by the performed tests, though there are no data to support this. Perhaps more advanced testing techniques are required to find behavioral discrepancies that are reflections of physical alterations in the rats. The only physical effect of CPF is impaired weight gain, but this only occurs at dosages exceeding 20 mg/kg/day (Padilla et al., 2002).
An informative study, such as the one performed by Aldridge et al. (2005), might serve as a useful tool in assessing the effects of CPF in the test rats studied here. Done without sufficient behavioral analyses, however, this study might be wasteful of both time and rats. For instance, when inferring behavior as a function of physiology, it could be necessary to couple behavioral analyses with histological studies. Had the rats in the present study exhibited differential performance in the mazes, then a subsequent microscopic analysis might be a useful follow-up study to confirm such findings.

Continued research in understanding the causes of autism might be aimed at testing a wider array of factors believed to be associated with this disorder. For example, altered serotonin levels may only play a small part in a larger cascade of effects, which could include genetic factors in humans (Bourgeron & Persico, 2006). Along with understanding the specific cues of autism, Bourgeron and Persico (2006) also suggest finding animal models that are suitably functional when applied to the genetic pathways in humans.
Literature Cited


