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ORGAN TOXICITY AND MECHANISMS

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Sensorimotor deficits and increased brain nicotinic acetylcholine receptors following exposure to chlorpyrifos and/or nicotine in rats

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Abstract Despite well-known adverse effects associated with cigarette smoking, approximately 20% of the US population continues to smoke and many more are exposed to environmental tobacco smoke. Many of the same individuals are also exposed to environmental neurotoxic chemicals such as the organophosphorus insecticide chlorpyrifos. In the present study, the effects of exposure to low doses of nicotine and chlorpyrifos alone and in combination, were studied on the central cholinergic system and sensorimotor performance in rats. Male Sprague-Dawley rats (250–300 g) were treated with nicotine (1 mg/kg s.c., in normal saline), chlorpyrifos (0.1 mg/kg dermally, in 0.1 ml 70% ethanol), or a combination of both, daily for 30 days. Control rats were treated with saline and dermally with ethanol. Sensorimotor behavior was evaluated 24 h following the last dose using a battery of tests. There was a significant deficit in incline plane performance, beam-walk score and beam-walk time following exposure to each chemical, alone or in combination. The deficit in incline plane performance was greater when the two chemicals were given in combination than with either compound alone. Biochemical analysis showed a decrease in cerebellar and an increase in midbrain acetylcholinesterase (AChE) activity following combined exposure. Exposure to nicotine alone resulted in a significant increase in AChE activity in brainstem and midbrain, whereas there was no significant change after exposure to chlorpyrifos, alone. A significant increase in ligand binding to nicotinic acetylcholine receptors (nAChR) was observed in brainstem and cortex following exposure to nicotine or chlorpyrifos. This was further augmented with combined exposure, which caused a modest but significant increase in m2 muscarinic acetylcholine receptors (m2-mAChR) ligand binding in the cortex. These data suggest that exposure to either nicotine or chlorpyrifos or a combination of the two may impair neurobehavioral performance and affect the central nervous system cholinergic pathways.

Keywords Organophosphorus Insecticides · Chlorpyrifos · Nicotine · Central nervous system · Acetylcholinesterase · Muscarinic acetylcholine receptor · Nicotinic acetylcholine receptor · Neurotoxicity · Combined exposure · Sensorimotor · Smoking

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Introduction

Despite well-known adverse health effects of cigarette smoking, approximately 20% of the US population continues to smoke (US DHHS 2000). In addition to individuals' exposure to nicotine through cigarette smoking, many are also exposed to this chemical through environmental smoke and transiently via various preparations used for smoking cessation (Benowitz 1996). The general population is also exposed to organophosphorus insecticides such as chlorpyrifos (Clegg and van Gemert 1999). Both chemicals exert their toxic effects primarily by affecting the cholinergic pathway (Slotkin 1999).

Nicotine (3-[1-methyl-2-pyrrolidinyl] pyridine) is a tertiary amine consisting of a pyridine and a pyrrolidine ring. Once absorbed, nicotine enters the circulation and distributes rapidly to different tissues including brain, where it is a direct cholinergic agonist at the nicotinic acetylcholine receptor (nAChR) site, resulting in behavioral (Martin and Becker 1970) and cellular effects (Slotkin 1999) consistent with cholinergic over-stimulation. Inhaled or dermally applied nicotine travels

directly to the brain, bypassing the liver, and readily crosses the blood–brain barrier (Benowitz 1996). Several metabolites of nicotine are found in rat brain after peripheral administration (Miskys et al. 2000).

Behaviorally, a single dose of nicotine causes biphasic effects on locomotion that are characterized by an initial decrease in activity followed by a period of hyperactivity (Stolerman et al. 1995). Following repeated exposure, nicotine results in an increase in locomotor activity because of a locomotor desensitization that is mediated by nAChR (Stolerman et al. 1995; Booze et al. 1999).

