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Imidacloprid Induces Neurobehavioral Deficits and Increases Expression of Glial Fibrillary Acidic Protein in the Motor Cortex and Hippocampus in Offspring Rats Following in Utero Exposure

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Imidacloprid, a neonicotinoid, is one of the fastest growing insecticides in use worldwide because of its selectivity for insects. The potential for neurotoxicity following in utero exposure to imidacloprid is not known. Timed pregnant Sprague-Dawley rats (300–350 g) on d 9 of gestation were treated with a single intraperitoneal injection (ip) of imidacloprid (337 mg/kg, 0.75 × LD_{50}, in corn oil). Control rats were treated with corn oil. On postnatal day (PND) 30, all male and female offspring were evaluated for (a) acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) activity, (b) ligand binding for nicotinic acetylcholine receptors (nAChR) and muscarinic acetylcholine receptors (m2 mAChR), (c) sensorimotor performance (inclined plane, beam-walking, and forepaw grip), and (d) pathological alterations in the brain (using cresyl violet and glial fibrillary acidic protein [GFAP] immunostaining). The offspring of treated mothers exhibited significant sensorimotor impairments at PND 30 during behavioral assessments. These changes were associated with increased AChE activity in the midbrain, cortex and brainstem (125–145% increase) and in plasma (125% increase). Ligand binding densities for [^{3}H]cytosine for α4β2 type nAchR did not show any significant change, whereas [^{3}H]AFDX 384, a ligand for m2mAChR, was significantly increased in the cortex of offspring (120–155% increase) of imidacloprid-treated mothers. Histopathological evaluation using cresyl violet staining did not show any alteration in surviving neurons in various brain regions. On the other hand, there was a rise in GFAP immunostaining in motor cortex layer III, CA1, CA3, and the dentate gyrus subfield of the hippocampus of offspring of imidacloprid-treated mothers. The results indicate that gestational exposure to a single large, nonlethal, dose of imidacloprid produces significant neurobehavioral deficits and an increased expression of GFAP in several brain regions of the offspring on PND 30, corresponding to a human early adolescent age. These changes may have long-term adverse health effects in the offspring.

Environmental exposure to neurotoxic chemicals during fetal development produces a cascade of effects in developing children, families and society, because of persistent neurological abnormalities of the central nervous system. Epidemiological studies have shown neurobehavioral and cognitive deficits and increased susceptibility to disease in offspring at various developmental stages, all ass ociated with maternal exposure to neurotoxic chemicals during pregnancy (Jacobson & Jacobson, 2002; Makri et al., 20 04; Berkowitz et al., 2003; Hanke & Jurewicz, 2004).

Imidacloprid was introduced in 1999 as the first member of a new generation of in secticides known as neonicotinoids. It acts as an agonist at the postsynaptic nicotinic acetylcholine receptor (nAChR) in insects (Tomizawa & Yamamoto, 1993; Tomizawa ˆ Casida, 2005). Nicotinic acetylcholine receptors that play important roles in sy naptic transmission in the central nervous system (CNS) belong to a superfamily of l igand-gated ion channels (Albuquerque et al., 1997; Dani & Biasi, 2001). There are three classes of nAChRs: (1) muscle subunits (α1, β1, γ, δ, ε); (2) neuronal αβ combinations of subunits (α2–α6 and β2–β4), including α4β2 (heteropentameric), which is the most abundant form

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of nAChR in the CNS; and (3) subunits (α7–α9) that are homomeric (and are inhibited by α-bungarotoxin), including α-7 cAChR (homopentameric), which is widely distributed in the CNS and is specifically involved in neuritic outgrowth (Girod et al., 1999).

Recent reports showed that many diseases involving nAChR dysfunction result from perturbation of cholinergic nicotinic neurotransmission (Cotti & Clementi, 2004). Activation of α7 nAChRs during development results in neuronal cell death, whereas stimulation in adult animals is neuroprotective (Laudenbach et al., 2002). Exposure of fetal brain to nicotine was found to increase the number of α7 nAChRs (Narayanan et al., 2002). Administration of nicotine to adolescent rats (30–45 d old) resulted in upregulation of α7 nAChRs but then a decrease to normal or subnormal levels occurred after stopping treatment at 50 d and through 75 d of age (Slotkin et al., 2004). Neonatal (postnatal days [PNDs] 4–9) nicotine exposure was shown to alter functional activity in the hippocampus of adult rats by Slawecki et al. (2000), who suggested that these changes were most likely produced by disorganization in the hippocampus and thus that neonatal exposure to nicotine elicits teratogenic effects on the hippocampus that persist into adulthood.

Imidacloprid is extensively used for both crop protection against termites, piercing-sucking pests, as well in animal health care, and is one of the fastest growing in sales of any insecticide worldwide because of its selectivity for insects and apparent safety for humans (Kagabu, 1997; Matsuda et al., 2001; Tomizawa & Casida, 2005). Matsuda et al., 2001). The selective toxicity of imidacloprid results from its higher affinity for insect nAChR compared to mammals (Liu & Casida, 1993; Chao & Casida, 1997; Zhang et al., 2000). Imidacloprid selectively binds to insect nAChR with a putative cationic subsite in the insect nAChR through its nitro group, compared to the anionic site in mammalian nAChR (Chao & Casida, 1997, Yamamoto et al., 1998; Tomizawa et al., 2000). A recent study in M10 cells expressing α4β2 nAChR showed that treatment with imidacloprid and its imine derivative induced upregulation of α4β2 nAChR, suggesting that these neonicotinoids act as agonists for nAChR (Tomizawa & Casida, 2000). One case of acute poisoning in humans was reported recently, following ingestion of an insecticide formulation containing ~10% imidacloprid (Wu et al., 2001), and two fatalities have resulted from imidacloprid intoxication (Proenca et al., 2005). Thus, increased potential neurotoxic hazards from imidacloprid may result from (1) global increase in the use of imidacloprid, (2) persistence in crops, vegetables and fruits, and (3) proximity to treated pets. Furthermore, the potential for neurotoxicity in humans resulting from developmental exposure is not known. In the present study, rat offspring on postnatal day (PND) 30 were evaluated for behavioral, biochemical, and neuropathological alterations following maternal exposure on gestational day (GD) 9 to a single large sublethal dose of imidacloprid.

MATERIALS AND METHODS

Imidacloprid (~99.5% pure) was obtained from Chem Service (West Chester, PA). [^{3}H]cytosine (specific activity 15 Ci/mmol) and [^{3}H]AF-DX 384[2,3dipropylamino-^{3}H] (specific activity 100 Ci/mmol) were obtained from Perkin Elmer Life Sciences (Boston). A polyclonal antibody against glial fibrillary acidic protein (GFAP) was obtained from Dako Laboratories (Carpinteria, CA). Reagent kits for avidin-biotin-peroxidase were obtained from Vector Laboratories (Burlingame, CA).

