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Hyperactivity Induced by Prenatal Nicotine Exposure Is Associated with an Increase in Cortical Nicotinic Receptors

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TIZABI, Y., E. J. POPKE, M. A. RAHMAN, S. M. NESPOR AND N. E. GRUNBERG. *Hyperactivity induced by prenatal nicotine exposure is associated with an increase in cortical nicotinic receptors.* PHARMACOL BIOCHEM BEHAV **58**(1) 141–146, 1997.—Prenatal exposure to nicotine may lead to hyperactivity. To evaluate possible involvement of central nicotinic receptors in this condition, pregnant Sprague–Dawley rats were implanted with osmotic minipumps to receive nicotine (6 mg/kg/day) or saline throughout gestation. A total of 222 pups (118 males and 104 females) from 24 dams were measured for locomotor activity. Male and female hyperactive and nonhyperactive offspring from each treatment group were selected and analyzed for nicotinic receptor concentrations in various brain regions. Hyperactive male offspring that were prenatally exposed to nicotine exhibited a significant increase in the cortical receptor densities without a change in binding affinity. Hyperactive offspring of saline-treated dams did not show an increase in cortical nicotinic receptors. These results suggest that hyperactive male offspring of nicotine-exposed dams are also susceptible to neurochemical effects of intrauterine nicotine exposure. © 1997 Elsevier Science Inc.

Prenatal Nicotine Nicotinic receptors Locomotor activity Hyperactivity Cortex Striatum Gender Rat

CONSIDERABLE data have accumulated during the past two decades indicating that cigarette smoking during pregnancy may be one of the causes of hyperactivity and learning deficits in children (4,9,10,16,25,44). It is now well established that nicotine is the primary pharmacological agent in tobacco that affects the central nervous system and underlies behavioral effects (2,14,33,40,42,45,48). Therefore, it has been suggested that hyperactivity in children of smoking mothers might result from effects of nicotine on the developing fetus (19,20). This suggestion is supported by several animal studies that report the presence of hyperactivity in offspring of nicotine-treated dams (11,19,22,35).

Recently, Richardson and Tizabi (31) reported that prenatal nicotine-induced hyperactivity is associated with changes in mesolimbic and nigrostriatal dopaminergic pathways. Because central actions of nicotine are mediated by specific nicotinic cholinergic receptors that are already present in fetal brain by midgestation (5,23,40,46), and because recent reports suggest that central nicotinic receptors play an important role in behavior (1,8,17,32,43,47), the present experiment examined the relationship between prenatal nicotine-induced hyperactivity and central nicotinic cholinergic receptor (nAChR) concentrations in discrete brain regions.

METHODS

Timed-pregnant Sprague–Dawley rats weighing 190–230 g were purchased from Charles River Laboratories (Kingston, NY, USA). The animals were maintained in an environmentally controlled room with a 12 L:12 D cycle (lights on at 1900 h), a temperature range of 22–24°C, and relative humidity of approximately 50%. Subjects were housed individually in 35.6 × 15.2 ×

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20.3-cm cages with absorbent Pine-Dri wood-chip bedding and had continuous access to standard laboratory chow (Agway Prolab 3000) and water.

On the fourth day of gestation, pregnant dams were randomly implanted (SC, in the intrascapular space) with osmotic minipumps (model 2002, Alza Corp., Palo Alto, CA, USA) to receive either nicotine dihydrochloride (6 mg/kg/day nicotine base dissolved in physiological saline) or saline. Methoxyflurane (Metophane), administered by inhalation, was used to anesthetize the dams during the implantation surgery. The minipumps, with a flow rate of 0.48 μl/h and a fill volume of 238 μl, had a total delivering capacity of approximately 23 days. It was determined previously that this treatment yields plasma nicotine concentrations of approximately 100 ng/ml (31).

All pups were born by day 23 of gestation. They were weaned on day 18 postnatally, and same-sex littermates were housed in groups of two to six pups per cage. A total of 222 pups (118 males and 104 females) from 24 dams were used in this study.

