Increased Susceptibility to Adult Paraoxon Exposure in Mice Neonatally Exposed to Nicotine

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Low-dose exposure of neonatal mice to nicotine has earlier been shown to induce an altered behavioral response to nicotine in adulthood. Organophosphorus insecticides are known to affect the cholinergic system by inhibition of acetylcholinesterase. This study was undertaken to investigate whether neonatal exposure to nicotine makes mice more susceptible to a known cholinergic agent. Neonatal, 10-day-old, male mice were exposed to nicotine-base (33 μg/kg body weight) or saline s.c. twice daily on five consecutive days. At 5 months of age the animals were exposed to paraoxon (0.17 or 0.25 mg/kg body weight [29% and 37% inhibition of cholinesterase, respectively]) or saline s.c. every second day for 7 days. Before the first paraoxon injection, the animals were observed for spontaneous motor behavior. The spontaneous motor behavior test did not reveal any differences in behavior between the treatment groups. Immediately after the spontaneous behavior test, the animals received the first injection of paraoxon and were observed for acute effects of paraoxon on spontaneous motor behavior. The acute response to paraoxon in the spontaneous motor behavior test was a decreased level of activity in mice neonatally exposed to nicotine. Control animals showed no change in activity. Two months after the paraoxon treatment, the animals were again tested for spontaneous motor behavior. Animals neonatally exposed to nicotine and exposed to paraoxon as adults showed a deranged spontaneous motor behavior, including hyperactivity and lack of habituation.

Key Words: nicotine; paraoxon; development; mice; cholinergic; behavior.

Whether early exposure of xenobiotics during critical periods of brain development has effects on adult susceptibility to other agents is an intriguing question. Some transmitter systems may be targets for certain xenobiotics. The cholinergic system is closely connected to many physiological processes and consciousness, such as memory, learning, wakefulness, audition, and vision (Lucas-Meunier et al., 2003). We have seen in earlier studies that a wide variety of agents can disturb the normal development of the brain (Eriksson, 1997; Eriksson et al., 2001). The agents used in the studies have been both specific cholinergic agents such as nicotine and organophosphorus insecticides (OPs), and agents such as DDT, bioalthrin, and various PCBs. The disturbances have been induced during the rapid growth and development of the brain, called ‘the brain growth spurt’ (Davison and Dobbing, 1968). In mice this period is neonatal, spanning from around day 0 to day 20, while in humans this development of the brain occurs from the third trimester of pregnancy until the child is about 2 years of age (Davison and Dobbing, 1968). During this time there is an extensive axonal and dendritic outgrowth, development of different transmitter systems, and synaptogenesis. During this period in rodents, the development of the cholinergic system peaks, and there is a rapid increase in the activity of choline acetyl transferase, acetylcholinesterase (AChE), and sodium-dependent choline uptake, as well as an increase in density of cholinergic receptors (Coyle and Yamamura, 1976; Falkeborn et al., 1983; Fiedler et al., 1987; Hohmann et al., 1995; Kuhr et al., 1980).

Nicotine is one of the most commonly used drugs in the world. Several studies have investigated nicotine’s effects on adult animals. It has been shown that nicotine is able to improve learning and memory in adult rodents in different behavioral tests (Decker et al., 1995; Levin et al., 1997; Levin and Torry, 1996). It has also been shown that chronic nicotine treatment in adult and prenatal animals upregulates the nicotinic receptors in the brain (Sparks and Pauly, 1999; van de Kamp and Collins, 1994; Wonnacott, 1990). Nicotine is a neuroteratogen, which mimics the actions of the endogenous transmitter acetylcholine (ACh). This may discoordinate the timing of trophic events linked to cholinergic nicotinic receptors that are present in the developing brain (Slotkin, 1998). Research concerning developmental exposure to nicotine is therefore of particular concern, as smoking during pregnancy and lactation is common and exposes the embryo/fetus and the infant to nicotine. It is well known that nicotine use during pregnancy causes lower birth weights, and SIDS (Sudden Infant Death Syndrome) is much more prevalent in children born to parents who are smokers (See Slotkin, 1998). It is also suggested that behavioral disturbances, such as ADHD (Attention Deficit Hyperactivity Disorder) are more prevalent in children of parents who are smokers (Biederman and Faraone, 2002; Hill et al., 2000; Mick et al., 2002).

