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REPRODUCTIVE TOXICOLOGY

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# In utero exposure to nicotine and chlorpyrifos alone, and in combination produces persistent sensorimotor deficits and Purkinje neuron loss in the cerebellum of adult offspring rats

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**Abstract** This study was carried out to investigate the effect of in utero exposure to the cholinotoxicants, nicotine and chlorpyrifos, alone or in combination on neurobehavioral alterations and neuronal morphology latter in adult age. In the present study, 90 days old (corresponding to a human adult age) male and female offspring rats were evaluated for neurobehavioral, and neuropathological alterations following maternal, gestational exposure to nicotine and chlorpyrifos (*O*,*O*-diethyl-*O*-3,5,6-trichloro-2-pyridinyl phosphorothioate), alone and in combination. Female Sprague-Dawley rats (300–350 g) with timed-pregnancy were treated with nicotine (3.3 mg/kg/day, in bacteriostatic water via s.c. implantation of mini osmotic pump), chlorpyrifos (1.0 mg/kg, daily, dermal, in 75% ethanol, 1.0 ml/kg) or a combination of both chemicals, on gestational days (GD) 4–20. Control animals received bacteriostatic water via s.c. implantation of mini osmotic pump and dermal application of 70% ethanol. The offspring at postnatal day (PND) 90 were evaluated for neurobehavioral performance, changes in the activity of plasma butyrylcholinesterase (BChE) and acetylcholinesterase (AChE), and neuropathological alterations in the brain. Neurobehavioral evaluations included beam-walk score, beam-walk time, incline plane performance and forepaw grip time. Male and female offspring from mothers treated with nicotine and CPF, alone or in combination showed impairments in the performance of neurobehavioral tests, indicating sensorimotor deficits. Female offspring from mothers treated with a combination of nicotine and chlorpyrifos showed significant increase in plasma BChE activity. Brain regional AChE activity showed differential increases in male and female offspring. Brainstem and cerebellum of female offspring from mothers treated with nicotine or chlorpyrifos, alone or in combination showed increased AChE activity, whereas brainstem of male offspring from mothers treated with nicotine alone or a combination of nicotine and chlorpyrifos showed increase in AChE activity. Also, male offspring exposed in utero to nicotine exhibited increased AChE activity. Histopathological evaluations using cresyl violet staining showed a decrease in surviving Purkinje neurons in the cerebellum in offspring of all treatments groups. An increase in glial fibrillary acidic protein (GFAP) immuno-staining was observed in cerebellum white matter as well as granular cell layer (GCL) of cerebellum following all exposures. These results indicate that in utero exposure to nicotine and chlorpyrifos, alone and in combination produced significant sensorimotor deficits in male and female offspring, differential increase in brain AChE activity, a decrease in the surviving neurons and an increased expression of GFAP in cerebellum in adult offspring rats at a corresponding human adult age. Collectively, this study demonstrates that maternal exposure to environmental neurotoxic chemicals, i.e., nicotine and chlorpyrifos leads to developmental abnormalities in the offspring that persist latter into adulthood.

**Keywords** Chlorpyrifos · Nicotine · Cerebellum · Purkinje cells · Behavioral · Maternal · Developmental · Adult · Offspring · Acetylcholinesterase · Glial fibrillary acidic protein

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## Introduction

While both genetic and environmental factors play a role in aberrant fetal central nervous system development (Pletnikov et al. 1999), neuronal cells in the brain are altered in offspring of rats exposed to nicotine during gestation. Cigarette smoking is a risk factor for adverse pregnancy outcomes, affecting the mother, fetus, and newborn (Ebrahim et al. 2000). In the U.S., approximately 24% of women 18 years and older are regular cigarette smokers and many of these women continue to smoke after becoming pregnant (Mayer et al. 1990; Floyd et al. 1993; Ebrahim et al. 2000; Centers for Disease Control and Prevention 2001). Cigarette smoking during pregnancy has been suggested to cause neurobehavioral and cognitive deficits, and susceptibility to diseases in the offspring (Naeye 1992; Johnson et al. 2000). These deficits may continue at all the developmental stages, including adulthood. Nicotine [3-(1-methyl-2-pyrrolidinyl) pyridine] is the main active constituent that has been associated with a variety of chronic diseases following cigarette smoking, including neurobehavioral toxicity (Hoffman and Hoffman 1997; Law et al. 2003). Nicotine is a direct cholinergic agonist at the nicotinic acetylcholine receptor (nAChR) site, resulting in behavioral (Martin and Becker 1970; Benowitz 1996) and cellular effects (Slotkin et al. 1986). Exposure to nicotine in early developmental stages, in utero, produces abnormal behavior in the adulthood (Nordberg et al. 1991; Slotkin 1998), and may lead to long-lasting neurological and neuropathological alterations in the offspring (Abdel Rahman et al. 2003, 2004).

Because of extensive use of pesticides in the general population, many women who smoke during pregnancy are also exposed to a variety of neurotoxic chemicals, such as the organophosphorus insecticide chlorpyrifos, (*O*,*O*-diethyl-*O*-3,5,6-trichloro-2-pyridinyl phosphorothioate) that is extensively used in house-hold and agriculture. Chlorpyrifos exposure can occur via inhalation or through dermal or oral routes from residues on floors and carpets, children toys, food and dust (Lemus and Abdelghani 2000). Chlorpyrifos is a broad-spectrum insecticide (Clegg and van Gemert 1999). It is one of the most commonly used insecticides in the U.S. today, although concerns about infant exposure have recently led to restriction of its domestic use (Fenske et al. 1990; US Environmental Protection Agency 2000). Chlorpyrifos exerts its cholinergic action via its active metabolite, chlorpyrifos oxon, which inhibits acetylcholinesterase (AchE) resulting in the accumulation of acetylcholine at acetylcholine receptors and subsequent development of cholinergic over-stimulation (Huff et al. 1994; Pope 1999). In utero exposure during gestational days (GD) 9–12 to sub-toxic levels of chlorpyrifos produced behavioral abnormalities in adult offspring, suggesting long-term developmental effects (Icenogle et al. 2004).