Chlorpyrifos (*O*,*O*-diethyl-*O*-[3,5,6trichloro-2-pyridinyl]phosphorothioate) is one of the most commonly used insecticides in the USA today. Concerns about adverse health effects of chlorpyrifos exposure in infants have recently led to restriction of its domestic use (Davis and Ahmed 1998; Gurunathan et al. 1998). Chlorpyrifos is metabolized to chlorpyrifos oxon, which inhibits acetylcholinesterase (AChE) resulting in the accumulation of acetylcholine at acetylcholine receptors and subsequent development of cholinergic over-stimulation (Abou-Donia 1992). Chlorpyrifos also exhibits cholinergic agonist-like actions, opening and then desensitizing nicotinic cholinergic receptor/ion channels (Katz et al. 1997). It interacts with signaling components, such as G proteins and adenylate cyclase involving muscarinic acetylcholine receptors (Huff et al. 1994). Large doses of chlorpyrifos produce organophosphate-induced delayed neuropathy (OPIDN) in humans (Aiuto et al. 1993) and other sensitive animal species, e.g., in the hen (Fikes et al. 1992; Abou-Donia 1992). Recently, impairments in various neurological functions of termiticide applicators exposed to low dose of chlorpyrifos have been reported (Steenland et al. 2000).

Combined neurotoxicity can result from simultaneous exposure to different chemicals. Although nicotine has been linked to a variety of harmful health effects associated with cigarette smoking, environmental exposures to other cholinotoxic chemicals such as organophosphorus insecticides could also play a role in tobacco-induced illnesses.

In the present study, we evaluated sensorimotor performance, brain regional AChE activity, and nicotinic and m2 muscarininc acetylcholine receptor ligand binding following daily treatment with nicotine and chlorpyrifos, alone and in combination, in adult male Sprague-Dawley rats. The results suggest that exposure to nicotine and chlorpyrifos, alone and in combination, results in sensorimotor impairments and affects the central nervous system cholinergic function.

Materials and methods

Materials

Nicotine bitartrate, butyrylthiocholine iodide, and acetylthiocholine iodide were obtained from Sigma Chemical Co (St. Louis, Mo., USA). Chlorpyrifos (~99% purity) was purchased from Chem Service (West Chester, Pa., USA). [^{3}H]Cytisine (specific activity 15 Ci/mmol) and [^{3}H]AF-DX 384[2,3-dipropylamino-^{3}H] (specific activity 100 Ci/mmol) was obtained from PerkinElmer Life Sciences formerly known as NEN (Boston, Ma., USA).

Procedures

Male Sprague-Dawley rats (200–250 g) were obtained from Zivic-Miller Laboratories (Allison Park, Pa., USA) and housed in Duke University Medical Center vivarium on 12 h dark–light cycle. The animals were allowed food and water ad libitum. The rats were treated between 7:30 and 11:00 a.m. daily. All treatments and procedures were carried out strictly according to Duke University Medical Center Institutional Animal Care and Use Committee guidelines.

Rats were randomly allocated into groups of five rats each. For dermal applications, the treatment was done at the back of the neck on a 1-square inch area preshaved with electric clippers. The rats were treated as follows:

Control	Animals received subcutaneously (s.c.) normal saline and dermal application of 70% ethanol, daily for 30 days ·
Nicotine	Animals received 1 mg/kg nicotine s.c. in normal saline, daily for 30 days ·
Chlorpyrifos	Animals received 0.1 mg/kg chlorpyrifos in 0.1 ml 70% ethanol by dermal application, daily for 30 days ·
Nicotine and chlorpyrifos	Animals received 1 mg/kg nicotine in normal saline s.c., and dermal application of chlorpyrifos (0.1 mg/kg) in 0.1 ml 70% ethanol, daily for 30 days.

After behavioral testing had been performed, the animals were anesthetized with 0.2 ml of ketamine/xylazine (100 mg/kg ketamine, 15 mg/kg xylazine) and blood was withdrawn into heparinized syringes. Brains were removed and washed thoroughly with ice-cold normal saline. Cerebral cortex, midbrain, cerebellum and brainstem were dissected on ice and rapidly frozen in liquid nitrogen. Plasma was separated and frozen at −80°C for enzyme studies.