Locomotor Activity Testing

Locomotor activity was measured using an Omnitech Electronics Digiscan infrared photocell system (Test Box model RXYZCM, 16 TAO, Omnitech Electronics, Columbus, OH, USA), where animals were placed singly in a 20 × 20 × 30-cm clear Plexiglas arena. The tests were conducted on days 21, 22, 24, and 25 postnatally during the dark portion of the rats' activity cycle (0800–1400 h). Males and females were tested separately. Spontaneous locomotor activity, determined by the total horizontal distance travelled (in centimeters), was automatically gathered and transmitted to a personal computer via an Omnitech model DCM-8-BBU analyzer. Animals were monitored continuously for 60 min on each testing day, with data recorded as cumulative activity over 5-min periods. Mean cumulative distances travelled for all test periods (activity score) were used to classify pups as "hyperactive = high activity" or "hypoactive = low activity." Hyperactivity was defined as mean activity scores of 1.3 SD above the mean for the saline pups of the same sex. Hypoactivity was defined as mean activity scores of 1.3 SD below the mean for the saline pups of the same sex. This classification criterion was used to select groups of subjects with clearly distinguishable behaviors and with sufficient sample size for receptor assays. A third group of offspring with mean activity score within 0.5 SD above and below the mean for the saline group of the same sex was also selected as the "middle activity" group for receptor binding analyses. Based on these criteria, five to nine animals in each behavioral category were obtained from each sex and from each treatment group for the receptor binding assays.

Tissue Collection

All pups were sacrificed at age 35 days, an age when the nicotinic binding capacities of rat brain membranes have achieved their adult levels and are no longer subject to changes with age (39). Following decapitation, brains were rapidly removed and frozen in powdered dry ice. The brains were kept at −80°C for several weeks before being thawed and dissected on an ice-cold plate under a magnifying lens. Cerebral cortex (left hemisphere), striatum, hippocampus, thalamus, and colliculi (superior and inferior) were separated and stored frozen at −80°C before measuring nicotinic receptor binding. These regions have been reported to play an important role in locomotor activity or contain relatively high levels of neuronal nicotinic receptors (6,7,26,27,50).

Nicotinic Receptor Assay

The assay procedure used to measure nicotinic cholinergic receptor binding in the selected brain regions was based on Pabreza et al. (27). Tissue was homogenized in ice-cold 50 mM Tris-HCl buffer (pH 7.0 at room temperature), then the tissue homogenate was centrifuged at 38,000 × *g* for 12 min at 4°C. The pellet was washed twice by suspension in fresh buffer and centrifuged again. Aliquots of homogenate equivalent to approximately 10–20 mg tissue were used in triplicate for total binding and in duplicate for nonspecific binding. For total binding, approximately 4 nM [^{3}H]cytisine (39.6 Ci/mmol, NEN, Boston, MA, USA) was incubated in a final volume of 0.25 ml at 2°C for 75 min. Nonspecific binding was obtained in the presence of 100 μM (−)-nicotine bitartrate. Membrane-bound [^{3}H]cytisine was separated from free ligand by filtration using Brandel GF/B filter paper and a Brandel cell harvester. The binding affinity was determined in cortical tissue using six concentrations (0.25–8 nM) of [^{3}H]cytisine. Scatchard plots to calculate B_{max} and K_d were obtained using the nonlinear least-squares regression LIGAND analysis (24). Protein concentration in the final homogenate was determined by the Bradford method (3).

Data Analysis

One-way analysis of variance followed by Duncan's post hoc test was used to examine the effects of prenatal nicotine exposure on nicotinic receptor binding in each of the brain regions studied. Because previous reports have indicated sex differences in effects of prenatal nicotine (12,13,18,19,28, 30,35), males and females were analyzed separately. All analyses were two-tailed and used an alpha of 0.05 or less to determine significance.

RESULTS

Hyperactivity in Offspring

Table 1 presents the activity score for various groups selected as having high, middle, or low activity levels. Each offspring group was obtained from a minimum of four dams (e.g., female hyperactives) and up to a maximum of seven dams (e.g., male hyperactives). Moreover, at least one offspring from each dam was included in the receptor assay. Analysis by the mean activity score between various groups did not reveal an overall treatment or gender effect (mean activity scores ± SEM for various groups: male saline, 474 ± 32; male nicotine, 510 ± 46; female saline, 444 ± 26; female nicotine, 450 ± 47). However, a higher percentage of hyperactive offspring were obtained from dams treated with nicotine (males 18.5%, females 16.1%) vs. dams treated with saline (males 9.4%, females 10.4%).