The cholinotoxicants nicotine and chlorpyrifos affect a variety of nervous system processes and pathways that impact on neuronal proliferation and differentiation, axonogenesis and synaptic functions (Slotkin 1998; Barone et al. 2000). Perturbation in any of these processes during fetal development due to exposure to neurotoxic chemicals and drugs may lead to neurological deficits into adult life (Zhang et al. 1998). Indeed, previous studies from our laboratory have shown that in utero (GD 4–20) combined exposure to nicotine (1.0 mg/kg/day, sc) and chlorpyrifos (0.1 mg/kg/day, dermal), produced differential alterations in cholinesterase in brain regions and neuronal cell loss in the hippocampus CA1 subfield and granular cell layer (GCL) of cerebellum on postnatal day (PND) 30 (corresponding to human preadolescent age) offspring (Abdel-Rahman et al. 2003). Furthermore, offspring at PND 60 (corresponding to human young adult age) showed persistent increase of glial fibrillary acidic protein (GFAP) and a decrease of surviving neurons in the cerebellum (Abdel-Rahman et al. 2004). In view of the epidemiological studies suggesting that prenatal exposure to nicotine produces neurological deficits in the offspring at late adolescence and adulthood (Fergusson et al. 1998; Johnson et al. 2000; Abdel-Rahman et al. 2005), we carried out the present study to investigate the effect of in utero exposure, during embryonic development of rats, to the cholinotoxicants, nicotine and chlorpyrifos, alone or in combination, on the development of neurological deficits on PND 90 (corresponding to human adult age) offspring. The results show that exposure during embryogenesis of brain neurons to nicotine and chlorpyrifos, alone or in combination produced neuronal cell loss and neurobehavioral changes in adulthood latter in life.

## Materials and methods

Nicotine bitartrate, butyrylthiocholine iodide, and acetylthiocholine iodide were obtained from Sigma Chemical Company (St. Louis, MO, USA). Chlorpyrifos (~99%; *O*,*O*-diethyl-*O*-3,5,6-trichloro-2-pyridinyl phosphorothioate) was obtained from Dow Elanco (Indianapolis, IN, USA). Polyclonal antibodies against GFAP was obtained from Dako Laboratories, Carpinteria, CA, USA. Avidin-biotin-peroxidase reagent kits were obtained from Vector Laboratories, Burlingame, CA, USA.

### Methods

Timed pregnant Sprague-Dawley rats (300–350 gm), obtained from Zivic-Miller Laboratories (Allison Park, PA, USA) were maintained on a 12-h dark/light cycle with free accesses to food and tab water. The animals were treated between 7:30 and 11:00 AM daily. All treatments and procedures were approved by Duke University Medical Center Institutional Animal Care and Use Committee. Rats were randomly divided into

groups of five rats each. Chlorpyrifos was applied on a 1-inch$^2$, pre-clipped area on the back of the neck. The treatments were carried out as follows.

*Control*

Rats were treated with bacteriostatic water via mini osmotic pump (type 2ML2) implanted s.c. on the back and dermal application of 70% ethanol (1 ml/kg, daily) during GD 4–20.

*Chlorpyrifos*

Rats were treated with 1.0 mg/kg/day chlorpyrifos in 70% ethanol (1 ml/kg) by dermal application, during GD 4–20.

*Nicotine*

Rats were treated with 3.3 mg/kg/day nicotine, in bacteriostatic water via s.c. implantation of mini osmotic pump, type 2ML2 and 1 ml/kg 70% ethanol (dermal) during GD 4–20.

*Chlorpyrifos and nicotine*

Rats were treated with nicotine (3.3 mg/kg, in bacteriostatic water via s.c. implantation of mini osmotic pump, type 2ML2) and dermal application of chlorpyrifos (1.0 mg/kg in 70% ethanol 1 ml/kg, daily) during GD 4–20.

Following parturition, the number and weight of the litters were recorded and the pops were reared by their respective mothers. Since there were no significant differences in the litter size between treated and control mothers, no culling was done. Rats at different ages are used to reflect different human developmental stages as follows: PND 9, corresponds to neonate, PND 15, to infant, PND 30 to preadolescent, PND 60 to young adult, and PND 90 to adult; the day of birth is counted as zero (Liptakova et al. 2000). In the present study, rats at PND 90, corresponding to adult humans' age were studied. In order to represent the population of control and treated animals, one offspring of each sex from each mother (treated and control) was included for biochemical and pathological evaluations. The behavioral evaluations on PND 90 were carried out on all ten offspring (two from each of the five mothers in every treatment) of each sex, following which five offspring were used for biochemical assays and five for pathological studies.

Thus, an $n = 5$ for biochemical and pathological evaluations each, represents offspring from five different mothers. For biochemical assays, the male and female offspring were anesthetized with ketamine (50 mg/kg)/xylazine (10 mg/kg) and decapitated. Brains were removed and washed thoroughly with ice-cold normal saline. Brain regions were dissected on ice and rapidly frozen in liquid nitrogen and stored at −80°C until further analysis.

Behavioral evaluations

On PND 90, male and female offspring ($n = 10$; two from each of the five mothers in every treatment) were evaluated for behavioral studies. The behavioral tests employed in these studies evaluate sensorimotor reflexes, motor strength, and coordination. All behavioral testing was performed by an observer blind to the animal's treatment status, and was carried out in a sound-proof room with subdued lighting (less than 10.76 lumens/m$^2$ ambient light).

*Beam walking and beam score*

The testing apparatus was a 2.5×122 cm$^2$ wooden beam elevated 75.5 cm above the floor with wooden supports (Goldstein 1993) and Abou-Donia et al. (2001). Beam-walking ability was measured with a seven point scoring system scale (Goldstein 1993): (1) the rat is unable to place the hind-paws on the horizontal surface of the beam; (2) the rat places the hind-paws on the horizontal surface of the beam and maintains balance for at least 5 s; (3) the rat traverses the beam while dragging the hind-paws; (4) the rat traverses the beam and at least once places a hind-paw on the horizontal surface of the beam; (5) the rat crosses the beam and places a hind-paw on the horizontal surface of the beam to aid less than half its steps; (6) the rat uses the hind-paws to aid more than half its steps and; (7) the rat traverses the beam with no more than two foot-slips. In addition, the latency until the animal's nose entered the goal box (up to 90 s) was recorded. Rats that fall off of the beam or did not enter the goal box were assigned latencies of 90 s. Beam-walk scores are based on an average of five trials separated by 1 h.

*Inclined plane*

Rats were placed on a flat plane in the horizontal position, with the head facing the side of the board to be raised (Yonemori et al. 1998; Abou-Donia et al. 2001). Incline plane performance was measured with a standard protractor to the nearest 5°. The trial ends when the rat begins to slip backward, therefore, there is no specific trial duration. The angle at which the rat began to slip downward was recorded. The results of two trials separated by 1 h were averaged.

*Forepaw grip time*

The rat's forepaw strength was assessed by having them grip a 5 mm diameter wood dowel that was held horizontally and raised so that the rat supported its body

weight (Andersen et al. 1991; Abou-Donia et al. 2001). The time to release grip was recorded in seconds. All rats attempt to grip for grip strength testing. The results of two trials separated by 1 h were averaged.