Methods

Timed pregnant Sprague-Dawley rats (300–350 g) were obtained from Zivic-Miller Laboratories (Allison Park, PA) and housed in the Duke University Medical Center vivarium in a temperature controlled room (21–22°C) with a 12-h dark–light cycle. The animals were supplied with Ralston Purina feed (St. Louis, MO) and water ad libitum. All treatments and procedures were carried out according to Duke University Medical Center Institutional Animal Care and Use Committee guidelines.

Treatment

Pregnant rats were randomly divided into control and imidacloprid-treated groups of 5 rats each, and treated as follows: (I) Controls: Maternal rats were treated with a single ip injection of corn oil on GD 9. (II) Imidacloprid-treated maternal rats were treated with a single ip injection of imidacloprid (337 mg/kg, in corn oil, 75% × LD_{50}) on GD 9. Animals were observed for overt signs of toxicity, such as weight change, changes in eating and drinking, locomotor changes, timing of birth, and recorded seizures. In the present study, the dose was chosen to simulate an acute, high-level, sublethal exposure scenario. Following parturition, the number and weight of the pups were recorded and then they were reared by their respective mothers. Because there were no significant differences in the litter size between the treated and control mothers, no culling was done. In experimental studies, rats at different ages are used to reflect different human developmental stages as follows: neonate, PND 4–9; preadolescence, PND 10–29; adolescence, PND 30–45; young adult, PND 46–60; and adult, PND 61–90 (Liptakova et al. 2000; Slotkin et al., 2004). The offspring were evaluated using various behavioral, biochemical, and pathological parameters on PND 30. Behavioral evaluations were carried out on 10 males and 10 females from the control and treated groups (n=10). Out of the 10 males and 10 females from each group used for behavioral studies, 5 offspring females and 5 males were used for the biochemical assays and 5 females and 5 males were used

for the pathology assessments. Thus, there was an n=5 for the biochemical and n= 5 for the pathological evaluations, with each representing offspring from 5 different mothers.

For the biochemical assays on PND 30, the male and female offspring were anesthetized with ketamine (50 mg/kg)/xylazine (10 mg/kg) and blood was drawn in a heparinized syringe, followed by decapitation. 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

The behavioral tests employed in these studies evaluate sensorimotor reflexes, motor strength, and coordination. All behavioral testing was performed by an observer who was 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).

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., 2001a, 2001b, 2004, 2006). Inclined plane performance was measured with a standard protractor to the nearest 5 degrees. A trial ended when the rat began to slip backward, therefore there was no specific trial duration. The angle at which the rat began to slip downward was recorded. The results of the two trials were averaged. Trials were separated by 1 h.

Forepaw grip time. The rat's forepaw strength was assessed by having it grip a 5-mm-diameter wood dowel that was held horizontally and raised so that the animal supported its body weight, as described by Andersen et al. (1991) and Abou-Donia et al. (2001a, 2001b). Time-to-release-grip was recorded in seconds. All rats attempted to grip the dowel during this grip strength testing. The results of the two trials were averaged. Trials were separated by 1 h.

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. (2001a, 2001b, 2004, 2006). Beam-walking ability was measured using a 7-point scale previously described by Goldstein (1993): (1) The rat was unable to place the hind paws on the horizontal surface of the beam; (2) the rat placed its hind paws on the horizontal surface of the beam and maintained its balance for at least 5 s; (3) the rat traversed the beam while dragging its hind-paws; (4) the rat traversed the beam and at least once placed a hind paw on the horizontal surface of the beam; (5) the rat crossed the beam and placed a hind paw on the horizontal surface of the beam to aid less than half its steps; (6) the rat used the hind paws to aid more than half its steps; and (7) the rat traversed 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 were based on an average of 5 trials, each separated by 1 h.

Statistical analyses. Data were compared among groups using a one-way analysis of variance (ANOVA). A p value of $<.05$ was considered statistically significant.

Biochemical Studies

Cholinesterase determination. Butyrylcholinesterase (BChE) activity in the plasma and acetylcholinesterase (AChE) activity in brain regions were determined according to the method of Ellman et al. (1961). To assess AChE activity, brain samples were homogenized in Ellman buffer, centrifuged for 5 min at 5000 × g, and the resulting supernatant used for AChE analysis. AChE activity was measured using acetylthiocholine as the substrate in a Molecular Devices UV Max Kinetic microplate reader at 412 nm, as described by Abou-Donia et al. (2004, 2006). The enzyme activities are expressed as nanomoles of substrate hydrolyzed per minute per milligram protein. Protein concentration was determined by the method of Smith et al. (1985).

Nicotinic acetylcholine receptor (nAChR) ligand binding assay. A single saturating concentration of [^{3}H]cytisine as the ligand for α4β2 nAChR was used to measure the ligand binding density in the membrane preparations in the cortex and midbrain of offspring from both control and imidocloprid-treated mothers. [^{3}H]Cytisine was used as the specific ligand for the α4β2 form of nAChR at 4°C for 75 min (Khan et al., 2000). Briefly, the tissue was homogenized by polytron in 50 mM Tris-HCl, pH 7.4, containing 120 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$ and 2.5 mM $MgCl_2$. The membranes were sedimented by centrifuging at 40,000 × g for 10 min. The resulting membrane pellet was resuspended in the same buffer, using a Teflon pestle and a glass homogenizer in a volume sufficient to give 1.5–2 mg/ml protein. An aliquot of membrane preparation containing ~200 µg protein was used to carry out the incubation with 1 nM [^{3}H]cytisine The labeled membranes were trapped on membrane filters using a rapid vacuum filtration system and the results were expressed as specific binding (fmol/mg protein, as percent of control).

Muscarinic acetylcholine receptor ligand binding. Muscarinic acetylcholine receptor (mAChR) ligand binding density assessment was carried out using an m2mAChR-specific ligand, [^{3}H]AF-DX, as described by Huff et al. (1994; Huff & Abou-Donia, 1995). Briefly, the tissue was homogenized by polytron in 10 mM phosphate buffer, pH 7.4, and the membranes were sedimented by centrifuging at 40,000 × g for 10 min. The resulting membrane pellet was resuspended in the same buffer at 1.5–2.5 mg/ml protein concentration. An aliquot of membrane preparation containing ~200 µg protein was used to carry out the incubation at room temperature for 60 min with 1 n–[^{3}H]AF-DX. Nonspecific binding was carried out in the presence of 2.2 µM atropine sulfate. Ligand-bound membranes were trapped on membrane filters presoaked with 0.1% polyethyleimine using a rapid vacuum filtration system and the results were expressed as specific binding (fmol/mg protein, as percent of control).