Nicotinic Receptor Binding

Hyperactive male pups that were exposed to nicotine prenatally had significantly higher nicotinic receptor concentrations in the cortex than did the other male pups [$F(5, 40) = 2.76$, $p < 0.05$] (Fig. 1). In particular, nicotinic receptor concentrations in male pups with high activity were 37% higher than in pups with middle activity and 51% higher than in pups with low activity following prenatal nicotine exposure (Duncan's post hoc test). Receptor concentrations in the striatum of hyperactive male offspring that were exposed to nicotine prenatally were also higher than in middle- or low-activity groups. However, these differences did not achieve statistical

TABLE 1

ACTIVITY SCORES OF OFFSPRING SELECTED AS HAVING HIGH, MIDDLE, OR LOW ACTIVITY

	Male			Female		
	High	Middle	Low	High	Middle	Low
Saline	823 ± 35 (8)	453 ± 11 (8)	255 ± 9 (8)	753 ± 79 (5)	424 ± 16 (6)	264 ± 30 (6)
Nicotine	890 ± 60 (90)	498 ± 23 (9)	276 ± 13 (9)	787 ± 98 (8)	412 ± 17 (8)	213 ± 10 (8)

Activity scores [mean cumulative distance travelled (in centimeters) for all test periods] are reported for offspring selected as having high, middle, or low activity according to the criterion described in the Methods section. Pregnant dams were administered nicotine (6 mg/kg/day) or saline throughout gestation. Offspring were tested on four different days at 21–25 days of age. Values are mean ± SEM. Number of animals is presented in parentheses.

significance (Fig. 2). There were no differences in receptor concentrations in the hippocampus, thalamus, or colliculi for male offspring that received saline or nicotine prenatally (data not shown). Hyperactive female offspring did not show any significant changes in nicotinic receptors in any of the examined regions compared with middle- or low-activity groups in either saline or nicotine prenatal treatment conditions (Figs. 1, 2; data for other brain regions not shown). Likewise, neither male nor female hyperactive offspring of saline-exposed dams showed any significant changes in receptor concentrations compared with middle- or low-activity groups of similar treatment (Figs. 1, 2; data for other brain regions not shown).

Scatchard analysis of the receptor binding in the cortex of hyperactive and hypoactive male groups also was performed. The results of this analysis confirmed the increase in receptor concentrations (B_{max}) in hyperactive offspring that were exposed to nicotine prenatally compared with other groups [$F(3, 20) = 3.5, p < 0.05$]. Binding affinity was similar in all groups (K_d values ranged from 0.58 to 0.63 nM, $n = 6$/group), suggesting that observed effects in nicotinic receptors were restricted to changes in receptor densities.

DISCUSSION

This study indicates that hyperactivity in male offspring induced by prenatal nicotine exposure is associated with an increase in neuronal nicotinic receptors in the cortex and possibly the striatum. Increases in central nicotinic receptors following