## Statistical analyses

Data for the behavioral tests were compared among groups by two-way ANOVA. If a significant difference was found, Fisher's LSD tests were used for post hoc, pair-wise comparisons. A two-tailed $P$ value of $< 0.05$ was considered statistically significant.

### *Biochemical assays*

For biochemical analyses, male and female offspring (total of 5; one from each of the five mothers in every treatment) on PND 90 were anesthetized with ketamine (50 mg/kg)/xylazine (10 mg/kg) and blood was drawn in heparinized syringe. Brains were removed and washed thoroughly with ice-cold normal saline. Brains were dissected into cortex, midbrain, cerebellum and brainstem on ice and rapidly frozen in liquid nitrogen. Plasma was separated and frozen at −80°C for enzyme studies.

### *Determination of cholinesterases*

Acetylcholinesterase in brain regions and BChE in plasma were determined according to the method of Ellman et al. (1961) modified for assay in a Molecular Devices UV Max Kinetic Microplate Reader as previously described (Khan et al. 2000; Abou-Donia et al. 2001). Protein concentration was determined by the bicinchoninic acid method according to Smith et al. (1985). The enzyme activities are expressed as $\mu$moles substrate hydrolyzed/min/mg protein for brain regions and $n$ moles substrate hydrolyzed/min/mg protein for plasma (percent of control).

## Statistical analysis

The results were analyzed by two-way ANOVA. If a significant difference was found, Fisher's LSD tests were used for post hoc, pair-wise comparisons. A two-tailed $P$ value of $< 0.05$ was considered statistically significant.

### *Histopathological analyses*

Male and female offspring (total of 5; one from each of the five mothers in every treatment) on the PND 90, were anesthetized with sodium pentobarbital (100 mg/kg) and perfused transcardially, first with normal saline containing 0.1% heparin and then with 4% paraformaldehyde in 0.1 M Tris–HCl (pH 7.2) over a period of 30 min. Brains were removed post fixed in 4% paraformaldehyde at 4°C for 18–24 h. The tissues were then blocked and embedded in paraffin according to the standard histological techniques. Six micrometer-thick coronal sections were cut through the cerebellum ($n = 5$). Sections were stained with cresyl violet for light microscopic examination (Abdel-Rahman et al. 2003).

To evaluate the astroglial pathology, polyclonal antibodies against GFAP were used (Abdel-Rahman et al. 2003). Avidin-biotin complex staining method of Hsu et al. (1981) was used to visualize the immunostaining. Briefly, the sections were incubated overnight at room temperature with anti GFAP antibodies (Dako Laboratories) at 1:10,000 dilution n 50 mM Tris–buffered saline (TBS) containing 1% normal goat serum. The sections were rinsed thoroughly with 50 mM TBS and incubated with biotinylated goat anti-rabbit IgG at 1:200 containing 1% normal goat serum for 1 h at room temperature. Following extensive rinsing with TBS, the sections were incubated with for 1 h in the avidin-biotin peroxidase complex solution diluted 1:25 in TBS. The sections were rinsed thoroughly with TBS and incubated with 3,3-diaminobenzene tetrahydrochloride (DAB) for 10 min and the reaction was stopped by several rinses with TBS. The sections were then dehydrated in alcohol, cleared in xylene, and cover-slipped with Permount for observations and analyses.

The quantification of surviving Purkinje neurons in the cerebellum and GFAP immunoreactivity of neighboring sections from cerebellar white matter as well as in the GCL was carried out according to previously described procedure (Abdel-Rahman et al. 2003). Briefly, the numerical density of surviving and dying neurons per square millimeter of tissue area was measured. All measurements were performed in a blind fashion using experimental codes. The coding was such that animal treatments were not known during the measurements; however, sections originating from the same animal were identified. The measurements were performed using a Nikon E600 microscope equipped with eyepiece grid. Both dying and surviving neurons were counted within a unit area of each section at 400× magnification. The unit area selected for measurements was 0.0063 $mm^2$ for the Purkinje cell layer of the cerebellum. Finally, the density of neurons per unit area was transformed to the numerical density per square millimeter of the cerebellum.

Glial fibrillary acidic protein-immunoreactive structure quantification in the GCL and white matter of the cerebellum were performed by using Scion Image for Windows, based on NIH Image for Macintosh (Scion Corporation, Fredrick, MD, USA). For every brain region, two sections were measured for each animal. The area occupied by GFAP-positive immunoreactive structures per unit area of tissue (0.176 $mm^2$) was determined. The microscopic image was transferred to the computer screen by focusing the appropriate area of immunostained section with a Nikon E600 microscope equipped with digital camera connected to an IBM computer (Abdel-Rahman et al. 2003).

*Statistical analysis*

The values were calculated separately for each animal before the means and standard errors were determined for the total number of animals in each group. Mean values between different groups of animals were compared using one-way ANOVA with Student's Newman–Keuls multiple comparison post hoc test. $P$ value $< 0.05$ was considered significant.

## Results

Clinical signs

The animals were observed for the development of clinical signs of toxicity throughout the experimental period. No animals died. There were no signs of overt toxicity in any group of mothers or offspring. There was no significant difference in body weight gain and litter size between control and treated groups.

*Effects on neurobehavioral performance in the offspring*

The data presented in Fig. 1a, b shows neurobehavioral performance using the beam walk test, of PND 90 male and female offspring, respectively. Treated animals of both sexes, did not differ from controls in beam walk scores. Male offspring exposed in utero to nicotine, alone or in combination with chlorpyrifos exhibited significant increase in beam walk time that was more profound in the combined exposure group (Fig. 1a). On the other hand, only female offspring exposed in utero to nicotine showed significant increase in beam walk time (Fig. 1b).

Incline plane evaluations (Fig. 1a, b) show that male offspring from nicotine-treated mothers and females from chlorpyrifos-treated mothers exhibited poorer performance than their matched controls ($P < 0.01$). Other treatment groups were not significantly different from control.

Forepaw grip time evaluations (Fig. 1a, b) show that all male groups performed poorer than their respective sex-matched controls ($P < 0.01$). On the other hand, only female offspring exposed in utero to chlorpyrifos alone or in combination with nicotine, had significantly less grip time compared to control (Fig. 1b).

*Effects on plasma BChE and brain AChE activity in the offspring*

Of all treatment groups, only female offspring exposed in utero to a combination of nicotine and chlorpyrifos exhibited significant increase (~140% of control) in plasma butyrylcholinesterase (BChE) activity. There was no significant change in BChE activity in female and all male offspring of other treated groups (data not shown).