Statistical analysis. The results were analyzed by one-way ANOVA. A p value <.05 was considered significant.

Neuropathological Studies

Male and female offspring (n=5, each) on the PND 30 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 and postfixed in 4% paraformaldehyde at 4°C for 18–24 h. The tissues were then blocked and embedded in paraffin according to standard histological techniques. Six-micrometer-thick coronal sections were cut through different brain regions. From every brain, representative coronal sections (n=5) through the motor cortex, the septal hippocampus, and the cerebellum were stained with cresyl violet for light microscopic examination, according to the procedures established in our laboratory (Abdel-Rahman et al., 2001, 2003).

Evaluation of astroglial alteration was carried out using polyclonal antibodies against GFAP (Abdel-Rahman et al., 2001). The 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 in 50 m*M* Tris-buffered saline (TBS) containing 1% normal goat serum. The sections were rinsed thoroughly with 50 m*M* TBS and incubated with biotinylated goat anti-rabbit immunoglobulin (Ig) G at 1:200 containing 1% normal goat serum for 1 h at room temperature. Following extensive rinsing with TBS, the sections were incubated for 1 h in an 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 using several rinses with TBS. The sections were then dehydrated in alcohol, cleared in xylene, and coverslipped with Permount for observation and analysis.

The quantification (n=5 sections from each rat, 5 rats in each group) of surviving neurons and GFAP immunoreactivity in the motor cortex and hippocampus subfields, surviving Purkinje neurons in the cerebellum, and GFAP immunoreactivity of neighboring sections from cerebellar white matter as well as in the granular cell layer was carried out according to previously described procedures (Abdel-Rahman et al., 2001). Briefly, the numerical density of surviving 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, the sections originating from the same animal were identified. The measurements were performed using a Nikon E600 microscope equipped with an eyepiece grid. Surviving neurons were counted within a unit area of each section at 400 × magnification. The unit area selected for measurement was 0.013 mm^2 for the motor cortex and 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 mm of each region.

GFAP-immunoreactive structure quantification in motor cortex layers III and V, the hippocampus subfields, and the granular cell layer and white matter of the cerebellum was performed using Scion Image for Windows, based on NIH Image for Macintosh (Scion Corporation, Fredrick, MD). Two sections were measured for each animal for each brain region. 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 on the appropriate area of immunostained section with a Nikon E600 microscope equipped with a digital camera connected to an IBM computer, as described in detail by Abdel-Rahman et al. (2001).

Statistical analysis. The mean values for each of the five brain regions were calculated separately for each animal (male and female) using the data from five sections each, before the mean and standard errors were determined for the total number of animals (n=5) included per group. The mean values between different groups of animals were compared separately for each of the five brain regions using a one-way ANOVA with a Student–Newman–Keuls multiple comparison post hoc test. A p value <.05 was considered significant.

RESULTS

There was no mortality among the mothers or the offspring in any group. There was no significant difference in the litter size or weight gain in the offspring between control and imidacloprid-treated mothers. The mothers were observed for signs of toxicity following treatment with imidacloprid. No overt sign of toxicity was observed in any group of mothers or offspring during the experimental period.

Effects on Neurobehavioral Performance in the Offspring

The data for beam-walk time, beam-walk score, inclined plane, and grip time from male offspring of mothers exposed to imidacloprid during gestation are presented in Figure 1. Beam-walk time showed a significant deficit following maternal treatment with imidacloprid (Figure 1A). There was no difference in beam-walk scores. The inclined plane test showed a significant effect following imidacloprid treatment (Figure 1B). Grip time also showed a significant deficit (Figure 1C). Similar results were obtained for female offspring of treated mothers. There were no gender differences in control animals. These results indicate that PND-30 offspring from mothers treated with imidacloprid had sensorimotor deficits.

Effects on Brain and Plasma Cholinesterase Activity in the Offspring

Brain regional AChE and plasma BChE activities are presented in Figure 2. In the cerebellum, there were no

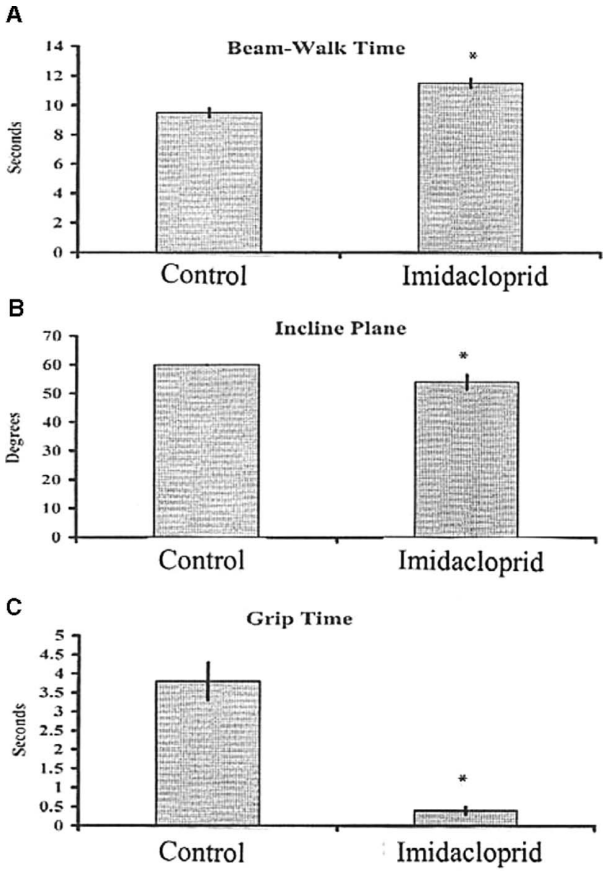

FIG. 1. Neurobehavioral evaluations of the male offspring on PND 30 following maternal exposure to imidacloprid. Timed pregnant rats were treated with a single ip injection of 33 7mg/kg imidacloprid in corn oil on GD 9. Neurobehavioral evaluations included beam-walk time (A), inclined plane performance (B), and grip time (C). Values represent mean ± SE (n=20/group). Analysis with a one-way ANOVA revealed that all of the above behavioral tests showed significant impairment (indicated by asterisks) compared to vehicle-treated controls, at $p < .05$.

significant changes in AChE activity (Figure 2A). Data demonstrated that midbrain and cortex AChE levels increased significantly in the male offspring of treated mothers (Figure 2, B and C). Female offspring from imidacloprid treated mothers showed a significant rise in AChE activity in the cortex and brainstem (Figure 2, C and D). Control enzyme activities expressed as nmoles of substrate hydrolyzed/min/mg protein were: males (cortex, 84.79 ± 6.23; brainstem, 39.13 ± 4.08; midbrain, 32.45 ± 1.6; cerebellum, 62.28 ± 3.78); females (cortex, 40.73 ± 5.6; brainstem, 82.03 ± 2.4; midbrain, 54.19 ± 9.83; cerebellum, 64.11 ± 5.63). Plasma BChE activity in the male offspring of mothers treated with imidacloprid showed a significant increase and there was a significant gender effect (Figure2E). The control enzyme activity in the males was 0.0104 ± 0.006 and in the females was 0.0125 ± 0.003 nmol substrate hydrolyzed/min/mg protein.

