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Persistent alterations in seizure susceptibility, drug responsiveness and comorbidities associated with chemical kindling after neonatal exposure to an organophosphate

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ABSTRACT

Developmental exposure to organophosphates (OPs), at doses that do not cause cholinergic crisis, induces profound and lasting alterations in different neurotransmitter systems, which contribute to several behavioral outcomes. The present work examines whether neonatal exposure to low dose of chlorpyrifos (CPF), a widely used OP insecticide, alters the general excitability of the adult brain, its responsiveness to drugs with antiepileptic properties, the process of chemical kindling and the kindling-induced behavioral outcomes. Neonatal rats were exposed to daily doses of CPF (1 mg/kg) or dimethyl sulfoxide (DMSO, vehicle) on postnatal days (PND) 1–4. On PND 60, a subgroup of animals from both CPF and DMSO groups were injected with additive doses of pentylenetetrazole (PTZ) to evaluate the latency time to the first seizure, the threshold of PTZ-induced convulsion, and to determine the anticonvulsive action of phenobarbital (20 mg/kg), ethosuximide (100 mg/kg) and scopolamine (0.6 mg/kg) when used as pretreatment. Rats in the other subgroups were kindled by repeated intraperitoneal injections of an initially subconvulsive dose of PTZ (37.5 mg/kg) at 48-h intervals for 4 weeks. Kindled rats were then subjected to radial arm maze, sweet taste preference and forced swim test. Neonatal exposure to CPF shortened the latency time to the first seizure after pretreatment with scopolamine in female rats and decreased the threshold for PTZ-induced clonic convulsions after phenobarbital pretreatment in male rats. Neonatal CPF exposure also decreased the rate of kindling progression in female rats during early stages of PTZ kindling. On the other hand, CPF exposure sex-selectively reduced the number of working memory errors after kindling only in male rats. Drug challenge with MK-801 induced more impairment in the working memory of female kindled rats, indicating more dependence of working memory on NMDA receptor activity in these animals. Female kindled rats from CPF exposed group also showed longer time of immobility in forced swim test, showing an increase in the depressive-like behavior. This difference was also observed in the second session of forced swim test, after treating with fluoxetine, a selective inhibitor of serotonin reuptake. The recent finding, together with lack of difference in the sweet taste preference, suggests that mechanism beyond the reduction of serotonergic activity underlie the increased depressive-like behavior in this animals. To our knowledge, this is the first report describing the potential contribution of developmental exposure to an OP in susceptibility to antiepileptic drug resistance and alteration of seizure-induced behavioral deficits.

1. Introduction

The interaction of environmental pollutants, especially during critical periods of brain growth and maturation, can potentially affect the complex processes of brain development. The perturbing effects of environmental chemicals adversely affect the normal wiring and activity of neural circuits, which is manifested by a variety of long-lasting neurobehavioral deficits (Aldridge et al., 2005; Ricceri et al., 2006; Johnson et al., 2009). The altered neuronal circuitry and activity can also affect the general excitability of the nervous system and contribute to the pathogenesis of epilepsy. Environmental insults may also contribute to special condition of resistance to antiepileptic drugs by

Abbreviations: CPF, chlorpyrifos; DMSO, dimethyl sulfoxide; FLX, fluoxetine; NMDA, *N*-methyl-D-aspartate; OP, organophosphate; PND, postnatal day; PTZ, pentylenetetrazol

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altering their targets and receptors; a condition called pharmacoresistant epilepsy (Remy et al., 2003; Potschka, 2012). Organophosphates (OPs) are a major group of insecticides that are well known for their developmental neurotoxicity and long-lasting behavioral deficits (Levin et al., 2001; Aldridge et al., 2005; Timofeeva et al., 2008; Chen et al., 2011). Although the primary mechanism of OPs toxicity is the inhibition of acetylcholinesterase (AChE), they are able to elicit developmental neurotoxicity at doses below the threshold for inhibition of AChE, suggesting that other mechanisms are involved in these actions (Jett et al., 2001; Ricceri et al., 2006; del Pino et al., 2015). Due to their effects on brain development, residential applications of OP insecticides have been limited in recent years, although some of them like chlorpyrifos (CPF) are still in use because of their lower systemic toxicity and higher stability. Several studies have shown that chronic exposure to CPF affects the development of nervous system by targeting gene expression, neurogenesis, neuronal differentiation and migration, synaptogenesis and neurotransmission (Qiao et al., 2003; Aldridge et al., 2005; Slotkin et al., 2007). It is well-known that dysregulation of neurotransmitter systems is central to the cognitive and emotional impairments associated with developmental exposure to low dose CPF in human and animal models (Levin et al., 2001; Aldridge et al., 2003, 2005). On the other hand, structural and functional modification in components involved in neurotransmitter release, reuptake, metabolism and their receptors on both pre- and postsynaptic membranes critically contribute to susceptibility to epilepsy and pharmacoresistance (Pisu et al., 2008; Mullins et al., 2015). We hypothesized that long-term disturbances of different neurotransmitter systems that have been shown following developmental exposure to low doses of CPF can potentially change the susceptibility to seizure and responsiveness to antiepileptic drugs. Furthermore, both epileptic patients and animal models of epilepsy are at high risk for cognitive and emotional disorders, and developmental exposure to CPF may affect the severity of these comorbidities. Kindling is a model of epilepsy that affords assaying the chronic epileptic state and consequent behavioral impairments. It is well known that acute OP poisoning may cause seizures due to cholinergic overstimulation, which can also lead to a range of neurological injuries and associated morbidities (Krishnan et al., 2016). The possible effects of developmental exposure to OPs on seizure susceptibility and their interaction with kindling-induced behavioral impairments have not been studied yet. Meanwhile, a higher prevalence of epilepsy in areas of greater use of pesticides (including OPs) has recently been reported, nevertheless a causal relationship is uncertain yet (Requena et al., 2018).

In the current study we assessed the effect of exposure to low doses of CPF during postnatal days (PNDs) 1–4 on the susceptibility to pentylenetetrazole (PTZ)-induced acute seizures and the effectiveness of some drugs with antiepileptic activity in adulthood. We also studied the effect of CPF exposure on the development of PTZ-kindling and kindling-induced memory impairment and depressive-like behavior.

2. Materials and methods

2.1. Animals

Male and female Wistar rats (180–240 g) were purchased from Razi institute and used as breeders. Animals were kept in a temperature-controlled room at 22 ± 2 °C with a 12 h light–dark cycle and free access to food and water, unless otherwise mentioned. Male and females were bred at a ratio of 1:2 and females' vaginal smears were examined every morning for sperm-positivity to confirm mating (Gestational day 0). Pregnant females were housed separately in standard condition and were checked for birth twice daily from gestational day 19. The day after birth (PND1) litters were adjusted as much as possible to obtain litters of the same size (7–8 pups). All procedures described here were in accordance with the guide for the care and use of laboratory animals of Shiraz University and were approved by the local animal ethics committee.