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OPs are AChE inhibitors and have been used in agriculture since World War II. Parathion is one of the compounds that replaced the organochlorine insecticide DDT in the 1950s, and it is still widely used. Parathion is bioactivated to paraoxon by oxidative desulfuration, a reaction that takes place in both insects and mammals. When an OP reaches the cholinergic synapse, it inhibits the AChE and thereby the degradation of acetylcholine. This causes an accumulation of acetylcholine in the synapse and an over-stimulation of cholinergic receptors. Chronic treatment with different OPs has been shown to decrease the number of muscarinic receptors in both the neonatal and adult rat brain (Liu et al., 1999; McDonald et al., 1988).

In earlier studies we have seen that exposure to different substances, including nicotine, during the rapid development of the brain, can cause behavioral disturbances and changes in cholinergic receptor configuration in mice (Ankarberg et al., 2001; Eriksson, 1997; Eriksson et al., 2000; Nordberg et al., 1991). Furthermore, it has also been found that exposure to low doses of toxicants during this neonatal period makes the animals more susceptible to adult exposure of different toxic agents (Eriksson and Talts, 2000; Johansson et al., 1996; Talts et al., 1998). For example, animals neonatally exposed to DDT were more susceptible to an adult exposure to low doses of paraoxon, seen as a changed spontaneous behavior and changes in muscarinic receptor density (Johansson et al., 1996).

The present study was undertaken to ascertain whether neonatal exposure to low doses of nicotine could modify the reaction to an adult exposure to paraoxon, an active metabolite of the short-acting insecticide parathion.

**MATERIALS AND METHODS**

**Chemicals and animals.** Paraoxon (diethyl p-nitrophenyl phosphate) and (−)-nicotine-bi- (+)-tartrate was obtained from Sigma, St. Louis, MO. [3H]-quinuclidinylbenzilate (QNB, 43.0 Ci/mole) was obtained from Amersham Pharmacia Biotech, U.K. Pregnant NMRI mice were obtained from Charles River, Uppsala, Sweden. Subsequently, each litter, with pups of both sexes were weaned, and the males were placed and raised in groups of 4–7 in a room for male mice only. At the age of 5 months the animals were exposed to paraoxon (0.17 or 0.25 mg/kg body weight) or saline, and were then observed for another 60-min period (60–120 min). Two months after the adult injections, the animals (aged 7 months) were again observed for spontaneous motor behavior (0–60 min).

**Cholinesterase activity.** Cholinesterase (ChE) activity was measured 1 h after the injections of paraoxon or saline. A crude synaptosomal P2 fraction (Gray and Whittaker, 1962) from the cerebral cortex was prepared as described by Eriksson and Nordberg (1986), with a protein content of 1–2 mg/ml determined by the method of Udenfried et al. (1972) as described in Lorenzen and Kennedy (1993). Analysis of the ChE activity was performed as described by Ehrman et al. (1961), and modified by Benke et al. (1974). Twenty μl of the P2 fraction was mixed with 50 μl 0.1 M acetylcholine iodide, 50 μl 1 mM 5,5-diethylbis-2-nitrobenzoic acid, and 0.1 M phosphate buffer (pH 8.0) to a total volume of 5 ml. The absorbance was measured immediately at 412 nm, and the tubes were then incubated at 27°C. After 30 min, the absorbance was measured again. The difference in absorbance was used to calculate the ChE activity (nmole/min × mg protein).