Effects on nAChR and m2mAChR Ligand Binding in the Offspring

The results for the nAChR and m2mAChR ligand binding densities are presented in Figure 3. No significant changes were observed in α4β2 nAChR ligand binding using [^{3}H]cytosine (Figure 3A). Both male and female offspring of imidacloprid-treated mothers showed a significant rise in the ligand binding for m2mAChR in the cortex and midbrain (Figure 3B). Ligand binding results for m2mAChR in the membrane preparations from control rats were: male cortex, 198.12 ± 3.25; male midbrain, 99.22 ± 6.32; female cortex, 147.92 ± 4.47; female midbrain, 100.44 ± 9.81 fmol/mg protein.

Histopathological Changes in the Motor Cortex of the Offspring

Histopathological changes in the motor cortex of the male offspring of imidacloprid- treated mothers on gestational day 9 are presented in Figure 4A. A1 and A2 are examples of cresyl violet staining, and B1 and B2 are examples of GFAP immunoassaying in the motor cortex layers I–VI. A1 and B1 are examples of control animals, and A2 and B2 are examples of treated animals. No neuronal degeneration was observed in layers III and V of the motor cortex in the male offspring of imidacloprid-treated mothers compared to controls (A2). The adjacent sections stained for GFAP, however, revealed a significant increase in GFAP immunostaining in male offspring from imidacloprid-treated mothers in comparison to their controls (B2). Similar pathological changes were observed in the female offspring. Figure 4, B and C, shows highly magnified pictures (20×) of Figure 4A. The densities of the surviving neurons in layers III (A) and V (C) are presented in Figure 4D. On the other hand, a significant rise in the GFAP immunoassay was seen in layers III (B) and V (D) of the offspring of imidacloprid-treated mothers (2607 ± 216, controls; 4187 ± 305, treated), expressed as GFAP immunoreactivity/0.176 mm^2. Similarly, GFAP immunoassaying in layer III of the female offspring of imidacloprid-treated mothers showed a significant increase (2701 ± 449, controls; 4132 ± 369, treated), expressed as GFAP immunoreactivity/0.176 mm^2 (Figure 4C).

Histopathological Changes in the Hippocampus of the Offspring

Histopathological alterations in the hippocampus of male offspring are presented in Figure 5. Figure 5A, panels A1–A2, shows examples of cresyl violet-stained (CV) sections of the septal hippocampus. Panels B1 and B2 show examples of GFAP immunostaining. A1 and B1 are examples from male controls, and A2 and B2 are examples from the male offspring of imidacloprid-treated mothers. No neuronal degeneration was observed in any regions of the hippocampus of male imidacloprid-treated mothers (A2) or controls (A1).

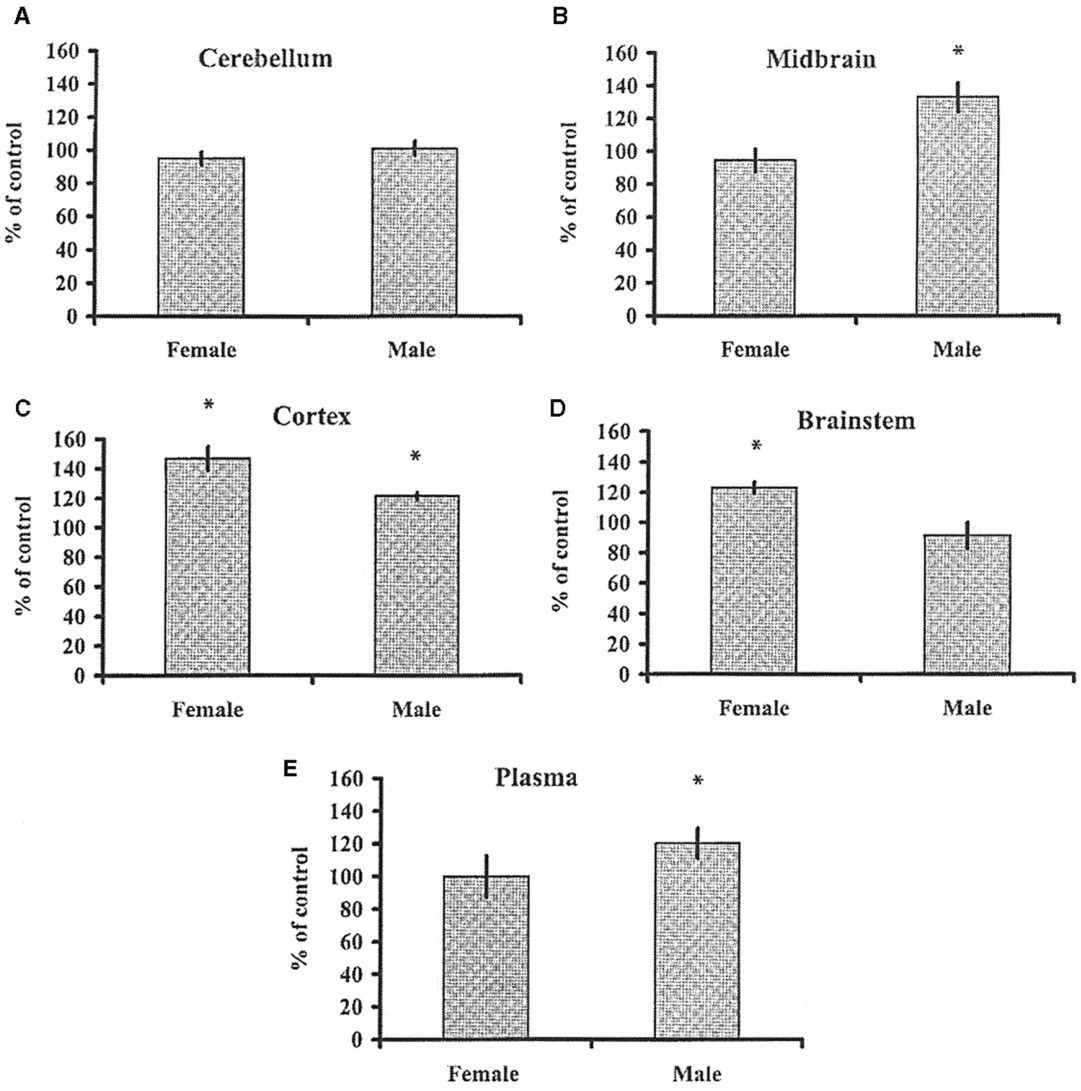

FIG. 2. Regional brain and plasma cholinesterase activity of the offspring on PND 30 following maternal exposure to imidacloprid. Timed pregnant rats were treated with a single ip injection of 337 mg/kg imidacloprid in corn oil on GD 9. Offspring regional brain acetylcholinesterase (AChE) activity are shown in the (A) cerebellum, (B) midbrain, (C) cortex, and (D) brainstem, as well as (E) plasma (ChE) activity. Values represent the mean plus standard error (n=5/group). Analysis with a one-way ANOVA revealed significant differences between the groups in both brain regions and plasma. The post hoc analysis revealed that male offspring of mothers treated with imidacloprid exhibited a significant increase in AChE in the midbrain and cortex; however, female animals exhibited a significant elevation in the cortex and brainstem (indicated by asterisks), at $p < .05$.