2.2. Chemicals and treatments

Chlorpyrifos (CPF, CAS No. 2921-88-2, purity 99.9%), pentylenetetrazole (PTZ), dimethyl sulfoxide (DMSO), scopolamine, MK-801, phenobarbital, ethosuximide and saccharin were purchased from Sigma Aldrich (St. Louis, MO, USA). Other chemicals were obtained from Merck (Darmstadt, Germany). On PND 1 litters were randomly assigned to either experimental or sham groups. CPF was dissolved in dimethyl sulfoxide (1 mg/ml) and was injected subcutaneously into neonatal rats in experimental group in a volume of 1 ml/kg/day on PNDs 1–4. Sham animals received equivalent injections of DMSO on the same schedule. All pups within a litter received the same treatments. Twenty-three litters were used in this study, of which 11 litters received DMSO and 12 received CPF. Animals were weaned on PND 23, separated by sex on PND 40 and maintained three to four per cage until the day of experiment. On PND 60 rats of each group were divided into two subgroups. One subgroup was subjected to chemical kindling and the consequent behavioral tasks, and the other subgroup was tested for PTZ-induced acute seizure and evaluation of anticonvulsant activity of some drugs. One male and one female from each litter were assigned to each experiment. Slight differences in the number of animals in each experiment were due to mortality or technical problems. The experimental timeline and animal subgrouping is illustrated in Fig. 1.

2.3. Latency time to PTZ seizure, threshold of convulsive seizure and the efficacy of antiepileptic drugs against PTZ-induced acute seizure

On PND 60, PTZ seizure threshold was determined in a subgroup from DMSO and CPF treated rats. The drugs, phenobarbital, ethosuximide and scopolamine were also tested on seizure threshold when used as pretreatments. Animals received pretreatments of 0.9% saline (vehicle, 15 min before PTZ injection), phenobarbital (20 mg/kg i.p., 60 min before PTZ injection), ethosuximide (100 mg/kg i.p., 45 min before PTZ injection) and scopolamine (0.6 mg/kg i.p., 15 min before PTZ injection). After each pretreatment, an initial dose of 40 mg/kg PTZ

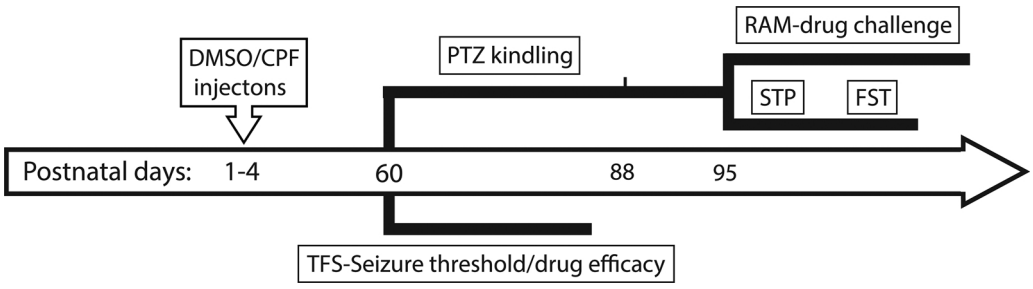

Fig. 1. Procedures timeline and subgrouping of animals. TFS, time to first seizure; RAM, radial arm maze; STP, sweet taste preference; FST, forced swim test.

was injected intraperitoneally and the latency time to the onset of any stage of seizure was recorded. Thereafter, additional doses of 10 mg/kg PTZ were administered to the rats every 12 min until the onset of stage 4 or 5 seizure, but the total dose did not exceed 80 mg/kg. Pretreatments were tested in counterbalanced order (within each group animals received pretreatments in different orders so that each drug was given first, as often as second, as third) to exclude the effect of treatment sequence. The washout time was 72 h between tests

to avoid residual drug effects.

2.4. Kindling

Rats from both CPF and DMSO (sham) groups were kindled by repeated i.p. injections of an initially subconvulsive dose of PTZ (37.5 mg/kg, dissolved in 0.9% sterile saline) at 48-h intervals for 4 weeks. After each PTZ injection the animal was placed in a chamber (60 × 40 × 42 cm) with soft bedding and its behavior was observed for 30 min. The seizures responses to PTZ administration were classified as follows: Stage 0, no response; Stage 1, ear and facial twitching; Stage 2, myoclonic jerks, convulsive waves through the body; Stage 3, myoclonic jerks with rearing; Stage 4, turn over into side position, clonic-tonic seizures; Stage 5, turn over into back position, generalized clonic-tonic seizures or death. Animals were considered to be fully kindled if they had at least three consecutive stages 4 or 5 seizures. Only kindled animals were used in the behavioral studies.

2.5. Radial arm maze

After completion of kindling, the diet of rats was restricted for a week to keep their body weight at approximately 90% of free feeding level, which was determined in rats having free access to food. Food restriction was done to increase motivation of rats to explore the maze for food. The maze consisted of eight arms (65 cm long x 10 cm wide with 17 cm side walls) extending radially from an octagonal central hub (32 cm in diameter). The apparatus floor was made of opaque gray PVC and the side walls were made of transparent Plexiglas. The maze was supported by a metal frame 40 cm above the floor in a quiet room containing many visual cues external to the maze. A food cup (5 cm x 5 cm and 2.5 cm deep) was placed near the distal end of each arm and peanut halves were used as food reward. Radial arm maze testing was performed in two phases: shaping and training. Shaping was conducted to familiarize rats with the apparatus and the food reward, which was initially done in groups and then individually, each for two days. During social shaping, 3 rats were placed in an opaque plastic cylinder (23 cm in diameter) on the central platform of the maze for 10 s to allow for orientation. Then the cylinder was removed and rats gained access to arms and peanut halves, which were scattered throughout all arms. Animals were removed as soon as peanuts were eaten or 10 min had elapsed. In individual shaping sessions, each rat was allowed 5 min to explore the maze with all eight arms baited. In each day of shaping, peanuts were placed further distal into each arm until they were finally placed at food cups. After shaping, rats were trained for 12 sessions on the radial arm maze, once per day. At the beginning of each training session four arms were baited and the other four arms were left unbaited. The pattern of baited and unbaited arms was consistent among rats and throughout testing for each rat, and no more than two consecutive arms were left unbaited. Each trial began by placing the rat in the opaque cylinder on the central platform for 10 s. The cylinder was then removed and rat was allowed to explore the maze and to eat peanuts. The trial was terminated and animal was removed as soon as all peanut halves were eaten or 5 min had elapsed. Between trials the maze was cleaned with damp paper towel to remove scent cues and the peanut halves were replenished. The training sessions were recorded by a video camera from the side of the maze for subsequent analysis. Arm entry was recorded when all paws entered an arm. Visit to an unbaited arm was recorded as a reference memory (RM) error and a working memory (WM) error was defined as return to an arm that had already been visited during that trial. Response time was calculated as total time taken to complete each session (in seconds)/number of arm entries.

2.6. Drug challenges in radial arm maze

After 12 sessions of usual training in radial maze, rats were trained for 12 more days in which they were challenged with different doses of antagonists of NMDA receptor, MK 801, and muscarinic receptor, scopolamine. Rats received i.p. injections of MK-801 (0.1 and 0.2 mg/kg), scopolamine (0.12 and 0.24 mg/kg) or 0.9% saline as vehicle (1 ml/kg) 30 min before the start of each RAM session. Drug challenges were counterbalanced among rats to exclude the effect of treatment sequence, and at least two non-drugged (vehicle) days were inserted between drugged sessions for drug washout.