Behavioral evaluations

The behavioral tests employed in these studies evaluate sensorimotor reflexes, motor strength, and coordination. All behavioral testing was performed 24 h after the last dose by an observer blind to the animal's treatment status, and was carried out in a soundproof room with subdued lighting (less than 10.76 lumens/m^2, ambient light) between 7:00 and 11:30 a.m.

Incline 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). The angle that the rat began to slip downward was recorded. The results of the two trials were averaged.

Forepaw grip time

The rats' 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 as described by Andersen et al. (1991) and Abou-Donia et al. (2001). Time to release grip was recorded in seconds. The results of the two trials were averaged.

Beam-walking

The testing apparatus was a 2.5×122 cm wooden beam elevated 75.5 cm above the floor with wooden supports, as described by Goldstein (1993) and Abou-Donia et al. (2001). Beam-walking ability was measured with a seven point scoring system scale as previously described by Goldstein (1993): at score 1, the rat is unable to place the hindpaws on the horizontal surface of the beam; 2, the rat places the hindpaws on the horizontal surface of the beam and maintains balance for at least 5 s; 3, the rat traverses the beam while dragging the hindpaws; 4, the rat traverses the beam and at least once places a hindpaw on the horizontal surface of the beam; 5, the rat crosses the beam and places a hindpaw on the horizontal surface of the beam to aid less than half its steps; 6, the rat uses the hindpaws to aid more than half its steps and; 7, the rat traverses the beam with no more than two footslips. 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.

Statistical analyses

Data for the behavioral tests were compared among groups by one-way analysis of variance (ANOVA). If a significant difference was found, Fisher's LSD tests were applied to permit post hoc, pairwise comparisons. A two-tailed P-value of <0.05 was considered statistically significant.

Cholinesterase determination

AChE in brain regions and butyrylcholinesterase (BChE) activities in plasma were determined according to the method of Ellman et al. (1961) modified for assay in a UV Max Kinetic Microplate Reader (Molecular Devices, Sunnyvale, Calif., USA) as previously described (Abou-Donia et al. 1996; Khan et al. 2000). Protein concentration was determined by the BCA method according to Smith et al. (1985). The enzyme activities are expressed as micromoles substrate hydrolyzed per minute per milligram protein for brain regions and nanomoles substrate hydrolyzed per minute per milligram protein for plasma (percentage of control).

Nicotinic acetylcholine receptor (nAChR) ligand binding assay

[^{3}H]Cytisine at saturating concentration was used as specific ligand for binding studies with nAChR (Slotkin et al. 1999; Abou-Donia et al. 2001). The results are expressed as specific binding (femtomoles per milligram protein, as percentage of control).

Muscarinic acetylcholine receptor (mAChR) binding assay

The m2-mAChR binding assay was carried out by using the m2-selective ligand, [^{3}H]AF-DX 384 (Slotkin et al. 1999; Khan et al. 2000). The results are expressed as specific binding (femtomoles per milligram protein, as percentage of control).

Statistical analysis

The results were analyzed by one-way ANOVA, followed by Dunnett's multiple comparison test. A P-value <0.05 was considered significant.

Results

Clinical signs

The rats were observed daily for the development of clinical signs of toxicity throughout the treatment period. No rats died. All animals from control and treated groups gained weight. The rats treated with nicotine alone, or in combination with chlorpyrifos, had a higher but non-significant body weight gain compared with either those treated with chlorpyrifos alone or the controls. No sign of overt cholinergic toxicity was observed during the entire period of exposure in any group of animals.

Behavioral assessments

Except for incline plane performance (in which only the combination differed from control), each chemical or the combination severely impaired each behavioral measure (Fig. 1) (beam-walk score $F_{(3,15)}=90$, $P<0.00001$, ANOVA; beam walk time $F_{(3,15)}=20$, $P<0.00002$, ANOVA; incline plane $F_{(3,15)}=5$, $P=0.015$, ANOVA; grip time $F_{(3,15)}=8$, $P=0.002$, ANOVA).