Immunostaining of neighboring sections for GFAP, however, demonstrated enhanced GFAP immunoreactivity in male imidacloprid-treated mothers (B2). The results (at high magnification) are presented in Figure 5B for the dentate gyrus, in Figure 5C for the CA1 subfield, and in Figure 5D for CA3. Similar pathological changes were observed in the female offspring (data not shown). Quantitative analyses of cresyl violet staining and GFAP immunostaining are presented in Figure 6. The histograms show the density of the surviving neurons: (A) in the dentate gyrus; (C) in the CA1 subfield; and (E) in the CA3 pyramidal neurons. The histograms (B, D, and G) show GFAP-immunoreactive elements in the dentate gyrus (B), CA1 (D) and CA3 (G). There was a significant increase in GFAP immunostaining in the dentate gyrus (3984 ± 195, controls; 4901 ± 198, imidacloprid-treated) expressed as GFAP immunoreactivity/0.176 mm^2, as well as in CA1 (3307 ± 330, controls; 4353 ± 185, imidacloprid-treated) expressed as GFAP immunoreactivity/0.176 mm^2 and CA3 (4322 ± 216, controls; 5021 ± 103, imidacloprid-treated) expressed as GFAP immunoreactivity/0.176 mm^2, in the offspring of the imidacloprid-treated mothers. Similarly, GFAP immunostaining of samples from the female offspring of imidacloprid-treated mothers showed a significant rise in the dentate gyrus (3799 ± 225, controls; 4744 ± 209, imidacloprid-treated) expressed as GFAP

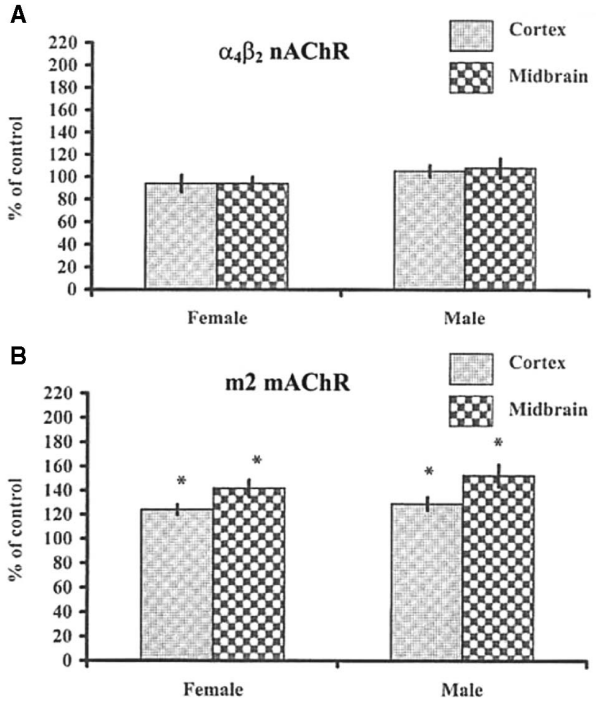

FIG. 3. Nicotinic (α4β2 nAChR) and muscarinic acetylcholine receptors (m2 mAChR) ligand binding in the cortex and midbrain of the offspring: Timed pregnant rats were treated with a single ip injection of 337 mg/kg imidacloprid in corn oil on GD 9. The cortex and midbrain from the pups were dissected and [^{3}H]cytisine and [^{3}H]AF-DX ligand bindings for (A) α4β2 nAChR and (B) m2 mAChR were evaluated. Data are presented as mean ± SEM (percent of control). Analysis with one-way ANOVA revealed significant differences between the groups only in the m2 mAChR receptor ($p < .05$). The post hoc analysis revealed that both male and female offspring exhibited a significant increase in m2 mAChR compared to vehicle-treated controls. However, α4β2 nAChR did not show any significant differences in comparison to the control animals. Asterisk indicates statistically significant difference from controls ($p < .05$), n=5 offspring (1 from each mother).

immunoreactivity/0.176 mm^2), and CA1 (3154 ±399, controls; 4568 ± 234, imidacloprid-treated) expressed as GFAP immunoreactivity/0.176 mm^2, and CA3 (4141 ± 102, controls; 4795 ± 196, treated) expressed as GFAP immunoreactivity/0.176 mm^2 (Figure 5 D).

Histopathological Changes in the Cerebellum of the Offspring

Male and female offspring of the treated mothers did not exhibit any significant changes in the density of surviving neurons or in GFAP immunoreactivity in the cerebellum compared to controls (results not shown).

DISCUSSION

The results of the present study show that gestational administration on GD 9 of a single large sublethal dose of imidacloprid produced neurobehavioral deficits that were evident on PND 30 (corresponding to adolescent human age) in these male and female offspring. A significant rise in AChE activity was observed in different brain regions and in plasma BChE activity, and a significant rise in m2mAChR ligand binding density in the cortex and midbrain of the offspring of imidacloprid-treated mothers. There were no significant changes in the number of surviving neurons in the motor cortex, hippocampus, or cerebellum. In contrast, there was an increase in GFAP immunoreactivity in motor cortex layer III, the hippocampus subfields, the dentate gyrus, and CA1 and CA3 in the offspring of the treated rats.

Widespread use of imidacloprid and its selectivity toward insect neuronal acetylcholine receptors suggested that this compound may be safe for the mammalian nervous system. Our results, however, show that maternal exposure to a large sublethal dose of imidacloprid during gestation produced significant sensorimotor impairments that were reflected in beam walk time, inclined plane performance, and forepaw grip in the offspring on PND 30. These neurobehavioral deficits may reflect dysfunction at multiple anatomical areas in the central nervous system, peripheral nervous system, or muscle. These effects are mediated by a complex array of multiple pathways. For example, beam walking is an integrated form of behavior necessitating pertinent levels of consciousness, memory, and sensorimotor, cortical, subcortical, cerebellar, spinal cord, peripheral nervous system, neuromuscular junction, and muscular functions (Abou-Donia et al., 2001a). The biochemical and pathological changes seen suggest that multiple brain region abnormalities may be involved in the sensorimotor deficits in the offspring due to maternal exposure to imidacloprid.

