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Effects of prenatal nicotine on expression of nicotine receptor subunits in the fetal brain

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Abstract

Previous studies have suggested that prenatal exposure to nicotine is associated with abnormal development in fetuses, including fetal brain damage. The present study determined the effect of maternal administration of nicotine during different gestational periods on brain nicotine receptor subunits in fetal rats. Subcutaneous injections of nicotine in maternal rats from the early and middle gestation decreased fetal blood PO₂, increased fetal blood PCO₂ and hemoglobin, and decreased fetal brain weight. The nicotinic acetylcholine receptor (nAChRs) mRNA abundance in the fetal brain was significantly changed by prenatal treatment with nicotine during pregnancy. Fetal $\alpha 2$, $\alpha 4$, $\alpha 7$, and $\beta 2$ units were significantly increased in the brain by prenatal exposure to nicotine in rat fetuses. However, the expression of mRNA of fetal brain $\alpha 3$, $\alpha 5$, $\beta 3$, and $\beta 4$ units were not changed. The results showed that prenatal nicotine can change the development of both α and β subunits of nAChRs in the fetal brain at gene level in association with restriction of fetal brain growth and in utero hypoxia.

Keywords

Nicotine α subunits; Nicotine β subunits; Fetal brain

1. Introduction

A number of studies have demonstrated that maternal smoking is associated with increased perinatal morbidity, sudden infant death syndrome, neuropsychiatric disorders, conduct disorders, and lower IQ, as well as reduced birth weight (Coleman et al., 2004; Cnattingius, 2004; Farkas et al., 2007; Ahlberg and Bodin, 1991). Cigarette smoke contains over 4700 chemicals, including a wide array of toxic chemicals such as nicotine (U.S. Environmental Protection Agency and Office of Health and Environmental Assessment, 1992). Nicotine as a principal psychoactive component in tobacco smoke can activate nicotinic acetylcholine receptors (nAChRs). Nicotine crosses the placenta, and maternal smoking causes accumulation of nicotine in fetal tissues (Ankarberg et al., 2001; Dempsey and Benowitz, 2001), resulting in fetal growth restriction and impaired brain development. Animal models have been used to study short- and long-term consequences of exposure to nicotine in an attempt to link nicotine to the observed adverse effects found in babies. Although nicotinic anorexic properties have

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been well documented in adults, the effect of prenatal exposure to nicotine during different gestational periods and the development of nAChRs in the fetal brain is not clear.

The presence of both choline acetyltransferase enzyme activity (Candy et al., 1985) and nAChRs in the early stage of embryo suggests a critical role for nAChRs in fetal development (Adams, 2003; Vizi and Lendvai, 1999; McGehee et al., 1995). The nAChRs distribute widely throughout the central nervous systems (CNS), and play an important role in brain development. Most of the nicotine receptor subunits, including $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 7$, and $\beta 2$, $\beta 3$, $\beta 4$ subunits, were detected in the CNS (Gotti et al., 2007). Whether, and to what extent, prenatal exposure to nicotine affects nAChR subunits in the fetal brain is not clear. Therefore, in the present study, a high dose of nicotine was used daily on maternal rats from the early, middle, and late gestation, in study of the effects of repeated episodes induced by nicotine on the genes of fetal nAChRs in the brain.

2. Methods

2.1. Experimental animals and nicotine treatment

Pregnant Sprague–Dawley rats were housed in plastic cages in a room maintained standard 12/12-h light/dark cycle at 22 °C. Pregnant rats were divided into six experimental groups randomly. Nicotine (Sigma, St. Louis, MO) was administered in a dose of 1.5 mg/kg to pregnant rats subcutaneously twice daily at 8:00 a.m. and 2:00 p.m. from gestational day (GD) 3 to 21 (the early group), from GD 8 to 21 (the middle group), and from GD 15 to 21 (the late group), respectively. For each treatment, control groups were injected subcutaneously with the same volume of saline (0.9% NaCl). All solution was freshly prepared.

At GD 21, 2 h after nicotine administration, animals were anesthetized and 1.0 ml maternal blood was collected from the abdominalis aorta. Fetal blood samples were collected from the heart directly by intracardiac puncture, and pooled in fetuses from the same dam. All blood samples were collected in chilled heparinized syringes. Maternal and fetal blood PO₂, PCO₂, hemoglobin, and the electrolyte concentrations were determined with a Nova analyzer (Nova Biochemical, Model pHox Plus L, Waltham, MA) at 39 °C. Fetal body and brain weight was measured before and after they were dried in oven at 80 °C for 24 h to remove water. All procedures used in this study were approved by the Institute Animal Care Service.