Chlorpyrifos and nicotine alone impaired beam-walk scores and beam-walk times (all $P<0.001$ and $P<0.005$, respectively compared with controls). Exposure to chlorpyrifos alone and chlorpyrifos plus nicotine caused poorer performances on these tests than nicotine alone. There was no significant difference, however, in chlorpyrifos alone versus chlorpyrifos plus nicotine for these parameters. There was no significant change on incline plane performance induced by chlorpyrifos or nicotine treatment alone; however, rats that received both chemicals had poorer performances compared with either controls ($P<0.003$, Fisher LSD) or each chemical given alone ($P<0.04$). Grip time was severely impaired by each treatment, with no significant differences among those given chlorpyrifos alone, nicotine alone, and chlorpyrifos plus nicotine.

Effect of treatment with nicotine and chlorpyrifos, alone and in combination, on brain regional AChE and plasma BChE activity

Given alone, chlorpyrifos exposure did not result in a significant change in the AChE activity in the various brain regions (Fig. 2). There was a significant increase in AChE activity in brainstem and midbrain (~141 and 129% of control, respectively, $P<0.05$) following treatment with nicotine alone. Co-exposure with chlorpyrifos and nicotine caused a significant increase (~142% of control, $P<0.05$) in midbrain and a decrease in AChE activity in cerebellum (~84% of control, $P<0.05$). Plasma BChE activity was not changed by any treatment.

Effect of treatment with nicotine and chlorpyrifos, alone or in combination, on nAChR ligand binding in the cortex and brainstem

Exposure to nicotine or chlorpyrifos alone resulted in a significant increase in the ligand binding densities for