2.7. Taste preference test

A week after the completion of kindling, another subgroup of rats from each group was subjected to taste preference test. The day before the experiment, each rat was housed in a separate cage that was supplied with food and two identical bottles, each containing 250 ml of tap water. On the next day (test day), water in one of the bottles was replaced with 0.1% saccharin diluted in tap water. The test was initiated at 8.00 a.m. and ran for 24 h. To avoid place preference in drinking behavior, the position of the bottles was switched after 12 h. Taste preference was expressed as percent of saccharin solution intake (ml) relative to the total water intake (saccharin plus regular tap water) over 24 h.

2.8. Forced swim test

A day after taste preference test, rats were subjected to forced swim test. Each rat was individually placed in a plastic cylinder (48 cm height x 25 cm diameter) containing 32 cm of water (25 ± 1 °C). Forced swim test was conducted in two sessions, a 15 min first session and a 5 min second session that was done 24 h later. To test the effect of the antidepressant fluoxetine (FLX) on rats' behavior, they were given three i.p. injections of FLX (10 mg/kg) at 23.5, 5 and 1 h before the test session. The first 5 min of the first session and the 5-min second session were video recorded by a digital camera fixed above the cylinder. After each forced swim session rats were towel-dried, placed under a heat lamp for 30 min in a separate cage and then returned to a clean home cage. The immobility time (sec) was later scored as an indicator of depressive-like behavior. Immobility was assigned when animal floated passively in the water and only made necessary movements to keep its head above the water.

2.9. Statistical analysis

Values are expressed as mean ± standard error of the mean (S.E.M.). Normal distribution was assessed using Shapiro-Wilk test. Statistical significance was determined using unpaired *t*-test or Mann-Whitney U, as appropriate. To analyze responses to acute drug challenges or pretreatments, Kruskal-Wallis H test followed by Mann–Whitney U test were used. To detect possible interactions between treatments and sex, initial comparisons were conducted by a global analysis of variance across treatments and sex. When a significant interaction of treatment with sex was identified, the data set was divided by sex and the analysis was done separately on the subdivisions, otherwise the data for males and females were combined. The accepted level of significance for main treatment effects was $p < 0.05$ and for interactions was $p < 0.1$.

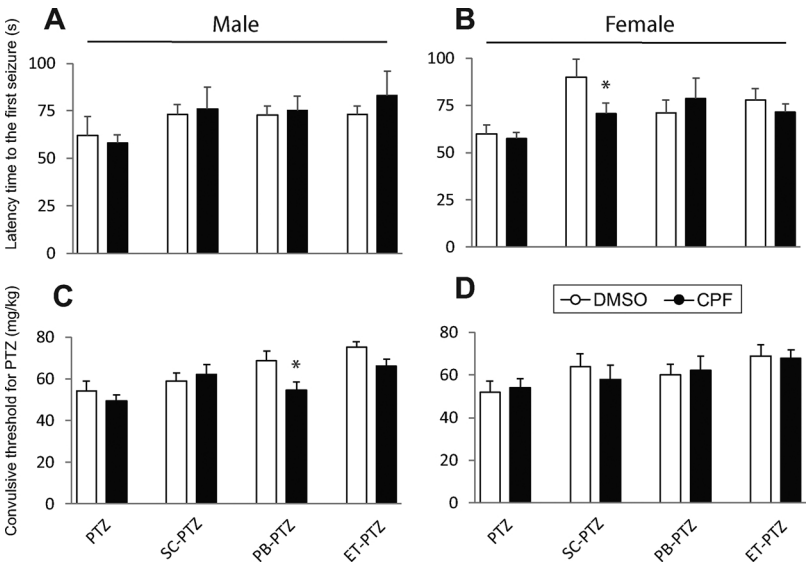

Fig. 2. The effects of neonatal CPF exposure on latency time to the onset of seizure, doses of pentylenetetrazole (PTZ) required for the induction of convulsion and the efficacy of scopolamine (0.6 mg/kg, SC), phenobarbital (20 mg/kg, PB) and ethosuximide (100 mg/kg, ET) to delay/suppress seizure when used as pretreatments. Scopolamine was less effective in increasing the latency time to seizure in female rats that were exposed to CPF (B). Male rats from CPF group showed lower threshold for PTZ-induced convulsion after PB pretreatment compared to the male rats that only received vehicle (dimethylsulfoxide, DMSO). (n = 9–12 per group). *p < 0.05 compared to DMSO group, Mann–Whitney U test.

3. Results

3.1. PTZ seizure threshold, efficacy of antiepileptic drugs against PTZ-induced acute seizure and kindling development

The effects of neonatal CPF exposure on the latency to onset of seizure and doses of PTZ required for the induction of convulsion showed a significant interaction with sex, so data were subdivided into males and females. The neonatal administration of CPF did not change the latency time to the onset of seizure (any type) elicited by a sub-convulsive dose of PTZ (40 mg/kg) in adult rats, but it shortened the latency time to the first seizure after pretreatment with scopolamine (0.6 mg/kg) in female CPF-exposed rats (Mann-Whitney U = 19, p = 0.035) (Fig. 2B). The threshold for pentylenetetrazole-induced clonic convulsions showed a significant reduction after phenobarbital pretreatment (20 mg/kg) in male CPF-treated rats compared to DMSO-exposed male rats (Mann-Whitney U = 21, p = 0.016) (Fig. 2C). Fig. 3 indicates seizure stages during chemical kindling elicited by repeated injection of PTZ (37.5 mg/kg). In both DMSO- and CPF-treated rats, the seizure activity showed a general progressive increase over time during the course of PTZ kindling. In female rats the seizure activity was significantly suppressed in CPF-exposed rats compared to DMSO-treated rats after fourth and sixth PTZ injections (Mann-Whitney U = 62, p = 0.021 and U = 69, p = 0.045 respectively) (Fig. 3B).

3.2. Radial arm maze and drug challenges

For reference memory errors during standard radial arm maze sessions, the initial evaluation by ANOVA revealed no significant interaction of treatment with sex (p > 0.1). So, the main treatment effect was assessed without subdividing each group by sex. Results showed that the number of reference memory errors after kindling in DMSO- and CPF-exposed groups do not significantly differ from each other (Fig. 4). The effect on working memory, on the other hand, was sex selective and neonatal exposure to CPF reduced the number of working memory errors after kindling only in male rats (Fig. 5). Comparing working memory between male and female within each group also showed that kindling significantly increased the number of errors in male rats compared to female rats and this effect was more pronounced in DMSO-injected rats than in CPF-exposed ones (Fig. 5). The response time did not show a significant difference between the two groups (data not shown), indicating that the difference in spatial memory, when detected, is not the result of alteration in locomotor activity or motivation to search for the reward.