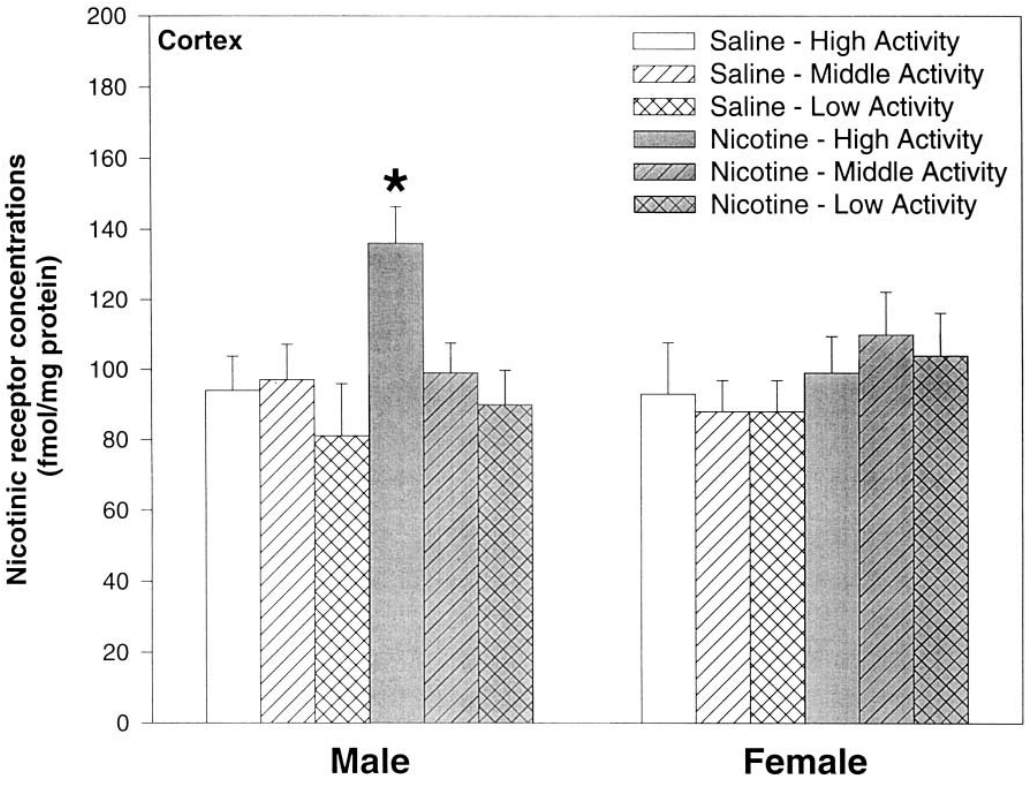

FIG. 1. Nicotinic receptor concentrations (fmol/mg protein) in the left cerebral cortex of 35-day-old male and female offspring with high, middle, or low activity as defined in the text. Dams were implanted with osmotic minipumps for administration of saline or nicotine (6 mg/kg/day) throughout the gestation period. Values are mean ± SEM ($n = 5$–9). *$p < 0.05$ compared with other male groups.