**Receptor assay.** The day after the last spontaneous behavior test, the 7-month-old mice were sacrificed by decapitation. A crude synaptosomal P2 fraction was prepared for eight mice from each treatment (as above). The P2 fractions were kept frozen (−70°C) until assayed. Measurements of muscarinic receptor density were performed following the method by Nordberg and Winblad (1981) and described by Eriksson and Nordberg (1986). Briefly, the assay was performed by measuring tritium-labelled quinuclidinylbenzilate (QNB, 0.2 nM in the density assay) specifically bound in the P2 fraction using atropine (10−6 M) for measuring the nonspecific binding. Specific binding was determined as the difference in the amount bound in the presence and in the absence of atropine. Specific binding constitutes about 95% of the total [3H]QNB binding.

**Statistical analysis.** The spontaneous behavior data were subjected to a split-plot analysis of variance (ANOVA), and pairwise testing between nicotine- and saline-treated groups was performed with Tukey’s HSD (honestly significant difference test) (Kirk, 1968) (α = 0.05). The statistical evaluation of ChE inhibition was made by one-way ANOVA and Tukey’s HSD test (α = 0.05). Muscarinic receptor density was statistically evaluated using one-way ANOVA and Tukey’s HSD test (α = 0.05).

**Behavioral testing.** In studying behavior in animals, spontaneous motor activity is especially meaningful because it reflects the animals’ ability to integrate the sensory input into a motory output. From the spontaneous motor behavior one can also view the animals’ habituation to a novel environment. The habituation index in this motor activity test chambers’ situations, over repeated test periods maybe assumed to provide a simple, nonassociative instance of learning.

Before the first injection of paraoxon, in the 5-month-old mice, the animals were observed for spontaneous motor behavior (0–60 min). Eight mice, randomly taken from 3–4 different litters, were tested from each treatment group. Motor activity was measured for 3 × 20 min in an automated device consisting of cages (40 × 25 × 15 cm) placed within two series of infrared beams (low level and high level) (Rat-O-Matic, ADEA Elektronik AB, Uppsala, Sweden) (Fredriksson, 1994).

Investigated parameters were:
- **Locomotion:** Registered when the mouse moved horizontally through the low-level grid of infrared beams.
- **Rearing:** Vertical movement was registered at a rate of four counts per second, whenever and as long as a single high-level grid was interrupted (i.e., the number of counts obtained was proportional to time spent rearing up).

**Total activity:** A pickup (mounted on a lever with a counter weight), with which the test cage was in contact, registered all types of vibration within the test cage, including those caused by mouse movements, shaking (tremors), and grooming.

The animals were tested between 8 A.M. and 12 P.M. under the same ambient light and temperature conditions as the housing. Immediately after the spontaneous motor behavior test, the animals received the first injection of paraoxon (0.17 or 0.25 mg/kg body weight) or saline, and were then observed for another 60-min period (60–120 min). Two months after the adult injections, the animals (aged 7 months) were again observed for spontaneous motor behavior (0–60 min).

Registered when the mouse moved horizontally through the low-level grid of infrared beams.

The spontaneous behavior data were subjected to a split-plot analysis of variance (ANOVA), and pairwise testing between nicotine- and saline-treated groups was performed with Tukey’s HSD (honestly significant difference test) (Kirk, 1968) (α = 0.05). The statistical evaluation of ChE inhibition was made by one-way ANOVA and Tukey’s HSD test (α = 0.05). Muscarinic receptor density was statistically evaluated using one-way ANOVA and Tukey’s HSD test (α = 0.05).
RESULTS

There were no clinical toxic signs in the treated mice throughout the experimental period. Nor were there any differences in body weight gain between the different treatment groups during the experimental period (data not shown).

Cholinesterase Inhibition

One h after the first paraoxon injection, ChE inhibition was approximately 29% in the animals exposed to 0.17 mg/kg body weight and 37% in the animals exposed to 0.25 mg/kg body weight as compared to saline-injected animals (Table 1).