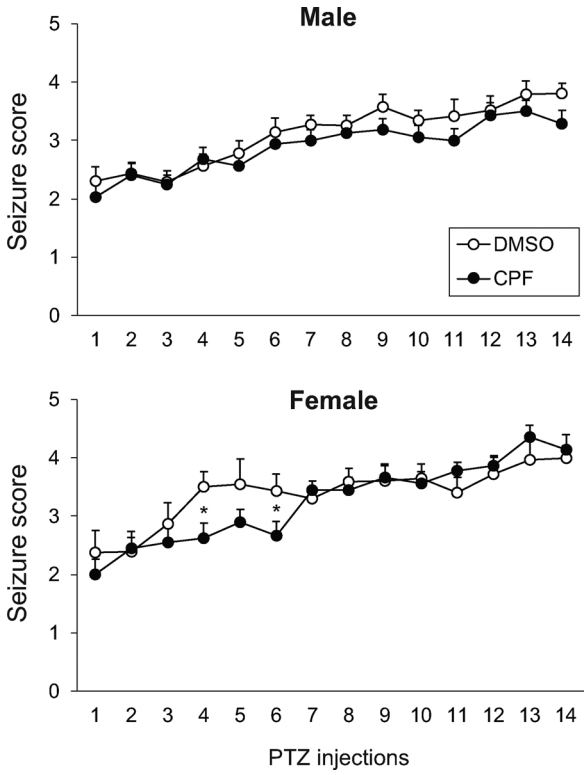

Fig. 3. The effects of CPF exposure on the development of PTZ kindling. CPF exposure selectively decreased the rate of kindling progression in female rats. (n = 15–18 per group). *p < 0.05 compared to DMSO group, Mann–Whitney U test.

During drug challenge tests, a significant interaction of CPF x sex was detected for both reference and working memory errors. Therefore, the effects of drug challenges on memory impairment induced after kindling in DMSO- and CPF-treated rats was examined separately for males and females. In both male and female rats, scopolamine did not differentially affect the number of either reference memory errors or working memory errors. The lower concentration of MK-801 significantly increased reference memory errors after kindling in CPF-exposed male rats compared DMSO-injected rats [Mann-Whitney U (17) = 20, p = 0.013] (Fig. 6). After kindling, working memory errors were significantly increased by pretreatment with higher concentration

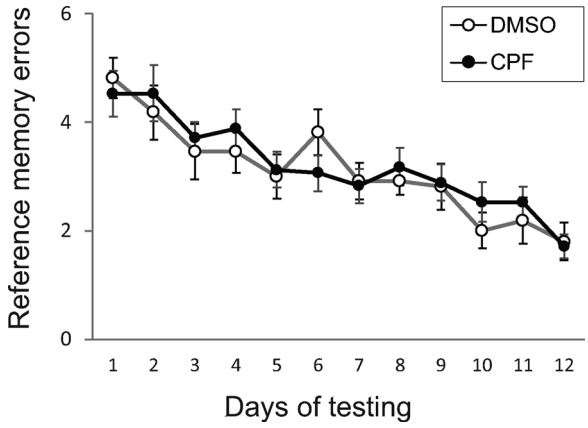

Fig. 4. Kindled animals from DMSO and CPF exposed groups did not show a significant difference in the number of reference memory errors in the radial arm maze. Data were pooled for both sexes as the interaction with sex was not significant. (n = 16–19 per group of both sexes).

of MK-801 in female rats from CPF-exposed group compared to the sex-matched rats from DMSO-exposed group [Mann-Whitney U(18) = 21, p = 0.016] (Fig. 6).

3.3. Forced swim test

On the first day of testing, female kindled rats from the CPF exposed group had longer times of immobility in the forced swim test, suggesting a higher level of behavioral despair [t(20) = 2.93, p = 0.008]. The longer time of immobility in these animals was still evident after FLX treatment in the second session of forced swim test [t(20) = 2.19, p = 0.04] (Fig. 7).

3.4. Sweet taste preference

The interaction of treatment with sex was not significant for sweet taste preference and data from male and female rats were pooled in each group. There was no significant difference in the preference for saccharin solution between kindled rats from DMSO- and CPF-exposed rats [92.3 ± 1.6 and 91.1 ± 1.3, respectively, t(40) = 0.77, p = 0.45].

4. Discussion

Our results showed that neonatal exposure to low dose CPF affects the progress of PTZ-induced kindling, responsiveness to drugs with antiepileptic activity and behavioral comorbidities associated with PTZ kindling, mostly in a sex-selective manner. Developmental exposure to some chemicals has been shown to have lasting effects on the excitability of neural circuits and seizure susceptibility (Gilbert and Llorens, 1993; Yuan, 2012; Bandara et al., 2017). Requena and colleagues have recently studied the association between environmental exposure to pesticides with prevalence of epilepsy and found a higher prevalence of hospital-diagnosed epilepsy in the population living in areas with greater pesticide use and exposure. They suggested that pesticides exposure may increase the risk of epilepsy, although they were not able to prove a causal relationship owing to the limitations of the study design (Requena et al., 2018). Here, we found that neonatal exposure to CPF sex-selectively shortens the latency time to the first seizure after pretreatment with scopolamine and retards the progression of PTZ kindling in female rats compared to the sex-matched DMSO-treated rats. On the other hand, the CPF exposed male rats had lower threshold for pentylenetetrazole-induced clonic convulsion after phenobarbital pretreatment than DMSO-treated rats that received the same pretreatment. An abnormal shift of synaptic systems contributes to the induction of chemical kindling and responsiveness to antiepileptic drugs (Bertram, 2013). A large number of studies have reported that neonatal exposure to low dose of CPF can affect the function of different neurotransmitter systems. Studies have shown that CPF exposure damages the cholinergic system. Acute and long-term exposure of basal forebrain cholinergic neurons to CPF induces cell death through apoptotic and necrotic pathways (del Pino et al., 2015; Moyano et al., 2017).

Neonatal administration of CPF during PNDs 1–4 also reduces the number of cholinergic neurons and the cholinergic synaptic function in young adulthood. This effect has been found to be much greater in female rats than in males, especially in the hippocampus and midbrain (Levin et al., 2001; Aldridge et al., 2005). Stimulation of the cholinergic system contributes to the development of kindling and endurable structural and functional alterations that are characteristic of the kindled state, while cholinergic antagonists limit the progression of kindling and reduce the kindling-induced effects (Adams et al., 2002). Accordingly, the retarded kindling rate and the reduced latency time to the first seizure after pretreatment with scopolamine in female rats parallels the expected female-selective suppression of cholinergic

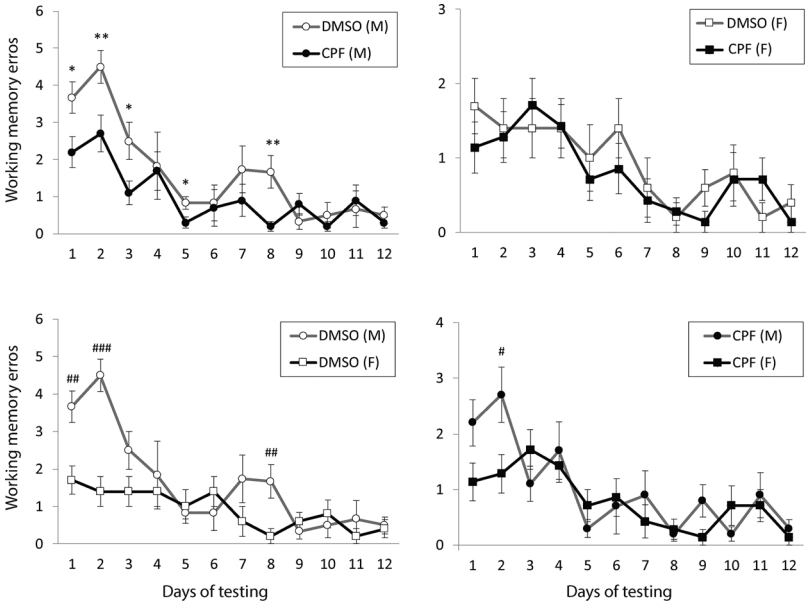

Fig. 5. Kindling sex-selectively affected working memory errors in both DMSO and CPF exposed rats. Male rats neonatally exposed to CPF showed more working memory errors after kindling than sex-matched rats from DMSO group, while such a difference was not observed in female rats. In both DMSO and CPF groups male (M) rats showed more vulnerability to kindling-induced working memory impairment than female (F) rats. (n = 8–11 per group). *p < 0.05, **p < 0.01 compared to CPF group. #p < 0.05, ##p < 0.01, ###p < 0.001 compared to female rats in the same group, Mann–Whitney U test.