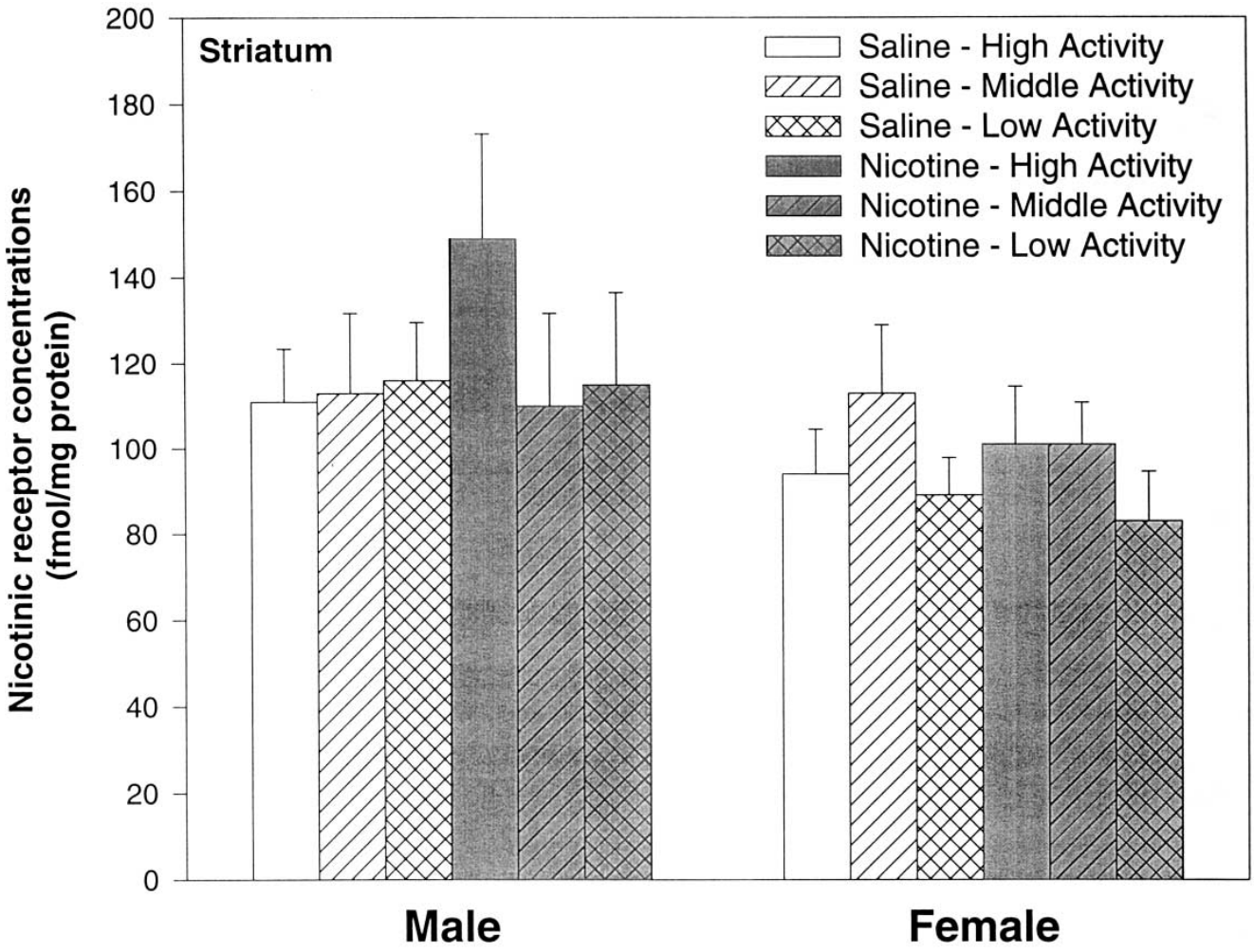

FIG. 2. Nicotinic receptor concentrations (fmol/mg protein) in the striatum of 35-day-old male and female offspring with high, middle, or low activity as defined in the text. Dams were implanted with osmotic minipumps for administration of saline or nicotine (6 mg/kg/day) throughout the gestation period. Values are mean ± SEM (n = 5–9).

prenatal exposure to nicotine have been reported in rats and mice (15,41,46). The present findings suggest that the increase in nicotinic receptors may occur primarily in offspring that manifest hyperactivity. It is important to note that hyperactive offspring of saline-treated dams did not show a significant increase in their cortical or striatal nicotinic receptor densities compared with low- or middle-activity groups. This apparent dissociation between nicotinic receptor densities and hyperactivity per se suggests that cortical or striatal nicotinic receptors may not be involved in this behavioral disorder. Therefore, the increases in nicotinic receptor densities in hyperactive male offspring of nicotine-treated dams may be due to the susceptibility of this particular group to nicotine. However, it remains to be determined whether nicotinic receptors in other specific brain regions (e.g., nucleus accumbens or mesolimbic system) may play a role in hyperactivity disorder.

Interestingly, prenatal exposure to nicotine seems to affect nicotinic receptor concentrations of male offspring only. Sex differences in behavioral and neurochemical effects of prenatal nicotine exposure have been reported by other investigators (12,13,18,19,29,30,35). In one study (35), in utero exposure to nicotine induced hyperactivity in 15-day-old male offspring only. Similarly, another investigation (13) found prenatal exposure to nicotine to result in decreases in dopamine D2 receptor concentrations in the striatum of 14-day-old male offspring only. These findings may indicate that: a) males are more sensitive to nicotine's prenatal effects on some behavioral and biological variables, b) the receptor changes in response to prenatal nicotine exposure differ qualitatively in males and females, or c) the sex differences result from different responses to nicotine abstinence. The third alternative is unlikely in the present experiment because of the amount of time (i.e., 35 days) after cessation of nicotine administration. Further empirical evaluations are necessary to assess the first two alternative explanations.

Increases in nicotinic receptor densities have been reported in adult rats and mice following chronic nicotine administration (21,36). This phenomenon is brain region specific (34) and is associated with a functional downregulation of the receptors (21,49). The numeric upregulation of nicotinic receptors following chronic administration of nicotine might be the result of a decrease in the rate of receptor turnover (28).

In prenatally exposed animals, upregulation of nicotinic receptors may be associated with initial supersensitvity followed by subsequent functional downregulation (37,38). Therefore, it remains to be elucidated whether the increase in nicotinic receptor densities observed in the present experiment is associated with a functional downregulation or represents receptor sensitization.

In humans, exposure of the fetus to nicotine through maternal smoking of cigarettes is associated with increased incidences of behavioral and cognitive abnormalities, including hyperactivity and impaired attention during childhood (16,44). The present finding of an increase in nicotinic receptor densities in specific brain regions in association with hyperactivity in male offspring that were exposed to nicotine prenatally suggests susceptibility of certain offspring to behavioral and neurochemical effects of intrauterine nicotine exposure. It would be of interest to examine whether behavioral and neurochemical effects of prenatal exposure to nicotine last into adulthood and whether there are differential responses to nicotine treatment in various groups.

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