Spontaneous Behavior, 5 Months

Before the first injection of paraoxon, the adult mice were observed for spontaneous motor behavior. The results from locomotion, rearing, and total activity variables in 5-month-old male mice after neonatal exposure to 33 μg nicotine-base/kg body weight or saline (10 mg/kg body weight) are shown in Figure 1. The spontaneous motor behavior test did not reveal any significant differences in behavior between the saline- and nicotine-treated mice. There was a significant decrease in activity over time in response to the diminished novelty of the test chambers in all animals. There were significant group × period interactions [F_{10,108} = 2.76; F_{10,108} = 1.94] for the variables ‘rearing’ and ‘total activity,’ respectively, but not for the ‘locomotion’ variable [F_{10,108} = 1.60]. Pairwise testing among the treatment groups did not reveal any significant difference in the three variables ‘locomotion,’ ‘rearing,’ or ‘total activity.’

TABLE 1
Cholinesterase Activity in Cerebral Cortex of 5-Month-Old Mice After Neonatal Exposure to Nicotine and Adult Exposure to Paraoxon

<table>
<thead>
<tr>
<th>Treatment at Day 10–14</th>
<th>Month 5</th>
<th>nmole/min × mg protein (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>Saline</td>
<td>72.19 ± 8.49 (7)</td>
</tr>
<tr>
<td>Saline</td>
<td>Paraoxon (0.17 mg/kg)</td>
<td>49.98 ± 15.01a (8)</td>
</tr>
<tr>
<td>Saline</td>
<td>Paraoxon (0.25 mg/kg)</td>
<td>49.88 ± 9.70a (8)</td>
</tr>
<tr>
<td>Nicotine</td>
<td>Saline</td>
<td>65.23 ± 11.14 (8)</td>
</tr>
<tr>
<td>Nicotine</td>
<td>Paraoxon (0.17 mg/kg)</td>
<td>52.99 ± 8.82 (8)</td>
</tr>
<tr>
<td>Nicotine</td>
<td>Paraoxon (0.25 mg/kg)</td>
<td>40.19 ± 18.44b (8)</td>
</tr>
</tbody>
</table>

Note. Male NMRI mice aged 10 days received 33 μg (-) nicotine-base/kg b.wt, or saline (10 ml/kg b.wt), sc twice daily for 5 days. At the age of 5 months they received one single paraoxon- (0.17 or 0.25 mg/kg b.wt) or saline injection (10 ml/kg b.wt.). One h after the injection the animals were sacrificed. ChE activity (mean ± SD) was assayed in the P2 fraction. The statistical evaluation was made by one-way ANOVA and Tukey’s HSD test.

*Significantly different from Saline-Saline, p < 0.05.

bSignificantly different from Nicotine-Saline, p ≤ 0.01.

Spontaneous Motor Behavior After Acute Paraoxon Exposure, 5 Months

Immediately after the spontaneous motor behavior test, the animals received the first injection of paraoxon or saline and were tested for acute reactions to paraoxon. There were significant group × period interactions [F_{10,108} = 5.99; F_{10,108} = 4.62; F_{10,108} = 2.93] for the variables ‘locomotion,’ ‘rearing,’ and ‘total activity,’ respectively. Pairwise testing did not reveal any differences in behavior in control animals exposed to paraoxon (0.17 or 0.25 mg/kg body weight) when compared with animals that received saline, except for time period 2 (80–100 min), where the control–high dose animals showed a significantly higher activity than the saline-injected controls.
Mice neonatally exposed to nicotine showed a decreased activity in response to the paraoxon injection both during the period 60–80 min and the period 80–100 min. There were no significant dose-response changes between the two paraoxon doses.