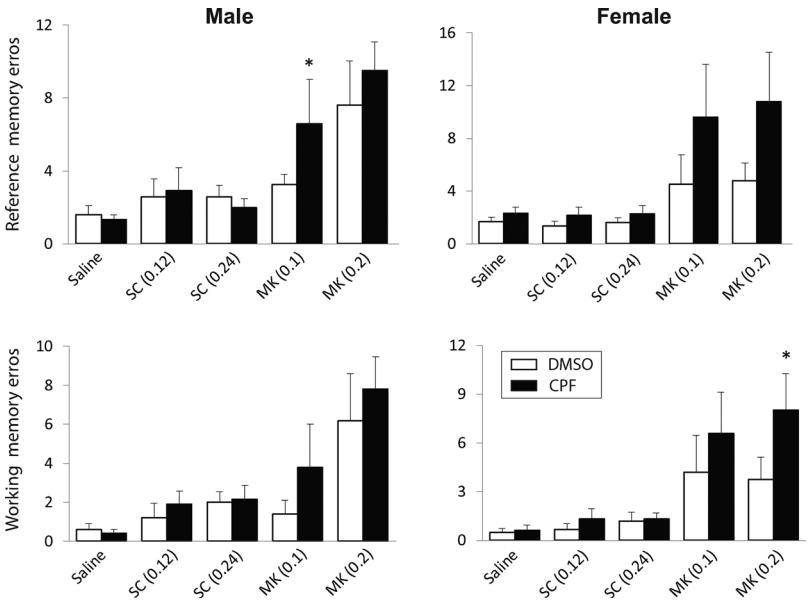

Fig. 6. The effect of challenging with different doses of scopolamine (SC) and MK-801 (MK) on reference and working memory errors in male and female kindled rats that were neonatally exposed to DMSO or CPF. Lower dose of MK (0.1 mg/kg) induced more impairment of reference memory in CPF-exposed male rats after kindling, while its higher concentration caused more deficit in working memory after kindling in CPF exposed female rats compared to the sex matched DMSO-exposed rats. (n = 8–11 per group). *p < 0.05 compared to DMSO group, Mann–Whitney U test.

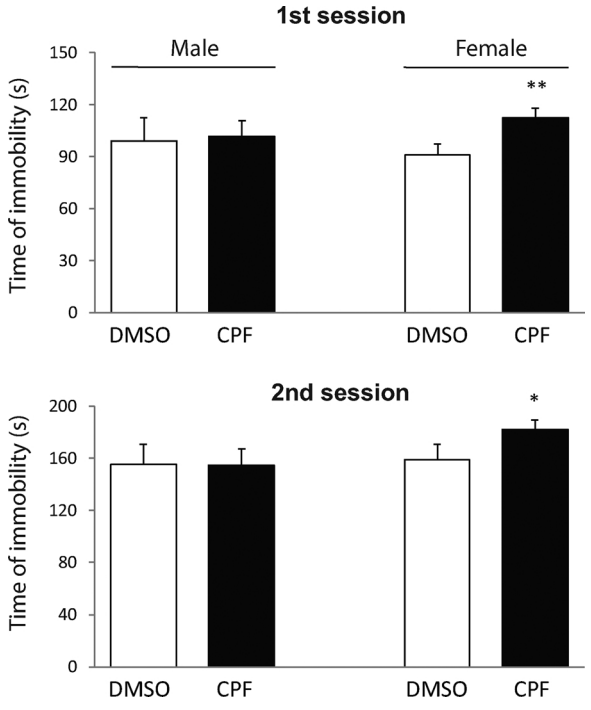

Fig. 7. Duration of immobility of male and female rats during 5 min of forced swim test in the first and second session. After kindling, CPF-exposed female rats showed longer time of immobility in the first session, showing higher levels of depressive-like behavior in these animals. These animals still had significantly more time of immobility even after treatment with fluoxetine (second session). (n = 10–12 per group). *p < 0.05, **p < 0.01 compared to DMSO group, unpaired *t*-test.

function. It has been shown that chronic CPF exposure during fetal and postnatal period in male mice reduces gene expression of GABAB receptors, which are critically involved in GABAergic synaptic transmission (Pallotta et al., 2017). A similar down-regulation of GABAB receptors may contribute to the higher sensitivity of CPF exposed male rats to PTZ after pretreatment with phenobarbital, while the basal excitation/inhibition balance could be maintained by adaptive response of other neurotransmitter systems. Furthermore, the serotonergic system has been reported to be persistently and sex-selectively affected by neonatal CPF exposure. Serotonin receptors are richly expressed in the neural circuits involved in epilepsy and their dysregulations is considered to be involved in epilepsy pathogenesis. The known effects of CPF on serotonergic synapses may also contribute to the sex-selective alterations in kindling development and responsiveness to antiepileptic drugs. The fact that many sex specific alterations in the neuronal circuits have been described following neonatal exposure to CPF, together with the multifactorial nature of kindling development makes it difficult to specify the functional significance of such alterations to delayed kindling progression in CPF exposed female rats.

No significant difference in reference memory errors was found either between kindled rats from DMSO- and CPF-exposed group or between male and female rats within each group. On the other hand, CPF exposure selectively reduced working memory errors in kindled male rats compared to DMSO-exposed group, while such a difference was not observed between female kindled rats from DMSO- and CPF-exposed groups. Female kindled rats from both DMSO- and CPF-exposed rats also made less working memory error compared to male kindled rats in the same group. It has been shown that neonatal exposure of rats to low dose CPF, a regimen similar to that used in this study, impairs reference and working memory in male rats while improves the performance of female rats (Slotkin et al., 2001). Almost similar sex-selectivity was reported by Johnson et al. (2009) and Gómez-Giménez et al., (2017) who administered CPF via oral route during postnatal period (Johnson et al., 2009; Gomez-Gimenez et al., 2017). Aldridge and colleagues (2005) found that CPF exposed female rats master the radial maze task without relying on cholinergic function but by relaying on serotonergic function, which is not seen in control animals and CPF exposed males (Aldridge et al., 2005). On the other hand, pentylenetetrazole kindling impairs spatial learning and memory, which has also been reported in epileptic patients (Fisher et al., 2000; Schubert et al., 2005). This effect is associated with reduced basal acetylcholine release in hippocampus and neuronal loss in different hippocampal and cortical regions that potentially can contribute to kindling-induced memory impairment (Cavazos et al., 1994; Serra et al., 1997). Meanwhile, our findings showed that PTZ-kindling of DMSO-exposed rats induces significantly more impairment of working memory in male rats than females. Accordingly, it seems that either neonatal CPF-exposure or PTZ-kindling similarly induce more impairment of memory in male rats. In this sense, with combined neonatal CPF exposure and PTZ-kindling an accumulative effect might be expected, whereas kindled rats from CPF-exposed group made significantly fewer working memory compared to kindled rats from DMSO-exposed group, and this effect was especially