2.2. Measurement of nAChRs mRNA

Total RNA was isolated from the fetal brain and was purified using a protocol adapted from published methods (YenPing et al., 2002). mRNA abundance of $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 7$ and $\beta 2$, $\beta 3$, $\beta 4$ subunits of nicotinic receptors in the fetal brain was determined using quantitative real-time RT-PCR. Primers were designed according to the GenBank database, and are shown in Table 1. Purification of total RNA from the fetal brain was performed using a protocol adapted from published methods (YenPing et al., 2002). To isolate total RNA, fetal brain tissue was treated with TRIZOL (Invitrogen Life Technologies, USA). RNA was then isolated, precipitated, and stored in 75% ethanol at –70 °C until analysis. RNA was only used if the ratio between spectrophotometer readings (260 nm:280 nm) were between 1.8 and 2.0, denoting minimum contamination from cellular proteins. One microgram of total RNA was used, a reverse transcription and first strand cDNA synthesis was performed using MMLV-RT reverse transcriptase (Invitrogen Life Technologies, USA). Samples were digested with DNase I to degrade any DNA present in the RNA isolation. The first strand cDNA was checked using the housekeeping gene to assess the quality of the reverse transcription. PCR products were run on a 1.5% agarose gel. First strand synthesis was achieved using 2 µg of total RNA. Each representative cDNA pool was aliquoted and stored at –20 °C until required.

nAChRs specific PCR assay was optimized using total RNA from the fetal brain in the absence of SYBY Green I (TaKaRa, Japan). CT is the threshold cycle that reflects the cycle number at which the fluorescence generated within a reaction crosses the threshold (baseline). The CT value was assigned to a particular PCR plate well, and thus reflects the point during the reaction at which a sufficient number of amplification has accumulated. CT values were collected at linearity and used to calculate the 18S rRNA corrected CT (or Δ CT) for nAChRs gene. Values from the control and experiment groups were then used to calculate the mean corrected difference in CT for nAChRs gene ($\Delta\Delta$ CT $\pm$ S.E.). The extent of the response is determined by $2 - \text{mean}(\Delta\Delta$ CT), while a negative value suggests repression of nAChRs gene expression, so the relative degree of response is calculated by $2 - \text{mean}(\Delta\Delta$ CT) (Kenneth et al., 2001). The internal control gene was used to normalize the PCR for the amount of RNA added to the reverse transcription reactions. Each assay contained three replicates and 18S rRNA as reference. After reverse transcription, Q-PCR was performed in a 96-well plate. 12.5 μ l 2 $\times$ SYBRGreen master mix (Takara, Japan), 0.5 μ l forward primer, 0.5 μ l reverse primer, 2.0 μ l cDNA, and 9.5 μ l dd water were added for a total volume of 25 μ l per well. The contents of the well were mixed by pipetting gently. The tubes were incubated in a thermocycler at 95 $^{\circ}$ C for 2 min. There were 45 cycles of PCR amplification performed consisting of denaturing at 95 $^{\circ}$ C for 30 s, annealing at 55 $^{\circ}$ C for 30 s, and extension at 72 $^{\circ}$ C for 30 s. Melt curves was run immediately after the last PCR cycle. Melt curves were produced by plotting the fluorescence intensity against temperature as the temperature was increased from 60 to 95 $^{\circ}$ C at 0.2 $^{\circ}$ C/s. Each assay contained three replicates and 18S rRNA as reference. Data was analyzed using the software and graphics programs provided with the iCycler (Bio Rad). For confirmation of amplification presence and purity, the PCR product was run on a 1.5% agarose gel.

2.3. Data analysis

Data are expressed as mean $\pm$ S.E.M., and the differences were evaluated for statistical significance ($p < 0.05$) using one-way analysis of variance (ANOVA) followed by a post-hoc test.

3. Results

3.1. Blood values

The nicotine treatment did not affect maternal blood PO₂ and PCO₂. However, both fetal PO₂ and PCO₂ were significantly decreased in nicotine-treated animals in all three groups, as compared to the controls (Fig. 1). Nicotine treatment starting from early and middle gestational stages, but not from the late gestation, significantly increased fetal blood hemoglobin (Fig. 2). Additionally, nicotine-increased fetal hemoglobin was significantly greater in the early group than that in the middle group (Fig. 2).

3.2. Fetal brain weight

Fetal body weight was significantly decreased by maternal nicotine administration starting from early, middle, and late gestational periods (Fig. 3). In contrast, fetal brain weight was significantly reduced only in the groups treated with nicotine from early and middle gestation, not from late gestation (Fig. 4).