**Spontaneous Motor Behavior, 7 Months**

Two months after the last injection of paraoxon, the animals were tested for spontaneous motor behavior. There were significant group × period interactions \( F_{10,108} = 98.09; F_{10,108} = 142.99; F_{10,108} = 100.94 \) for the variables 'locomotion,' 'rearing,' and 'total activity' respectively.

Pairwise testing among the treatment groups showed significant differences in all three test variables. In the control mice there was a distinct decrease in activity in all behavioral variables over the 60-min period. Mice that had received nicotine neonatally and paraoxon (0.17 or 0.25 mg) at 5 months of age displayed significantly less activity than the controls during the first 20-min period (0–20 min), but during the third 20-min period (40–60 min) they were significantly more active than the controls.

**Muscarinic Receptor Assay**

Analysis of the muscarinic receptors in the cerebral cortex with QNB did not reveal any significant differences between the treatment groups (data not shown).
DISCUSSION

Our study shows that a substance known to affect the cholinergic system, such as nicotine, in doses that apparently do not cause direct effects, can make the animals more susceptible to another cholinergic agent, such as paraoxon, given at doses that have no pronounced effects in adult untreated animals. This was seen in the acute reaction to paraoxon in the spontaneous motor behavior test in 5-month-old animals and as a delayed effect in spontaneous motor behavior 2 months after the paraoxon exposure, manifested as a lack of habituation to a novel environment. This development of alterations in spontaneous motor behavior indicates some kind of time-dependent effect.

The effect on the spontaneous motor behavior indicated two different responses to paraoxon in the adult animals, the first observed in the nicotine-paraoxon groups at 5 months of age, and the second at 7 months of age. At 5 months of age there were no differences in the spontaneous motor behavior between the control animals and the neonatally nicotine-treated animals. But when the animals were given the first paraoxon injection, the neonatally nicotine-treated animals responded with a hypoactive behavior. This behavior reaction is similar to that seen in an earlier study where mice were exposed to nicotine neonatally (33 or 66 mg/kg body weight) (Ankarberg et al., 2001; Eriksson et al., 2000). In these animals a normal spontaneous motor behavior and habituation was seen at an adult age of 4 months, but when challenged by nicotine (40 or 80 mg/kg body weight), they showed the same hypoactive response as the animals exposed to nicotine neonatally in this study did when exposed to a single dose of paraoxon as adults. This indicates that an increased concentration of ACh (which may be an effect of nicotine and the inhibition of AChE by paraoxon) may have effects on behavior. The acute reaction to paraoxon was reversible, since the animals showed differences in behavior only during the first two time periods. The second effect was observed when the animals had reached 7 months of age (2 months after the termination of the paraoxon exposure), when the animals showed significant different spontaneous motor behavior when compared to the controls. The neonatally nicotine- and adult paraoxon-treated animals were hypoactive at the beginning of the test period and hyperactive at the end of the test period, when compared to the control animals (i.e., there was a lack of habituation). This type of behavior indicates a difficulty in habituating to a novel environment and thereby acquiring and processing new information.

Johansson et al. (1996) showed that a low neonatal dose of DDT, a substance that increases neuronal activity, caused behavioral changes in adult animals. When these animals were exposed to paraoxon as adults, they developed additional behavioral disturbances that worsened with age. In that study, as also seen in the present, the behavior defect was not seen directly after the paraoxon exposure was terminated, but appeared 2 months later. These animals showed an especial lack of habituation in the rearing variable, which is often associated with exploratory behavior and can be interpreted with a nonassociative type of learning process. These behavioral disturbances were accompanied by additional changes in muscarinic cholinergic receptors.