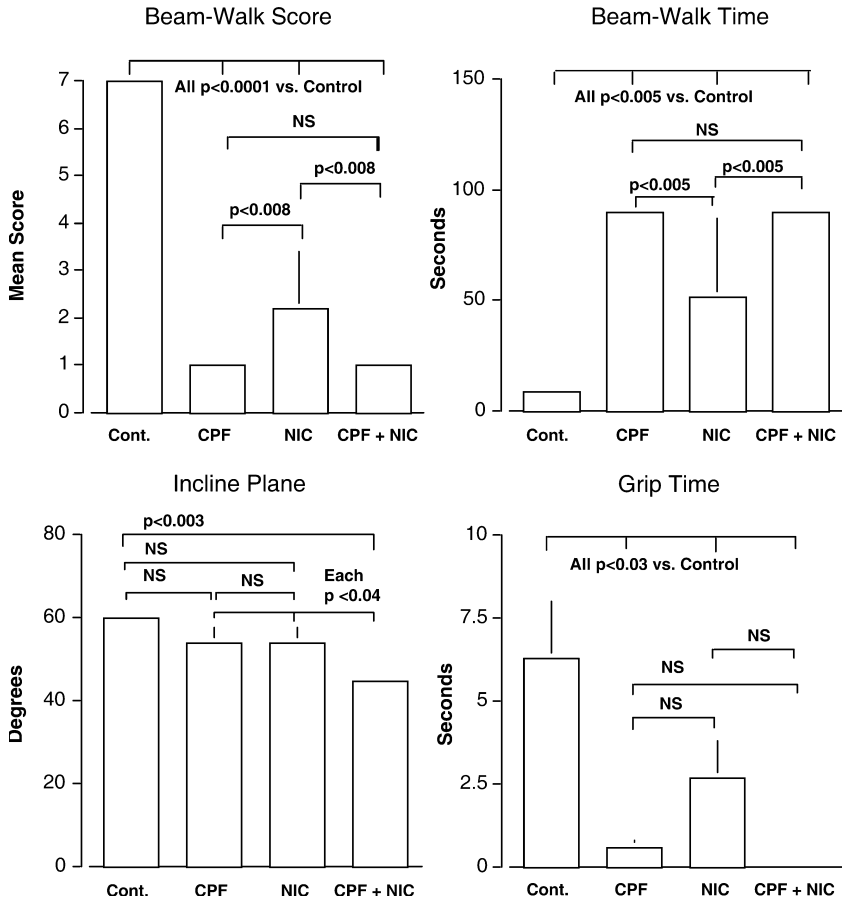

Fig. 1 Effect of treatment with chlorpyrifos and nicotine, alone or in combination on sensorimotor performance. Rats were treated with nicotine (*CPF*, 1 mg/kg s.c.) and chlorpyrifos (*NIC*, 0.1 mg/kg dermally), alone or in combination (*CPF+NIC*), for 30 days. Twenty-four hours after the last dose, the animals were tested for Beam-Walk Score, Beam-Walk Time, Incline Plane performance and Grip Time. At least one treatment on each behavioral test resulted in a significant impairment ($P < 0.05$, ANOVA, see text) as compared with controls (*Cont.*). The data are presented as means ± SE, $n = 5$. *P*-values represent post hoc tests by pair-wise Fisher LSD test (*NS* not significant)

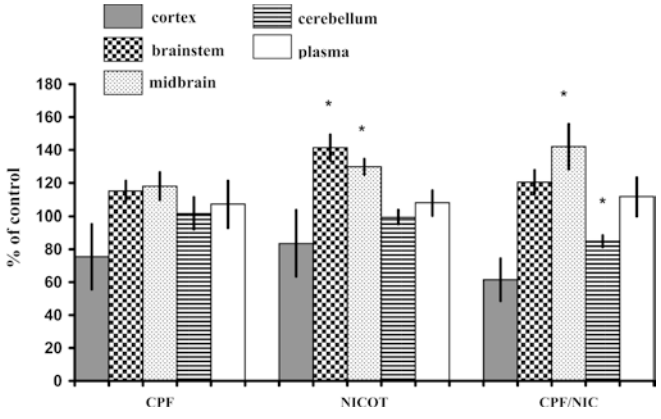

Fig. 2 Effect of treatment with chlorpyrifos and nicotine, alone or in combination, on acetylcholinesterase activity in different brain regions. Rats were treated with nicotine (*NICOT*, 1 mg/kg s.c.) and chlorpyrifos (*CPF*, 0.1 mg/kg dermally), alone and in combination (*CPF*/*NIC*), for 30 days. Twenty-four hours after the last dose, the animals were killed and enzyme activity in plasma and brain regions was determined. The control activity expressed as nanomoles acetylthiocholine hydrolyzed per minute per milligram protein was 860 ± 180 for cortex, 618 ± 70 for brainstem, 543 ± 29 for midbrain, and 239 ± 12 for cerebellum, and the plasma control activity was 21 ± 2 nmol butyrylthiocholine hydrolyzed/min per mg protein. Data are presented as means ± SE (percentage of control), $n = 5$. *Statistically significant compared with control