The results on brain regional AChE activity from male and female offspring are presented in Fig. 2a, b. Male offspring from mothers treated with either nicotine alone or a combination of nicotine and chlorpyrifos showed a significant increase (~140% of control) in the AChE activity in the brainstem, whereas midbrain AChE activity in male offspring exposed to nicotine showed a significant increase compared to control. Male offspring in all treatment groups, did not exhibit any changes in cortex or cerebellum AChE activity. On the other hand, female offspring exposed in utero to all treatments, sowed significant increase in brainstem (~125–137% of control) and cerebellum (~120–135% of control) AChE activity compared to control.

*Histopathological assessments*

All regions of the brain from treated and control animals underwent histopathological examinations. The cerebellum was the only region that exhibited neuropathological alterations. The results of the histopathological examination of the cerebellum from male and female offspring are presented in Figs. 3 and 4, respectively. In the cerebellum the most conspicuous injury following in utero exposure to nicotine and chlorpyrifos, alone or in combination was in the Purkinje cell layer. Degenerated neurons were observed in animals with all treatments (Fig. 3, $A_2$–$A_4$). Quantitative analyses of Purkinje cells in male offspring presented in Fig. 3, right panel, show that animals in all treatment groups, exhibited significant decrease in surviving neurons, compared to control animals (top panel, ANOVA $F_{3,32} = 27.04$, $P < 0.00001$). Fig. 3, middle and bottom panels represent the GFAP immunostaining in GCL and white matter of the cerebellum, respectively. The measurement of GFAP-immunoreactive structures per unit area of the GCL demonstrated a significant upregulation in GFAP-positive elements in all treatment groups (ANOVA $F_{3,32} = 98.25$, $P < 0.000001$). Similar results were obtained in the cerebellar white matter (ANOVA $F_{3,32} = 21.98$, $P < 0.000001$). There was no significant difference; however, among the treated groups.

The pathological alterations in the female offspring are presented in Fig. 4. Quantitative analyses of Purkinje cells in male offspring are presented in right panel. A significant decrease in the number of surviving Purkinje neurons in the cerebellum was observed in offspring from mothers in all treatment groups compared to controls (top panel, ANOVA $F_{3,32} = 27.04$, $P < 0.00001$). Middle and bottom panel represent the GFAP immunostaining in GCL and white matter of the cerebellum, respectively. The results show significant increase in GFAP immunostaining in the GCL (ANOVA $F_{3,32} = 98.25$, $P < 0.000001$). Also, the cerebellar white matter showed significant upregulation in the GFAP immunostaining (ANOVA $F_{3,32} = 21.98$, $P < 0.000001$). There was no significant difference among the treated groups and no effects of sex and sex and treatment interaction.