Imidacloprid, like other neonicotinoid insecticides, acts as an agonist at the postsynaptic nicotinic acetylcholine receptor (nAChR) of insects (Tomizawa & Yamamoto, 1993; Tomizawa & Casida, 2005). Most animal studies involving imidacloprid focused on effects on nicotinic acetylcholine receptors such as α4β2 nAChR, because of selective targeting of nAChR (Lansdell & Millar, 2000; Tomizawa & Casida, 2000, 2003). Midbrain and cortex AChE activity was significantly increased in these male offspring, whereas cortex and brainstem AChE activity showed significant elevation in the female offspring of imidacloprid-treated rats. Increased AChE activity in various brain regions may contribute to the neurotoxic effects in the offspring resulting from decreased ACh and less than optimal function of ACh receptors. Furthermore, studies showed that increased expression of AChE produces neurodegeneration in vivo and in vitro (Sberna et al., 1998; Yang et al., 2002; Day & Greenfield, 2003). This possible mechanism involves influx of calcium ions following activation of α7 nAChR and induction of neuronal apoptosis (Day & Greenfield, 2000; Zbarsky et al., 2004). In addition, it was suggested that an increase in AChE protein may reflect enhanced axonal repair and synaptic

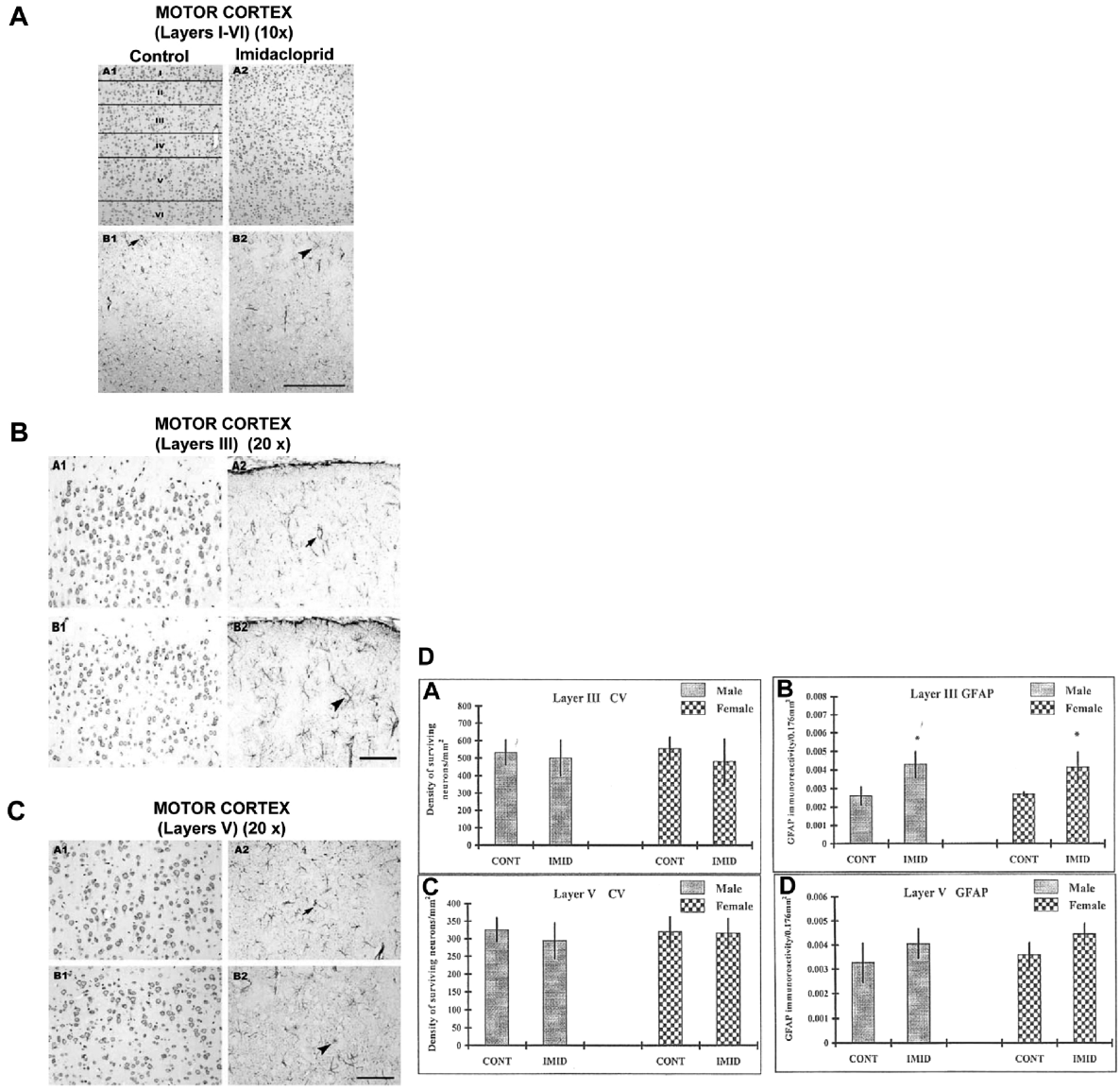

FIG. 4. (A) Histopathological alterations in the motor cortex (layers I–VI) of the offspring following maternal exposure to imidacloprid. (A1–A2) cresyl violet (CV) staining, (B1–B2) GFAP immunostaining. (A1, B1) Examples from control animals; (A2, B2) examples from one pup after maternal exposure to a single (ip) injection of imidacloprid (IMI). Note that the offspring of imidacloprid treated mothers did not show any change in the form of degenerating neurons (A2). However, the GFAP immunoreactivity of astrocytes is significantly enhanced (arrows in B2). Scale bar=200 µm. (B) Highly magnified (20 ×) alterations in the superficial layers (layers I–III) of the motor cortex. Scale bar=200 µm. (C) 20× Magnification of the alterations in the deeper layers of layers I-III of the motor cortex. Scale bar=200 µm. (D) Histogram showing the density of surviving neurons (A1 and A2) per square millimeter in layers III and V of the motor cortex. Values represent mean plus standard error (n = 5 per group). The histograms in (A) and (C) show the density of the surviving neurons in layer III and layer V of the motor cortex. (B) and (D) show the area of GFAP-immunoreactive elements (in mm^2) per unit area (0.176 mm^2) of layers III and V of the motor cortex. Values represent the mean plus standard error (n = 5 per group). There is significant upregulation in GFAP positive elements ($p < .05$) in both male or female offspring following maternal exposure to a single ip injection of imidacloprid. Asterisk indicates statistically significantly different from controls.

modeling (Guizzetti et al., 1996; Sternfeld et al., 1998; Bigbee et al., 2000).