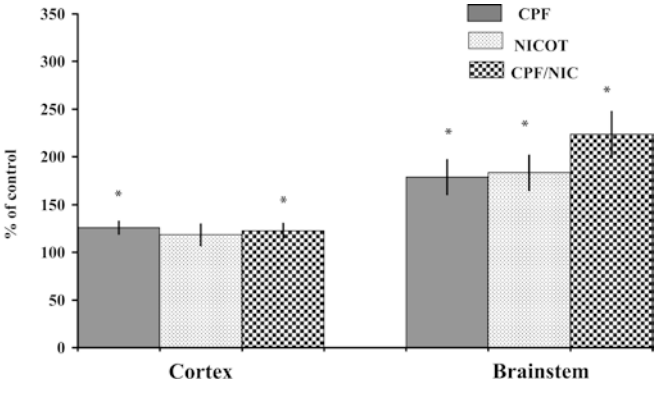

Fig. 3 Effect of treatment with chlorpyrifos and nicotine, alone or in combination, on nicotinic acetylcholine receptor ligand binding in the cortex and brainstem. Rats were treated with nicotine (*NICOT*, 1 mg/kg s.c.) and chlorpyrifos (*CPF*, 0.1 mg/kg dermally), alone and in combination (*CPF*/*NIC*), for 30 days. Twenty-four hours after the last dose, the animals were killed and ligand binding in the membrane fraction of brainstem and cortex was determined using [^{3}H]cytisine. The control [^{3}H]cytisine binding was 19.5 ± 0.62 and 6.9 ± 1.7 fmol/min per mg protein for cortex and brainstem, respectively. Data are presented as means ± SE (percentage of control), $n = 5$. *Statistically significant compared with control

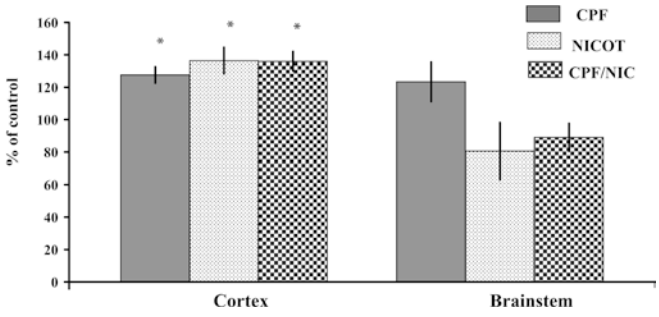

Fig. 4 Effect of treatment with chlorpyrifos and nicotine, alone or in combination, on m2 muscarinic acetylcholine receptor ligand binding in the cortex and brainstem. Rats were treated with nicotine (*NICOT*, 1 mg/kg s.c.) and chlorpyrifos (*CPF*, 0.1 mg/kg dermally), alone and in combination (*CPF*/*NIC*), for 30 days. Twenty-four hours after the last dose, the animals were killed and ligand binding was determined using [^{3}H]AF-DX 384. The control [^{3}H]AF-DX 384 binding was 855 ± 92 and 36 ± 9.1 fmol/min per mg protein for the cortex and brainstem, respectively. Data are presented as means $\pm$SE (percentage of control), $n = 5$. *Statistically significant compared with control

nAChR in the brainstem (~183 and 178% of control, respectively, $P < 0.05$; Fig. 3). Co-exposure to both agents resulted in a significant increase in the ligand binding in the cortex as well as brainstem (~123 and 223% of control, respectively, $P < 0.05$).

Effect of treatment with nicotine and chlorpyrifos, alone or in combination, on m2-mAChR ligand binding in the cortex and brainstem

Treatment with nicotine and chlorpyrifos, alone and in combination resulted in a significant increase in m2-mAChR ligand binding in the cortex (~127–135% of control, $P < 0.05$) (Fig. 4). No significant changes were observed in the brainstem following any treatment.

Discussion

Many individuals are exposed to a variety of chemicals at doses below the threshold that causes any acute toxicity. Tobacco smokers are directly exposed to nicotine. The same individuals may also be exposed to chlorpyrifos, either because of environmental contamination or occupational exposure. The present study was carried out to determine whether there is the possibility that neurotoxic effects following co-exposure to low doses of nicotine and chlorpyrifos could result in health consequences that are different from those caused by exposure to either chemical alone. Our results show that exposure to low doses of nicotine and chlorpyrifos given alone may cause neurobehavioral deficits as well as differential changes in the central cholinergic system, whereas combined exposure did not result in augmented deficits in most of the parameters studied.