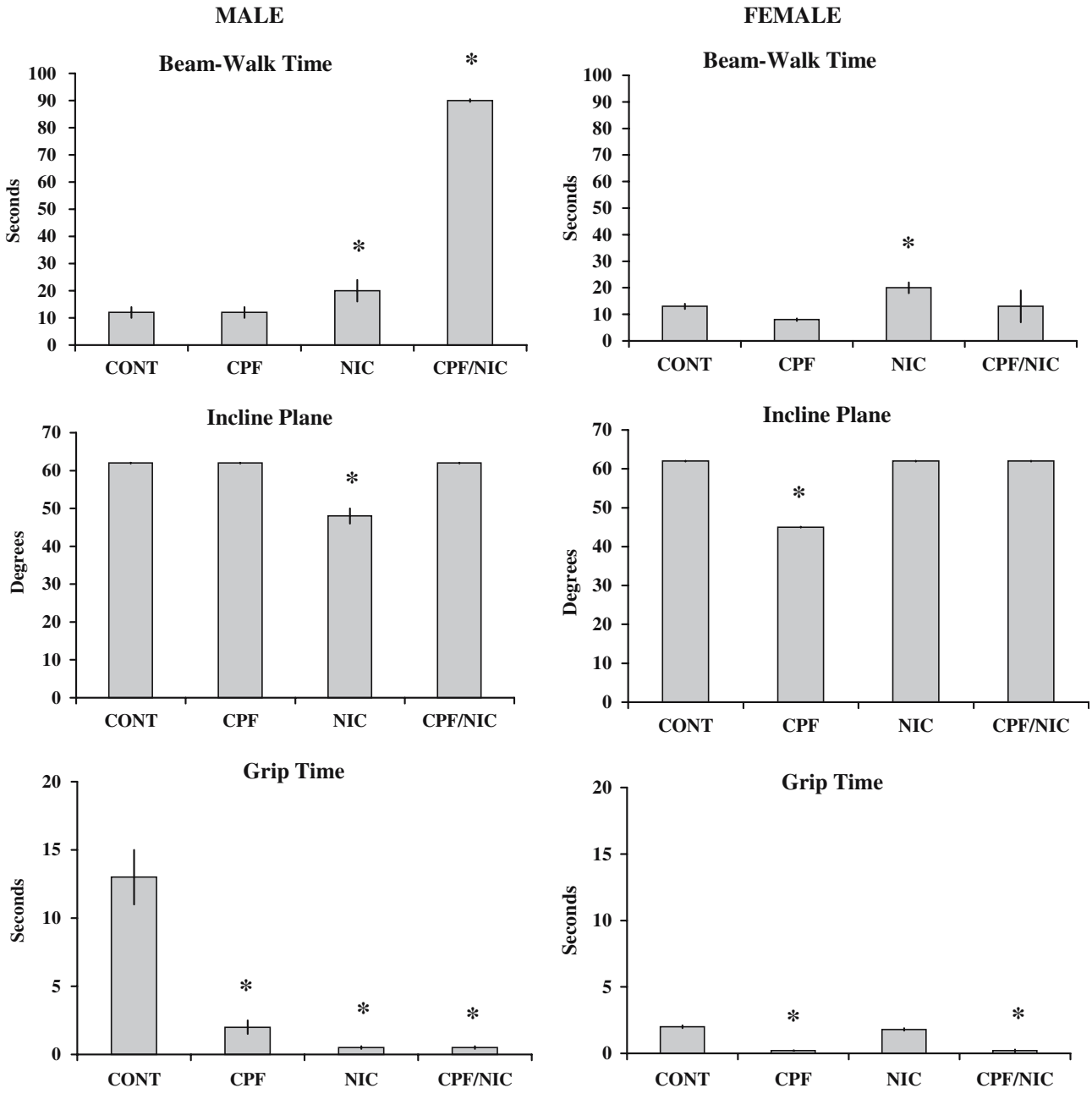


**Fig. 1** Neurobehavioral evaluations of the male and female adult offspring. Male and female offspring on PND 90 were evaluated for neurobehavioral deficits (beam-walk score, beam-walk time, inline plane performance and grip time) as described in Materials and methods. *Asterisk* indicates statistically significant difference from control ($P < 0.05$). *CONT* Control, *CPF* chlorpyrifos, *NIC* nicotine, *CPF*/*NIC* chlorpyrifos and nicotine. Beam walk time shows a significant effect of treatment group (ANOVA $F_{3,32} = 392.6$, $P < 0.0001$), a significant effect of sex (ANOVA $F_{1,32} = 382.6$, $P < 0.0001$) and a significant treatment × sex interaction (ANOVA $F_{3,32} = 360.6$, $P < 0.0001$). Incline plane test shows a significant effect of treatment group (ANOVA $F_{3,32} = 27.7$, $P < 0.0001$), no effect of sex (ANOVA $F_{1,32} = 1.0$, $P = 0.32$) but a significant treatment × sex interaction (ANOVA $F_{3,32} = 54.3$, $P < 0.0001$). Forepaw grip time evaluation shows a significant effect of treatment group (ANOVA $F_{3,32} = 60.0$, $P < 0.0001$), a significant effect of sex (ANOVA $F_{1,32} = 57.5$, $P = 0.0001$) and a significant treatment × sex interaction (ANOVA $F_{3,32} = 48.9$, $P < 0.0001$)

## Discussion

The present study shows that in utero exposure to nicotine and chlorpyrifos, alone or in combination impairs sensorimotor function, increases brain AChE activity, causes Purkinje cell death and upregulates GFAP in the cerebellum of PND 90 (corresponds to human adult age) male and female offspring rats. Alterations that accompany the differentiation of precursor neurons (birth of the neurons) may be modified by in utero intervention of chemicals such as nicotine

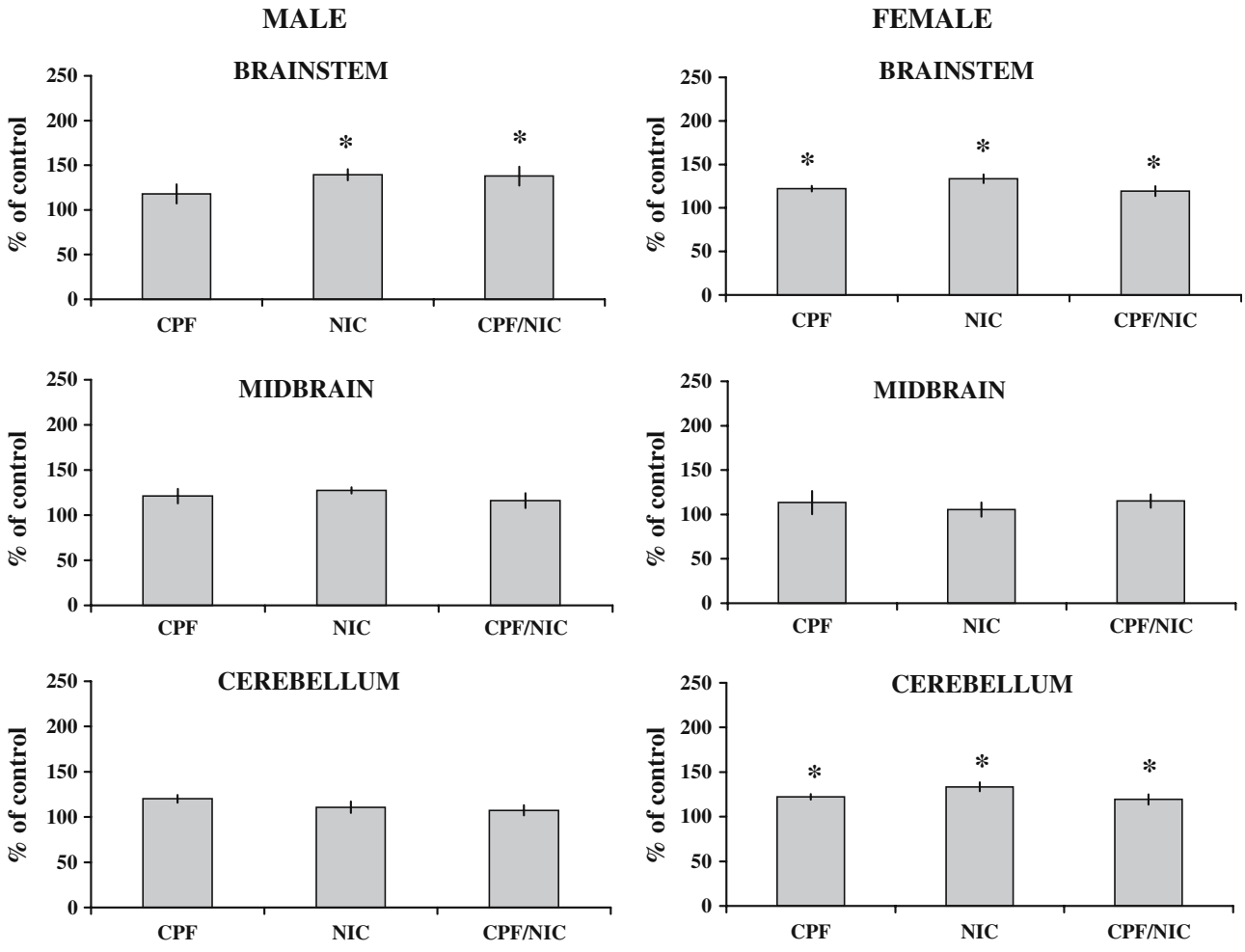


**Fig. 2** Brain regional acetylcholinesterase activity of the male and female adult offspring. Brain regional acetylcholinesterase activity on PND 90 male and female offspring. *Asterisk* indicates statistically significant difference from control ($P<0.05$), $n=5$ offspring, one from each mother. *CONT* Control, *CPF* chlorpyrifos, *NIC* nicotine, *CPF/NIC* chlorpyrifos and nicotine. Control enzyme activities expressed as $\mu$moles acetylthiocholine hydrolyzed/min/mg protein were: males: cortex $0.72+0.062$; brainstem $1.42+0.06$; midbrain $1.4+0.16$, cerebellum $0.57+0.050$; females: cortex $1.56+0.17$; brainstem $1.19+0.06$; midbrain $1.12+0.05$; cerebellum $0.482+0.012$. Two-way ANOVA showed a significant effect of treatment (ANOVA $F_{3,32}=16.6$, $P<0.00001$) and a significant effect of sex (ANOVA $F_{1,32}=41.2$, $P<0.00001$). Two-way ANOVA of midbrain AChE activity in male and female offspring did not show significant effect of treatment, but showed a significant effect of sex (ANOVA $F_{1,32}=46.28$, $P<0.0001$). Only female offspring cerebellum showed a significant increase in AChE activity from mothers treated with nicotine and CPF, alone and in combination. Two-way ANOVA showed a significant effect of treatment (ANOVA $F_{1,32}=46.28$, $P<0.0007$) and a significant effect of sex (ANOVA $F_{1,32}=7.83$, $P<0.008$)

and chlorpyrifos, so that the life-long viability and population of the neurons are reduced resulting in neurobehavioral deficits.