Signs observed in relation to OP exposure are often correlated to the degree of AChE inhibition. At higher degrees of AChE inhibition it is also seen that the cholinergic muscarinic receptors can be downregulated. Repeated or chronic exposure to OPs has been shown to cause downregulation of muscarinic receptors in both young and adult animals (Liu et al., 1999; Moser and Padilla, 1998; Zheng et al., 2000). It has been proposed that this can be a result of the increasing amount of acetylcholine that accumulates in the synapses after OP exposure and that the downregulation of the muscarinic receptors is a sign of tolerance toward the OP (Costa et al., 1982). In the present study, no differences in QNB binding were seen. However, one cannot exclude changes in the muscarinic receptors, since QNB does not distinguish between different receptor subtypes but binds to all equally well. Whether changes can be seen on cholinergic nicotinic receptors is yet to be investigated, since earlier studies have shown that neonatal exposure to nicotine affects the nicotinic low affinity-binding sites (i.e., α7 receptors) in cerebral cortex, an effect that is persistent into adult age (Eriksson et al., 2000).

Many studies report that young and immature animals are more sensitive to OP exposure than adult animals (Benke et al., 1974; Olivier et al., 2001; Zhang et al., 2002; Zheng et al., 2000). This sensitivity is especially pronounced after acute exposure. In the study by Zheng et al. (2000), the degree of AChE inhibition was 1–10 times higher in neonatal rats than in adult rats after acute exposure to chlorpyrifos. Despite this difference in AChE inhibition, they could not find any changes in the muscarinic or nicotinic receptors, measured with QNB or epibatidine, regardless of treatment, when they compared neonatal and adult animals. The sensitivity in neonatal animals may be due to maturational differences in the feedback inhibition of acetylcholine release (Disko et al., 1998) or to differences in detoxification of the OPs (Benke and Murphy, 1975). However, in a study by Ahlbom et al. (1995), it was shown that a single oral dose of diisopropylfluorophosphate (DFP) on postnatal day 3 or 10 caused alterations in adult spontaneous motor behavior and also a decrease in muscarinic receptor density, measured with QNB. In this study, mice exposed at postnatal day 19 did not show any changes in adult spontaneous motor behavior or receptor density. Neonatal exposure to paraoxon, at a subclinical oral dose giving 45–50% AChE inhibition, has been shown to cause changes in spontaneous motor behavior in adult mice, observed as a significant decrease in activity during the first 20-min period of a 60-min observational period (Ahlbom, 1995). In the present study, a dose of paraoxon causing less AChE inhibition, only about 30% in adult mice, could more adversely affect the spontaneous behavior in mice neonatally exposed to nicotine. This indicates that early exposure to nicotine, during neonatal life,
can cause an adult susceptibility to OPs that can be more pronounced than the sensitivity to OPs found in neonatal animals.

In the present study there were no significant differences in ChE inhibition between the saline-paraoxon and the nicotine-paraoxon groups. Whether the cholinesterase-inhibiting effect of paraaxon (29% and 37%) is the main cause of the observed effects on spontaneous motor behavior remains to be further investigated. There are reported effects of OPs not connected with AChE inhibition that induce working memory deficits (Bushnell et al., 1991).

Cholinergic dysfunctions have been shown to cause impairments in learning and memory functions (Bartus et al., 1982). Ageing is also known to cause impairment of learning and memory functions (Gage et al., 1984; Gallagher et al., 1993; Lamberty and Gower, 1990). The study by Lamberty and Gower (1990) showed that, from the age of 9 months, normal NMRI mice exhibited changes in spontaneous behavior and learning. In our study it seems possible that neonatal nicotine exposure and adult paraaxon exposure may have brought this ageing process forward, since the behavioral changes appear earlier in life and worsen over time.

In conclusion, this study indicates that a paraaxon exposure, causing low AChE inhibition can induce permanent effects on behavior in adult animals neonatally exposed to low doses of nicotine. No effects on spontaneous motor behavior were seen in adult mice neonatally exposed to the vehicle and, as adults, to the same paraaxon doses. The results indicate that differences in adult susceptibility to environmental pollutants are not necessarily an inherited condition. Rather, they might well be acquired by low-dose exposure to different toxic agents during early life.

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