pronounced in male rats. The acquisition of PTZ kindling is associated with reorganization of several synaptic connections especially the recurrent excitation of dentate gyrus granular cells by sprouted mossy fibers and axons of pyramidal neurons in proximal CA3 subfield (Zhang et al., 2012). Although such excessive excitatory circuits also commonly develop in epileptic patients, but their functional significance in the pathogenesis of epilepsy is uncertain (Sutula et al., 1996; Timofeeva and Peterson, 1999). Nevertheless, such alterations are most likely involved to the behavioral comorbidities, including memory impairment and anxiety-like behaviors in animal models of kindling and epileptic patients (de Oliveira et al., 2008). This idea has been proposed that many developmental behavior disorder that may result from interfering factors are not induced by direct and localized impairment of neuronal circuits function, but rather results from the whole brain adaptive responses to the initial alterations (Slotkin et al., 2015). Aside from its involvement to the kindling progression, cholinergic system also contributes to the neuronal remodeling and axonal sprouting in the hippocampus of kindled animals (Adams et al., 2002). It may be speculated that the sex-selective reduction of cholinergic function in female CPF-treated rats, which was reported in earlier studies and also indirectly evidenced in the current work, can suppress the cholinergic dependent adaptive responses to kindling and the consequent spatial memory impairment. Further studies are required to find out to what extent this mechanism may contribute to the more protection of female CPF-treated rats against kindling-induced memory impairment.

Drug challenge with scopolamine did not differentially affect reference and working memory of kindled rats from DMSO and CPF-exposed groups. In male kindled rats, the number of reference memory errors showed more increase after pretreatment with lower dose of MK-801. On the other hand, while kindled female rats from DMSO- and CPF-exposed groups did not show significant difference in the number of working memory errors, pretreatment with higher dose of MK-801 (0.2 mg/kg), induced more increase in the number of working memory error in female kindled rats than those in the CPF exposed group. The current finding suggests that in these animals, the working memory is more dependent on glutamatergic neurotransmission. During early phase of PTZ kindling development, glutamate release is increased but during late stages and after completion of kindling, the density of excitatory amino acid binding sites is enhanced (Lason et al., 1998; Schroeder et al., 1998). The increase in the expression of glutamate receptors in the rat hippocampal and neocortical regions has been associated with the development of PTZ-induced kindling (Schroder et al., 1993). Meanwhile, kindled rats show a decrease in learning and memory, accompanied by a long-lasting decline in the brain concentrations of glutamate (Szyndler et al., 2008). Even one month after the establishment of PTZ-kindling, a significant increase of *N*-methyl-D-aspartate (NMDA) receptor binding in the dentate gyrus and CA3 region of the hippocampus could be detected (Ekonomou and Angelatou, 1999). Kraus and his colleagues (1994) showed that a marked and long-lasting increase in the expression of a novel NMDA receptor in the CA3 hippocampal region may underlie the increased sensitivity to NMDA observed in the kindled animals and contribute to the maintenance of hyperexcitable state (Martin et al., 1992; Kraus et al., 1994). This effect may also contribute to the saturation of long term potentiation of synapses in the hippocampal circuit, which has been suggested as an underlying mechanism of spatial memory impairment in kindled animals (Leung and Shen, 2006). To our knowledge, there is no report available on the lasting alteration of glutamatergic system after neonatal exposure to CPF. It is plausible, however, that the well-known lasting aberration of different neurotransmitter systems following to neonatal CPF exposure can also affect the kindling development, as evidenced in our study, and the consequent alteration of glutamatergic system. It seems that neonatal exposure to CPF reduces the kindling-induced impairment of glutamatergic system, which is evidenced by a more reliance of spatial memory on this system.

We have earlier showed that neonatal exposure to CPF significantly increases the expression of neuronal nitric oxide synthase (nNOS) in the hypothalamic nuclei, while reduces the number of nNOS containing neurons in the basolateral nucleus of amygdala, CA1 and CA3 hippocampal subfields and many cortical regions of adult rats (Naseh et al., 2013; Vatanparast et al., 2013; Naseh and Vatanparast, 2014). It has been suggested that kindling is a type of use-dependent synaptic plasticity, sharing many mechanisms of induction and progress including dependency on NO (Gilbert, 2001). Considering the involvement of NO in PTZ kindling progression, the reduction of nNOS expressing neurons in the cerebral cortex, amygdala and hippocampus can also contribute to the lower kindling rate in CPF treated rats although this mechanism cannot explain the sex-dependent effect. Zhu and colleagues recently showed that hippocampal over expression of nNOS during PTZ-induced kindling in mice is also involved to the memory impairment (Zhu et al., 2017). Hippocampus, cerebral cortex and amygdala are essential substrates of cognitive and affective functions and their neuronal structure, connectivity and function are affected by kindling (Schubert et al., 2005). With the known role of NO in these alterations (Zhu et al., 2017), the pre-reduction of nNOS expressing neurons in these regions of CPF-exposed rats is expected to affect the response to kindling-induced nNOS overexpression and behavioral outcomes.

In the current study, we found that PTZ kindling selectively increased depressive-like behavior in female rats from CPF-exposed group. This significant difference was not eliminated after treatment with FLX. Both kindling and developmental exposure to CPF have been reported to induce depressive-like behavior (Aldridge et al., 2005; Mazarati et al., 2008). Aldridge and colleagues showed that a similar regimen of neonatal CPF exposure elicits sex-dependent hallmarks of functional impairment of 5 H T synapses that most likely contribute to CPF-induced depressive-like behavior in adult rats (Aldridge et al., 2004). Our results showed that PTZ- kindling does not induce any significant difference in the expression of depressive-like behavior between male and female rats from DMSO group. So, it is reasonable to assume that sex selective alterations of serotonergic system, and other neurotransmitter systems, following CPF-exposure may underlie the sex dependent outcomes in the depressive-like behavior after PTZ kindling. It has been shown that the development of chronic epileptic state is associated with increased immobility time under conditions of forced swim test and loss of taste preference in saccharin solution consumption test, which are associated with compromised serotonergic transmission (Mortazavi et al., 2005; Mazarati et al., 2008). We found that even after FLX treatment the immobility time of kindled female rats from CPF-treated group was still significantly longer than those from DMSO-treated group, suggesting that mechanism beyond the reduction of serotonergic activity are involved in the induction of depressive-like behavior in this animals. As serotonergic pathways also contribute to hedonic response to sweet taste (Harrison et al., 2001; Mortazavi et al., 2005), the founding that sweet taste preference was not different between kindled rats from the two groups also support the idea that non-serotonergic mechanisms underlie the induction of the observed depressive-like behavior.