Male offspring exposed in utero to nicotine showed deficits in beam walk time, forepaw grip time and incline plane performance, whereas, exposure to chlorpyrifos alone, or in combination with nicotine caused deficits in beam walk time and forepaw grip time. On the other hand, female offspring on PND 90 showed significant deficits in beam walk time following maternal exposure to nicotine; and a deficit in incline plane and forepaw grip time following in utero exposure to chlorpyrifos; whereas in utero exposure to both chemicals caused deficits in forepaw grip time. Generally, all male groups performed poorer than their respective sex-matched controls, except female offspring from chlorpyrifos treated mothers. Several factors play a role in gender-dependent differences in response to nicotine exposure, such as route (Booze 1999); dose (Harrod et al. 2004); duration (Elliott et al. 2004); strain and age (Collins et al. 2004), and hormonal status (Kanyt et al. 1999). The gender dependent deficit in the male offspring is consistent with the findings of Trauth et al. (2000) and Levin et al. (2002) that direct treatment of nicotine and chlorpyrifos, respectively to young rats also resulted in gender-specific behavioral deficits. Pervious reports Romero and Chen (2004) also reported gender-related response in the PND 60 offspring in open field activity following developmental exposure to nicotine during gestation. Similarly, Levin et al. (2002) observed

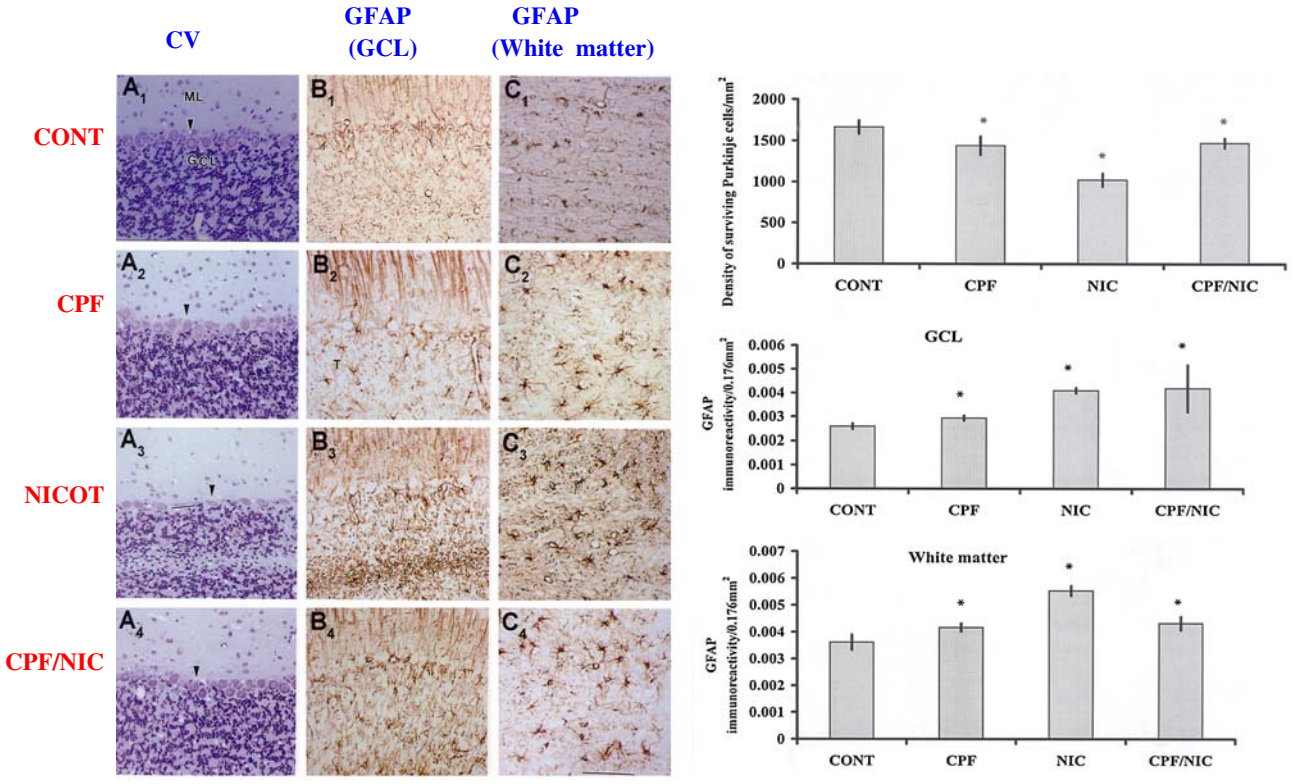


**Fig. 3** Histopathological changes in the cerebellum of the adult male offspring. Pathological alterations showing cresyl violet staining (*CV*) ($A_1$–$A_4$); GFAP immunostaining in the granular cell layer (*GCL*) of the cerebellum ($B_1$–$B_4$); GFAP immunostaining in the cerebellar white matter ($C_1$–$C_4$). $A_1$, $B_1$ and $C_1$ Control; $A_2$, $B_2$ and $C_2$ CPF; $A_3$, $B_3$ and $C_3$ nicotine; $A_4$, $B_4$ and $C_4$ CPF/nicotine ($n=5$). *Left Panel* Photomicrograph; *Right Panel* Bar graph showing quantification of surviving Purkinje neurons (*upper panel*), GFAP immunostaining in GCL (*middle panel*) and GFAP immunostaining in the white matter (*lower panel*). *CONT* Control, *CPF* chlorpyrifos, *NIC* nicotine, *CPF/NIC* chlorpyrifos and nicotine. *Arrow heads* in the cresyl violet staining indicate Purkinje cells; *bar* 100 $\mu$m. Bar graph presented as mean ± SE, $n=5$, *asterisk* indicates statistically significant

persistent behavioral and cognitive deficits at adulthood following neonatal exposure to chlorpyrifos. Adriani et al. (2003), recently reported neurobehavioral deficits after exposure to nicotine during pre-adolescence in rats. Vaglenova et al. (2004) also observed significant long-term developmental and behavioral teratogenic effects in the offspring following 6 mg/kg/day nicotine exposure to mothers during gestation.