We found that each chemical caused significant impairments of sensorimotor performance, presumably through the central cholinergic effects. However, previous work (Nostrandt et al. 1997) did not show a correlation between the extent of AChE inhibition and behavioral abnormalities. Although our studies were carried out using a low dose of chlorpyrifos (0.1 mg/kg for 30 days) by dermal application, the studies by Nostrandt et al. (1997) were carried out with oral dose of relatively higher doses of chlorpyrifos. High-dose nicotine exposure for 8–10 weeks has been shown to cause inhibition of AChE activity (Larsson et al. 1980). The low dose of nicotine (1 mg/kg s.c.) used in our studies may have caused metabolic changes leading to the increased activity. Repeated dosing with chlorpyrifos caused slow accumulation of AChE protein in rat brain (Chiappa, et al. 1995). Thus, it is possible that co-exposure to nicotine and chlorpyrifos might have resulted in neurobehavioral changes due to an increase in AChE. It has also been shown that increased AChE protein may reflect an increased axonal repair and synaptic modeling (Sternfeld et al. 1998). Therefore, it is also possible that an increased activity in midbrain following co-exposure to nicotine and chlorpyrifos may cause subtle changes that are reflected in increased synaptic modeling and repair.

Neuronal nicotinic receptors are large ligand-gated ion channels comprised of various combinations of α and β subunits (McGehee 1999). The predominant high affinity nicotinic receptor protein is composed of $\alpha4\beta2$ receptor subtypes (Flores et al. 1992), and the subunit composition determines the rate of desensitization following low-level nicotine exposure (Fenster et al. 1997). Upregulation of this receptor subtype is initiated by receptor desensitization after chronic exposure to nicotine (Fenster et al. 1999). It is believed that both acute and chronic nicotine treatment cause differential regulation of various nAChRs that may be relevant for the nicotine-induced tolerance, dependence, and withdrawal symptoms (Dani and Heinmann 1996). Consistent with the published reports (Benwell et al. 1988; Slotkin et al. 1999), we also observed a significant increase in the ligand binding for $\alpha4\beta2$ nAChR subtype (Fig. 3). It is also possible that other subtypes, such as $\alpha7$ may also be upregulated, as reported earlier (Hellstrom-Lindahl et al. 2001), because [^{3}H]cytisine has highest affinity for nicotinic acetylcholine receptors containing $\alpha4\beta2$ subunits.

Our results also show that treatment with chlorpyrifos, alone or in combination with nicotine, resulted in an increased ligand binding for m2-mAChR in the cortex, whereas treatment with nicotine alone did not cause a significant change (Fig. 4). We have previously shown that chlorpyrifos and its bioactivated species (chlorpyrifos oxon) bind directly to m2-mAChR, and activate down stream signaling events (Huff et al. 1994). Studies by Ward et al. (1993) have also shown that organophosphate anticholinesterases selectively regulate m2-mAChR ligand binding. Chaudhuri et al. (1993) reported an increased m2-mAChR ligand binding in response to chlorpyrifos exposure. It is known that

treatment with muscarinic antagonists induce receptor upregulation (Ben-Barak and Dudai 1980). Wang et al. (1996) reported an increase in muscarinic receptor ligand binding by repeated exposure to nicotine. Increased ligand binding for m2 muscarinic receptor results in inhibition of adenylate cyclase activity through a pertussis toxin-sensitive G protein, resulting in an inhibitory postsynaptic response (Wess 1996). Increased m2-mAChR receptor ligand binding density in the cortex in response to treatment with chlorpyrifos, alone and in combination with nicotine, could also reflect a compensatory mechanism for a reduced ability of these receptors to bind their respective ligands due to desensitiztion.

In summary, these results suggest that daily exposure for 30 days to low-dose chlorpyrifos and/or nicotine results in sensorimotor impairments with co-exposure causing a deficit in incline plane performance not present when each chemical is given alone. These changes may be related to the capacity of these chemicals to directly affect the cholinergic system in a brain region-specific manner. Furthermore, our data show that sub-chronic exposure to low doses of nicotine and chlorpyrifos, alone or in combination, may lead to a differential alteration in the cholinergic system that may be relevant in assessing the health risk associated with exposure to each of these agents in general population. Further work is necessary in order to elucidate the mechanism of neurobehavioral deficits following exposure to these compounds.

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