The lack of effect of imidacloprid on ligand binding for α4β2 nAChR is consistent with previous reports (Buckingham et al., 1997; Tomizawa et al ., 2000; Tomizawa & Casida, 2003), although studies by Tomizawa and Casida (2000) found that imidacloprid and its imine derivative upregulated α4β2 nAChR in M10 m ouse fibroblast cells. An increase in the ligand binding density for m2 mAChR in the co rtex and midbrain of offspring following gestational exposure to imidacloprid suggests a role for neuronal m2mAChR in its neurotoxicity. Muscarinic acetylcholine receptors (mAChR) are known to play important roles in f acilitating memory and cognition (Coyle et al., 1993; Iversen, 1997). The mAChR in the CNS is

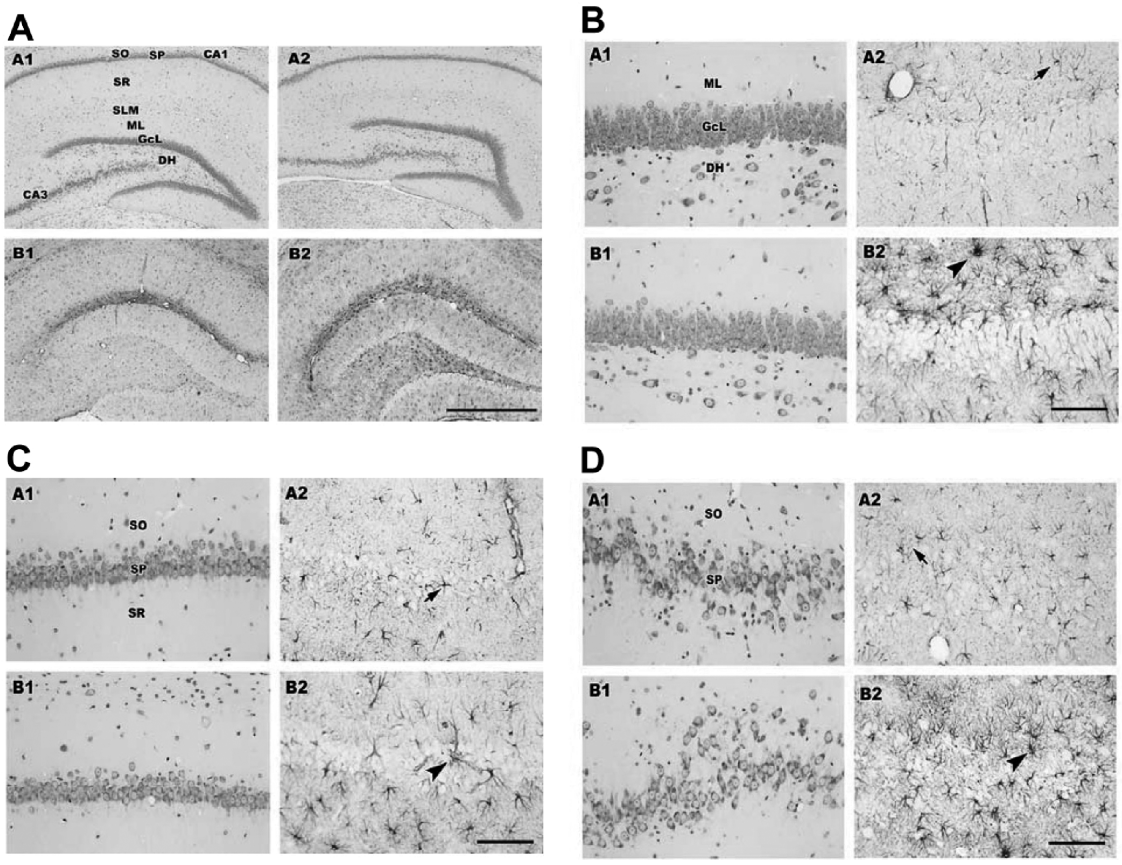

FIG. 5. Histopathological alterations in different regions of the hippocampus of male offspring at PND 30, following maternal exposure to a single ip injection of 337 mg/kg imidacloprid in corn oil on GD 9. (A) A1 and A2 are examples of cresyl violet staining; B and B2 are examples of GFAP immunostaining; A1 and B1 are examples of control animals; and A2 and B2 are examples from the offspring of imidocloprid-treated mothers. Note that there were no changes in the thickness (cell packing density) in any hippocampal region. However, GFAP immunoreactivity was upregulated in all three regions of the hippocampus (the dentate gyrus and the CA1 and CA3 subfields). SO, striatum oriens; SP, striatum pyramidale; SR, striatum radiatum; SLM striatum lacunosum moleculare; GCL, granular cell layer; DH, dentate hilus. Scale bar = 200 µm. (B) Alterations in the dentate gyrus at 20× magnification. A1 and B1 show CV staining; A2 and B2 show GFAP immunostaining. A1 and A2 are examples of control offspring and A2 and B2 are examples of the offspring of imidacloprid-treated mothers. Note that GFAP-immunoreactive structures exhibit a significant increase in the offspring of mothers treated with a single ip injection of imidocloprid. Scale bar = 100 µm. (C) Alterations in the CA1 subfield of the hippocampus at 20× magnification. A1 and B1 show CV staining; A2 and B2 show GFAP immunostaining. A1 and A2 are examples of control offspring and A2 and B2 are examples of offspring of imidacloprid-treated mothers. Note: GFAP-immunoreactive structures exhibit a significant increase in the offspring of mothers treated with a single ip injection of imidacloprid. SO, striatum oriens; SP, striatum pyramidale; SR, striatum radiatum. Scale bar =100 µm. (D) Changes in the CA3 subfield of hippocampus at 20× magnification. A1, B1: CV staining; A2, B2: GFAP immunostaining. A1 and A2 are examples of control offspring and A2 and B2 are examples of offspring of treated mothers. Note: GFAP- immunoreactive structures exhibit a significant increase in the offspring of mothers treated with a single ip injection of imidacloprid (striatum oriens; SP, striatum pyramidale). Scale bar = 100 µm.

comprised of five classes of receptors (m1–m5), which have distinct structural and pharmacological features and which show different cellular localization (Levey et al., 1995). The potential roles of the individual mAChRs in learning and memory are not well understood, although pharmacological agents were developed to act as specific agonists or antagonists for each individual class of receptor (Wess, 1996). The m2mAChRs are presynaptic and are localized on both cholinergic and noncholinergic nerve terminals in the mammalian brain (Rouse et al., 2000). These receptors are located on the cholinergic nerve endings in the hippocampus and cerebral cortex and are known to mediate autoinhibition of acetylcholine release (Quirion et al., 1995; Zhang et al., 2002). Normal functional levels of m2mAChR were reported to be essential for working memory, behavioral flexibility, and hippocampal plasticity (Seeger et al., 2001) . Furthermore, increased ligand binding was found in the temporal cortex and cingulated cortex in patients with neuropsychiatric symptoms of dementia with Lewy bodies (Teaktong et al., 2005). Increased m2mAChR-ligand binding in the cortex and midbrain of offspring following in utero treatment with imidacloprid suggests that signaling events downstream of the receptor might be impaired and may lead to memory deficits in exposed individuals.