The results from the present study show that only female offspring from mothers treated with combined nicotine and chlorpyrifos showed a significant increase in plasma BChE activity, in agreement with previous report in PND 60 offspring (Abdel-Rahman et al. 2004). There was a differential increase of brain AChE activity in male and female offspring. Thus, brainstem AChE activity in offspring exposed in utero to nicotine alone or a combination with chlorpyrifos, exhibited significant increase in both sexes. Also male offspring exposed in utero to chlorpyrifos showed increased brainstem or midbrain AChE activity. On the other hand, female offspring following all treatments in utero exhibited increased brainstem and cerebellum AChE activity. An increase in the AChE activity in the adult offspring following maternal exposure to chlorpyrifos, alone or in combination with nicotine would result in increased hydrolysis and depletion of acetylcholine at acetylcholine receptors in the brain, with subsequent diminishing in brain functions that can explain, at least in part, neurobehavioral deficits.

Neuropathological alterations in adult male and female offspring are characterized by significant decrease in the number of surviving Purkinje neurons, and increase in expression of GFAP in the cerebellum GCL and cerebellar white matter following in utero exposure to nicotine and chlorpyrifos, alone and in combination. These results are consistent with our previous finding that maternal exposure combined to nicotine (1.0 mg/kg) and chlorprifis (0.1 mg/kg/day, dermal) caused neuronal cell death in the cerebellum GCL of offspring at PND 60 (Abdel-Rahman et al. 2004), and the report that maternal exposure to nicotine during gestational period caused a substantial decrease in the cell size in the hippocampal CA3 region and dentate gyrus (Roy et al. 2002). These results are in agreement with the suggestion that cerebellar maturation is acutely sensitive to a variety of neurotoxicants such as nicotine (Hauser et al. 2003). Furthermore, early developmental deficits produced by maternal exposure to these neurotoxic agents, in the cerebellum are not repaired, because of later ontogenic development of cerebellar structures (Altman

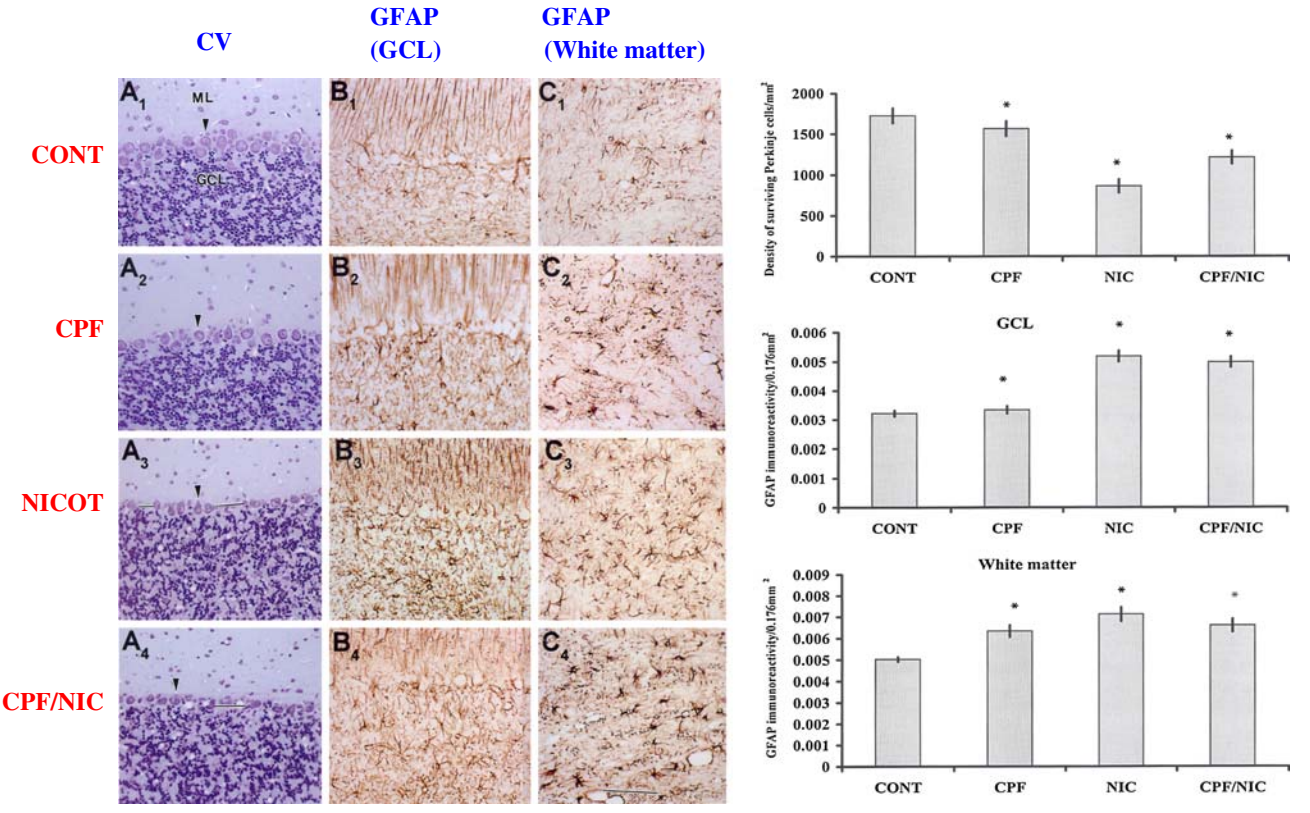


**Fig. 4** Histopathological changes in the cerebellum of female offspring. Pathological alterations showing cresyl violet staining (*CV*) ($A_1$–$A_4$); GFAP immunostaining in the granular cell layer (*GCL*) of the cerebellum ($B_1$–$B_4$); GFAP immunostaining in the cerebellar white matter ($C_1$–$C_4$). $A_1$, $B_1$ and $C_1$ Control; $A_2$, $B_2$ and $C_2$ CPF; $A_3$, $B_3$ and $C_3$ nicotine; $A_4$, $B_4$ and $C_4$ CPF/nicotine ($n=5$). *Left panel* Photomicrograph, *right panel* bar graph showing quantitation of surviving Purkinje neurons (*upper panel*), GFAP immunostaining in GCL (*middle panel*) and GFAP immunostaining in the white matter (*lower panel*). *CONT* Control, *CPF* chlorpyrifos, *NIC* nicotine, *CPF/NIC* chlorpyrifos and nicotine. *Arrow heads* in the cresyl violet staining indicate Purkinje cells; *bar* 100 $\mu$m. Bar graph presented as mean ± SE, $n=5$, *asterisk* indicates statistically significant

and Bayer 1990). Such alterations in the cerebellum are consistent with sensorimotor deficits in offspring rats.