In summary, these results indicate that neonatal exposure to low doses of CPF sex-selectively reduces the susceptibility to PTZ-induced kindling in adulthood; while in parallel diminishes the efficacy of some drugs with anti-seizure activity. Our results put more emphasis on the potential contribution of early-life chemical exposure to the development of pharmacoresistance in epilepsy. CPF exposure also sex selectively reduces memory impairment and depressive-like behavior following to chemical kindling. The CPF-induced impairment of different neurotransmitters seems to reduce their functional adaptation to kindling that is known to play a crucial role in the kindling-induced behavioral deficits. Epileptogenesis, pharmacoresistance and comorbities associated with epilepsy are multifactorial processes, so more studies are required to address the extent and mechanisms that environmental OP exposure may interact with other intrinsic factors.

5. Transparency document

The Transparency document associated with this article can be found in the online version.

References

Adams, B., Vaccarella, L., Fahnestock, M., Racine, R.J., 2002. The cholinergic system modulates kindling and kindling-induced mossy fiber sprouting. Synapse 44, 132–138.

Aldridge, J.E., Seidler, F.J., Meyer, A., Thillai, I., Slotkin, T.A., 2003. Serotonergic systems targeted by developmental exposure to chlorpyrifos: effects during different critical periods. Environ. Health Perspect. 111, 1736–1743.

Aldridge, J.E., Seidler, F.J., Slotkin, T.A., 2004. Developmental exposure to chlorpyrifos elicits sex-selective alterations of serotonergic synaptic function in adulthood: critical periods and regional selectivity for effects on the serotonin transporter, receptor subtypes, and cell signaling. Environ. Health Perspect. 112, 148–155.

Aldridge, J.E., Levin, E.D., Seidler, F.J., Slotkin, T.A., 2005. Developmental exposure of rats to chlorpyrifos leads to behavioral alterations in adulthood, involving serotonergic mechanisms and resembling animal models of depression. Environ. Health Perspect. 113, 527–531.

Bandara, S.B., Sadowski, R.N., Schantz, S.L., Gilbert, M.E., 2017. Developmental exposure to an environmental PCB mixture delays the propagation of electrical kindling from the amygdala. Neurotoxicology 58, 42–49.

Bertram, E.H., 2013. Neuronal circuits in epilepsy: do they matter? Exp. Neurol. 244, 67–74.

Cavazos, J.E., Das, I., Sutula, T.P., 1994. Neuronal loss induced in limbic pathways by kindling: evidence for induction of hippocampal sclerosis by repeated brief seizures. J. Neurosci. 14, 3106–3121.

Chen, W.Q., Yuan, L., Xue, R., Li, Y.F., Su, R.B., Zhang, Y.Z., Li, J., 2011. Repeated exposure to chlorpyrifos alters the performance of adolescent male rats in animal models of depression and anxiety. Neurotoxicology 32, 355–361.

de Oliveira, D.L., Fischer, A., Jorge, R.S., da Silva, M.C., Leite, M., Goncalves, C.A., Quillfeldt, J.A., Souza, D.O., e Souza, T.M., Wofchuk, S., 2008. Effects of early-life LiCl-pilocarpine-induced status epilepticus on memory and anxiety in adult rats are associated with mossy fiber sprouting and elevated CSF S100B protein. Epilepsia 49, 842–852.

del Pino, J., Moyano, P., Anadon, M.J., Garcia, J.M., Diaz, M.J., Garcia, J., Frejo, M.T., 2015. Acute and long-term exposure to chlorpyrifos induces cell death of basal forebrain cholinergic neurons through AChE variants alteration. Toxicology 336, 1–9.

Ekonomou, A., Angelatou, F., 1999. Upregulation of NMDA receptors in hippocampus and cortex in the pentylenetetrazol-induced "kindling" model of epilepsy. Neurochem. Res. 24, 1515–1522.

Fisher, R.S., Vickrey, B.G., Gibson, P., Hermann, B., Penovich, P., Scherer, A., Walker, S., 2000. The impact of epilepsy from the patient's perspective I. Descriptions and subjective perceptions. Epilepsy Res. 41, 39–51.

Gilbert, M.E., 2001. Does the kindling model of epilepsy contribute to our understanding of multiple chemical sensitivity? Ann. N. Y. Acad. Sci. 933, 68–91.

Gilbert, M.E., Llorens, J., 1993. Delay in the development of amygdala kindling following treatment with 3,3'-iminodipropionitrile. Neurotoxicol. Teratol. 15, 243–250.

Gomez-Gimenez, B., Llansola, M., Hernandez-Rabaza, V., Cabrera-Pastor, A., Malaguarnera, M., Agusti, A., Felipo, V., 2017. Sex-dependent effects of developmental exposure to different pesticides on spatial learning. The role of induced neuroinflammation in the hippocampus. Food Chem. Toxicol. 99, 135–148.

Harrison, A.A., Liem, Y.T., Markou, A., 2001. Fluoxetine combined with a serotonin-1A receptor antagonist reversed reward deficits observed during nicotine and amphetamine withdrawal in rats. Neuropsychopharmacology 25, 55–71.

Jett, D.A., Navoa, R.V., Beckles, R.A., McLemore, G.L., 2001. Cognitive function and cholinergic neurochemistry in weanling rats exposed to chlorpyrifos. Toxicol. Appl. Pharmacol. 174, 89–98.

Johnson, F.O., Chambers, J.E., Nail, C.A., Givaruangsawat, S., Carr, R.L., 2009. Developmental chlorpyrifos and methyl parathion exposure alters radial-arm maze performance in juvenile and adult rats. Toxicol. Sci. 109, 132–142.

Kraus, J.E., Yeh, G.C., Bonhaus, D.W., Nadler, J.V., McNamara, J.O., 1994. Kindling induces the long-lasting expression of a novel population of NMDA receptors in hippocampal region CA3. J. Neurosci. 14, 4196–4205.

Krishnan, J.K.S., Arun, P., Appu, A.P., Vijayakumar, N., Figueiredo, T.H., Braga, M.F.M., Baskota, S., Olsen, C.H., Farkas, N., Dagata, J., Frey, W.H., Moffett, J.R., Namboodiri, A.M.A., 2016. Intranasal delivery of obidoxime to the brain prevents mortality and CNS damage from organophosphate poisoning. Neurotoxicology 53, 64–73.

Lason, W., Turchan, J., Przewlocka, B., Labuz, D., Machelska, H., Przewlocki, R., 1998. Effects of pentylenetetrazol kindling on glutamate receptor genes expression in the rat hippocampus. Brain Res. 785, 355–358.

Leung, L.S., Shen, B., 2006. Hippocampal CA1 kindling but not long-term potentiation disrupts spatial memory performance. Learn. Mem. 13, 18–26.

Levin, E.D., Addy, N., Nakajima, A., Christopher, N.C., Seidler, F.J., Slotkin, T.A., 2001. Persistent behavioral consequences of neonatal chlorpyrifos exposure in rats. Brain Res. Dev. Brain Res. 130, 83–89.

Martin, D., McNamara, J.O., Nadler, J.V., 1992. Kindling enhances sensitivity of CA3 hippocampal pyramidal cells to NMDA. J. Neurosci. 12, 1928–1935.

Mazarati, A., Siddarth, P., Baldwin, R.A., Shin, D., Caplan, R., Sankar, R., 2008. Depression after status epilepticus: behavioural and biochemical deficits and effects of fluoxetine. Brain 131, 2071–2083.