Histopathological findings in our study showed a significant increase in GFAP immunostaining in motor cortex layer III, the dentate gyrus, and hippocampal CA1 and CA3 subfields in

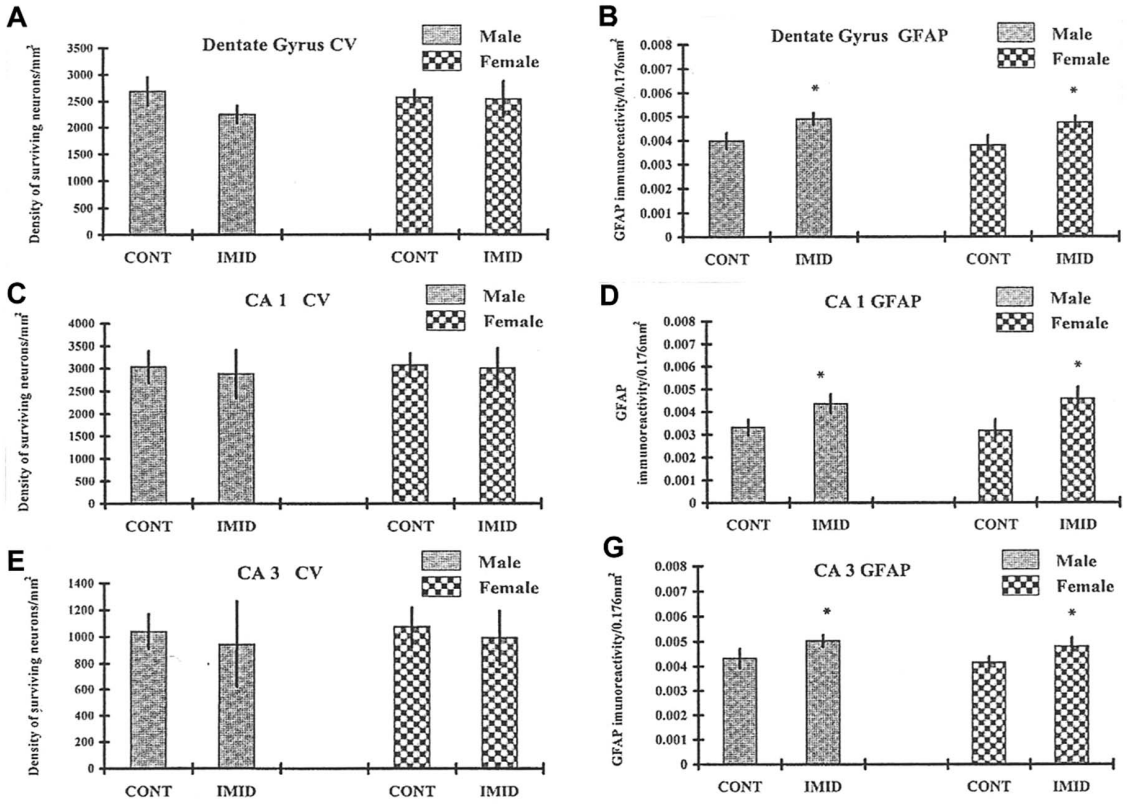

FIG. 6. Histogram showing the density of surviving (A, C, and E) neurons per square millimeter of area of different cell layers in the hippocampal formation (i.e., dentate gyrus and CA1 and CA3 subfields) in both males and females of the control and treated groups. Histograms (B, D, and E) show GFAP immunostaining of different cell layers in the hippocampal formation. Note: There was no change in the density of surviving neurons in the offspring of mothers treated with a single ip injection of imidacloprid. However, there was a significant increase in GFAP immunoreactivity in both male and female offspring of the above treated animals. Asterisk indicates statistically significantly different from controls.

offspring following maternal exposure to imidacloprid. GFAP plays an important role in the long-term maintenance of brain cytoarchitecture (Liedtke et al., 1996), proper functioning of the blood–brain barrier (Penky et al., 1998), and modulation of neuronal functions (Shibuki et al., 1996). GFAP is expressed primarily by astrocytes, and these cells are known to play a critical role in neuromodulation, neuroprotection, and axon guidance control during development, as well as in homeostasis preservation and blood-brain barrier maintenance (Ridet et al., 1997). In the developing rodent brain, vimentin is initially the major glial filament protein (Dahl et al., 1981), which changes to expression of GFAP as development progresses. A major abrupt expression in GFAP occurs around the second week of the postnatal period (Giulian et al., 1988; Faivre-Sarrailh et al., 1991; Tsuneishi et al., 1991); this is the period when glial maturation also occurs. Reactive gliosis accompanied by increased accumulation of GFAP is a characteristic response of both the immature and adult brain to a variety of neurotoxic insults (O'Callaghan, 1993; Burtrum & Silverstein, 1993; Clarke et al., 1996; Fattore et al., 2002; Garcia et al., 2002; Little et al., 2002). Previous studies from our laboratory showed an increase in GFAP immunostaining in the CA1 subfield of the hippocampus and the granular cell layer as well as in cerebellar white matter in offspring following maternal treatment with nicotine (Abdel-Rahman et al., 2003, 2005). Thus, it appears that maternal exposure to nicotine or neonicotinoid insecticides, such as imidacloprid produces widespread gliosis in the offspring brain. An increased GFAP immunoreactivity reflects reactive astrocytic activation in motor cortex layer III as well as in the hippocampal formation. Glial activation may result from axonal degeneration of motor cortex layer III and the CA1 subfield of the hippocampus (Atarashi et al., 2001). Furthermore, there are several reports of elevated levels of GFAP in the cerebrospinal fluid of children with developmental neuropsychiatric disorders (Ahlsen et al., 1993; Singh et al., 1997; Kristjansdottir et al., 2001). In conclusion, the present findings indicate that in utero exposure to a large sublethal dose of imidacloprid produced significant developmental neurobehavioral and neuropsychiatric abnormalities in rats at a corresponding an early adolescent age. It would be useful to expand the present studies to include an investigation of a wide range of doses using an extensive battery (sensory, motor, affective, and cognitive tests) to determine the chronic effects resulting from exposure to imidacloprid.

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