Glial fibrillary acidic protein, a major component of astrocytic neurofilaments, is up regulated in response reactive gliosis resulting from such insults as trauma, neurodegenerative disease, and exposure to neurotoxicants (Eng and Ghirnikar 1994). Reactive gliosis, accompanied by increased accumulation of GFAP is a characteristic response of both immature and adult brain to injury by variety of neurotoxic insults, including chlorpyrifos (O'Callaghan 1993; Burtrum and Silverstein 1993; Clark et al. 1996; Fattore et al. 2002; Garcia et al. 2002; Little et al. 2002). GFAP plays an important role in the long-term maintenance of brain cytoarchitecture (Liedtke et al. 1996; Ridet et al. 1997), proper functioning of the blood-brain barrier (Penky et al. 1998), and modulation of neuronal functions (Shibuki et al. 1996). The present study shows significant increase in GFAP immunostaining in the cerebellar GCL and white matter in male and female adult offspring that were exposed in utero to nicotine and chlorpyrifos, alone and in combination. Previous studies from our laboratory, showed that male pups at PND 30 from the mothers treated with a combination of nicotine (1.0 mg/kg) and chlorpyrifos (0.1 mg/kg) exhibited increased GFAP immuno-staining in cerebellar GCL as well as white matter, but to a lesser extent than the females (Abdel-Rahman et al. 2003). Previous reports that children with developmental neuropsychiatric disorders have elevated levels of GFAP in the cerebrospinal fluid (Ahlsen et al. 1993; Kristjansdottir et al. 2001) suggest that in utero exposure to test compounds may lead to the development of neuro-psychiatric abnormalities in adulthood. Such prolonged glial activation in the white matter may result from axonal degeneration in the cerebellar white matter (Atarashi et al. 2001; Tsuneishi et al. 1991). The neuropathological alterations might be related to oxidative stress caused by exposure to chlorpyrifos (Bagchi et al. 1995; John et al. 2001; Verma and Srivastava 2001) and nicotine (Bhagwat et al. 1998; Guan et al. 2003).

Neuronal degeneration following low-level, in utero exposure to the cholinotoxicants nicotine and chlorpyrifos in PND 90 offspring rats, corresponding to a human adult age is significant. Nicotine is a direct cholinergic agonist at nicotinic acetylcholine receptors, delivered by cigarette smoking. An organophosphorus insecticide, chlorpyrifos has three neurotoxic actions.

The primary action is the irreversible inhibition of AChE resulting in the accumulation of ACh and subsequent over-stimulation of nicotinic and muscarinic receptors resulting in cholinergic effects (Abu-Qare et al. 2001). Another action arises from exposure to large toxic doses of chlorpyrifos resulting in the development of organophosphorus ester-induced delayed neurotoxicity (OPIDN) in humans (Lotti et al. 1986) and in sensitive animal species such as the hen (Fikes et al. 1992; Abou-Donia and Wilmarth 1995; Abou-Donia et al. 1998) and the cat (Capodicasa et al. 1991). The third neurotoxic action induced by chlorpyrifos is a long-term neurological and neurobehavioral deficits accompanied by brain neuronal cell death that has been characterized recently as organophosphorus ester-induced chronic neurotoxicity (OPICN, Abou-Donia 2003). Although such an effect may result from exposure to high doses that cause acute cholinergic neurotoxicity, it can also be induced by low-level, sub-clinical doses of organophosphorus compounds, consistent with the present results that in utero exposure to very small doses (1.0 mg/kg/day) of chlorpyrifos, resulted in neurobehavioral deficits and biochemical alterations, accompanied by brain neuronal cell death in the cerebellum, in offspring rats, at corresponding human adult age. Although the mechanism of this neurodegenerative disorder is yet to be established, available information suggest that sub-clinical exposures produce delayed apoptotic neuronal cell death that involves oxidative stress (Abou-Donia 2003). This suggestion is consistent with increased AChE activity in offspring rats, following in utero exposure to chlorpyrifos, reported in this and previous studies (Abdel-Rahman et al. 2003; 2004). This increase in AChE activity might have resulted from increased AChE gene expression similar to that reported by the organophosphate, nerve agent sarin in the same regions of the brain that exhibited neuronal degeneration (Damodaran et al. 2003). AChE has recently been proposed to play an important role in neurogenesis that includes mitosis, axonal guidance, neurite outgrowth, synaptogenesis and cell adhesion (Brimijoin and Koenigsberger 1999; Bigbee et al. 2000). Over-expression of AChE produced enhanced neurite growth and synapse development in transgenic mice (Sternfeld et al. 1998).

Several studies have implicated AChE in brain neuronal cell death in some neuronal disorders (Saberna et al. 1998). (1) AChE has been shown to be neurotoxic in vivo and in vitro; it accelerates assembly of amyloid peptide in Alzheimer's fibrils, resulting in death via apoptosis (Yang et al. 2002). (2) Increased AChE expression has been demonstrated in apoptotic neuroblastoma SK-N-SH cells after long-term culturing (Lie et al. 2002; Yang et al. 2002). (3) Brain AChE has been shown to be toxic to neuronal (Neuro2a) and Glial-like (B12) cells (Calderon et al. 1998). (4) Transgenic mice over-expressing human AChE in brain neurons undergo progressive cognitive deterioration (Andres et al. 1998). Recently, Day and Greenfield (2003) have demonstrated that a peptide derived from AChE (AChE-peptide) caused a continuum of apoptotic and necrotic cell death in rat hippocampal organotypic cultures. They proposed that the following events caused cell death, initiated by 1 nM AChE-peptide: *N*-methyl-D-aspartate (NMDA) receptor activation, opining of the L-type voltage gated calcium channel, activation of calcium/calmodulin kinase II, generation of reactive oxygen species and caspase activation. Taken all together, it seems that chlorpyrifos results in increased AChE in brain cells, followed by events leading to delayed apoptotic brain neuronal cell death.

This study demonstrates that sub-clinical, low-level in utero exposure of rats to nicotine and/or the insecticide chlorpyrifos, that does not induce signs of neurotoxicity or body weight change, resulted in persistent neurological disorder characterized by sensorimotor performance deficits, biochemical alterations and accompanied by brain neuronal cell death in the offspring at PND 90, a corresponding human adult age.

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