Mortazavi, F., Ericson, M., Story, D., Hulce, V.D., Dunbar, G.L., 2005. Spatial learning deficits and emotional impairments in pentylenetetrazole-kindled rats. Epilepsy Behav. E&B 7, 629–638.

Moyano, P., Del Pino, J., Anadon, M.J., Diaz, M.J., Gomez, G., Frejo, M.T., 2017. Toxicogenomic profile of apoptotic and necrotic SN56 basal forebrain cholinergic neuronal loss after acute and long-term chlorpyrifos exposure. Neurotoxicol. Teratol. 59, 68–73.

Mullins, R.J., Xu, S., Pereira, E.F., Pescrille, J.D., Todd, S.W., Mamczarz, J., Albuquerque, E.X., Gullapalli, R.P., 2015. Prenatal exposure of guinea pigs to the organophosphorus pesticide chlorpyrifos disrupts the structural and functional integrity of the brain. Neurotoxicology 48, 9–20.

Naseh, M., Vatanparast, J., 2014. Enhanced expression of hypothalamic nitric oxide synthase in rats developmentally exposed to organophosphates. Brain Res. 1579, 10–19.

Naseh, M., Vatanparast, J., Baniasadi, M., Hamidi, G.A., 2013. Alterations in nitric oxide synthase-expressing neurons in the forebrain regions of rats after developmental exposure to organophosphates. Neurotoxicol. Teratol. 37, 23–32.

Pallotta, M.M., Ronca, R., Carotenuto, R., Porreca, I., Turano, M., Ambrosino, C., Capriglione, T., 2017. Specific Effects of Chronic Dietary Exposure to Chlorpyrifos on Brain Gene Expression-A Mouse Study. Int. J. Mol. Sci. 18.

Pisu, M.G., Mostallino, M.C., Dore, R., Mura, M.L., Maciocco, E., Russo, E., De Sarro, G., Serra, M., 2008. Neuroactive steroids and GABAA receptor plasticity in the brain of the WAG/Rij rat, a model of absence epilepsy. J. Neurochem. 106, 2502–2514.

Potschka, H., 2012. Animal models of drug-resistant epilepsy. Epileptic Disord. 14, 226–234.

Qiao, D., Seidler, F.J., Tate, C.A., Cousins, M.M., Slotkin, T.A., 2003. Fetal chlorpyrifos exposure: adverse effects on brain cell development and cholinergic biomarkers emerge postnatally and continue into adolescence and adulthood. Environ. Health Perspect. 111, 536–544.

Remy, S., Gabriel, S., Urban, B.W., Dietrich, D., Lehmann, T.N., Elger, C.E., Heinemann, U., Beck, H., 2003. A novel mechanism underlying drug resistance in chronic epilepsy. Ann. Neurol. 53, 469–479.

Requena, M., Parrón, T., Navarro, A., García, J., Ventura, M.I., Hernández, A.F., Alarcón, R., 2018. Association between environmental exposure to pesticides and epilepsy. Neurotoxicology 68, 13–18.

Ricceri, L., Venerosi, A., Capone, F., Cometa, M.F., Lorenzini, P., Fortuna, S., Calamandrei, G., 2006. Developmental neurotoxicity of organophosphorous pesticides: fetal and neonatal exposure to chlorpyrifos alters sex-specific behaviors at adulthood in mice. Toxicol. Sci. 93, 105–113.

Schroeder, H., Becker, A., Hoellt, V., 1998. Sensitivity and density of glutamate receptor subtypes in the hippocampal formation are altered in pentylenetetrazole-kindled rats. Exp. Brain Res. 120, 527–530.

Schubert, M., Siegmund, H., Pape, H.C., Albrecht, D., 2005. Kindling-induced changes in plasticity of the rat amygdala and hippocampus. Learn. Mem. 12, 520–526.

Serra, M., Dazzi, L., Cagetti, E., Chessa, M.F., Pisu, M.G., Sanna, A., Biggio, G., 1997. Effect of pentylenetetrazole-induced kindling on acetylcholine release in the hippocampus of freely moving rats. J. Neurochem. 68, 313–318.

Slotkin, T.A., Cousins, M.M., Tate, C.A., Seidler, F.J., 2001. Persistent cholinergic presynaptic deficits after neonatal chlorpyrifos exposure. Brain Res. 902, 229–243.

Slotkin, T.A., Seidler, F.J., Fumagalli, F., 2007. Exposure to organophosphates reduces the expression of neurotrophic factors in neonatal rat brain regions: similarities and differences in the effects of chlorpyrifos and diazinon on the fibroblast growth factor superfamily. Environ. Health Perspect. 115, 909–916.

Slotkin, T.A., Skavicus, S., Levin, E.D., Seidler, F.J., 2015. Prenatal nicotine changes the response to postnatal chlorpyrifos: Interactions targeting serotonergic synaptic function and cognition. Brain Res. Bull. 111, 84–96.

Sutula, T., Koch, J., Golarai, G., Watanabe, Y., McNamara, J.O., 1996. NMDA receptor dependence of kindling and mossy fiber sprouting: evidence that the NMDA receptor regulates patterning of hippocampal circuits in the adult brain. J. Neurosci. 16, 7398–7406.

Szyndler, J., Maciejak, P., Turzynska, D., Sobolewska, A., Lehner, M., Taracha, E., Walkowiak, J., Skorzewska, A., Wislowska-Stanek, A., Hamed, A., Bidzinski, A., Plaznik, A., 2008. Changes in the concentration of amino acids in the hippocampus of pentylenetetrazole-kindled rats. Neurosci. Lett. 439, 245–249.

Timofeeva, O.A., Peterson, G.M., 1999. Dissociation of mossy fiber sprouting and electrically-induced seizure sensitivity: rapid kindling versus adaptation. Epilepsy Res. 33, 99–115.

Timofeeva, O.A., Sanders, D., Seemann, K., Yang, L., Hermanson, D., Regenbogen, S., Agoos, S., Kallepalli, A., Rastogi, A., Braddy, D., Wells, C., Perraut, C., Seidler, F.J., Slotkin, T.A., Levin, E.D., 2008. Persistent behavioral alterations in rats neonatally exposed to low doses of the organophosphate pesticide, parathion. Brain Res. Bull. 77, 404–411.

Vatanparast, J., Naseh, M., Baniasadi, M., Haghdoost-Yazdi, H., 2013. Developmental exposure to chlorpyrifos and diazinon differentially affect passive avoidance performance and nitric oxide synthase-containing neurons in the basolateral complex of the amygdala. Brain Res. 1494, 17–27.

Yuan, Y., 2012. Methylmercury: a potential environmental risk factor contributing to epileptogenesis. Neurotoxicology 33, 119–126.

Zhang, W., Huguenard, J.R., Buckmaster, P.S., 2012. Increased excitatory synaptic input to granule cells from hilar and CA3 regions in a rat model of temporal lobe epilepsy. J. Neurosci. 32, 1183–1196.

Zhu, X., Dong, J., Han, B., Huang, R., Zhang, A., Xia, Z., Chang, H., Chao, J., Yao, H., 2017. Neuronal nitric oxide synthase contributes to PTZ kindling-induced cognitive impairment and depressive-like behavior. Front. Behav. Neurosci. 11, 203.