3.3. nAChRs mRNA abundance

Expression of the nicotine subunits (α 2, α 3, α 4, α 5, α 7 and β 2, β 3, β 4) mRNA was detected in the fetal forebrain and hindbrain of both control and nicotine-treated animals by using real-time RT-PCR.

The nicotine treatment from the early gestation caused a significant increase in mRNA abundance of $\alpha 2$, $\alpha 4$, $\alpha 7$, and $\beta 2$ subunits in the fetal forebrain and hindbrain (Fig. 5). In contrast, $\alpha 3$, $\alpha 5$, $\beta 3$, and $\beta 4$ mRNA levels were not significantly changed by nicotine in the fetal forebrain and hindbrain (Fig. 5).

For rats treated with nicotine from the middle and late gestational periods, mRNA expression of the fetal brain α and β subunits also were changed according to their types. Only $\alpha 2$, $\alpha 4$, and $\alpha 7$ in α type, and $\beta 2$ in β type of tested subunits in both forebrain and hindbrain were increased significantly by fetal exposure to nicotine. In contrast, mRNA levels of fetal brain $\alpha 3$, $\alpha 5$, $\beta 3$, and $\beta 4$ were not changed significantly by nicotine (Figs. 6 and 7).

4. Discussion

The present study demonstrates that fetal exposure to nicotine not only induced brain growth restriction associated with fetal hypoxia, but also affected the brain development evidenced by differential changes in the nAChR subunits expression pattern in the fetus. Previous studies have demonstrated that high doses of nicotine can cause fetal growth restriction (Vik et al., 1996; Vogt, 2004). Three experimental periods of maternal nicotine administration ranged from 6 to 18 days during rat pregnancy were chosen in the present study in attempt to test which gestational stage is most risk for nicotine exposure to fetuses. We have demonstrated that the longer fetal exposure to nicotine, the severer of restriction of fetal body growth. Fetal growth restriction in human (Regnault et al., 2007), and in large and small animals (Bauer et al., 2007), can be caused by multiple mechanisms, including in utero hypoxia (Viswanathan et al., 2007). It has been shown that maternal nicotine administration decreases fetal blood PO_2 in animals including rats (Socol et al., 1982; Mao et al., 2007). Notably, fetal brain weight was also significantly decreased by nicotine treatment in the present study. After removing water from the brain tissue, the brain weight was still significantly lower than that in the control. This is consistent with the finding from other studies (Liu et al., 2004).

Although a previous study has shown that fetal exposure to nicotine increases nAChRs binding sites (Falk et al., 2005), to our knowledge, the present study was the first to demonstrate that chronic exposure to nicotine for different gestational periods differentially regulated nAChR subunits expression pattern in fetal forebrain and hindbrain. Heteromeric and homomeric nAChRs are widely distributed in the embryonic brain, and play a role in the maturation of brain structures during development. In the developing somatosensory cortex, $\alpha 7$ subunit mRNA expression is dependent on the thalamocortical innervation (Broide et al., 1996). In the present study, 3 α and 1 β subunits ($\alpha 2$, $\alpha 4$, $\alpha 7$, and $\beta 2$) were significantly changed in the fetal brain by chronic fetal exposure to nicotine. These changes of nAChRs expression in the brain is of great importance in both physiological and/or pathophysiological significance, because brain nAChRs have been demonstrated to play an important role in multiple central functions. For example, in utero exposure to nicotine led to behavioral, neurochemical, and cognitive abnormalities in animal offspring (Robinson et al., 2002; Tizabi and Perry, 2000; Tizabi et al., 2000). Nicotine-induced release of dopamine from striatal synaptosomes is mediated by both $\alpha 4$ and $\beta 2$ nAChRs, and hence the increase of these subunits in the fetal brain observed in the present study is likely to alter dopamine release in the CNS (Kulak et al., 1997; Oli et al., 2002). Consistent with the present finding, previous studies have shown that $\alpha 4$, $\alpha 7$, and $\beta 2$ nAChRs are increased by chronic nicotine treatment (Flores et al., 1992; Huang and Winzer-Serhan, 2006; Brody et al., 2006; Wada et al., 2007). Additionally, prenatal nicotine treatment increased levels of nicotine binding at birth in the hypothalamus, hippocampus, and the cortex (Van de Kamp and Collins, 1994). In addition to $\alpha 4$, $\alpha 7$, and $\beta 2$ subunits, the $\alpha 2$ subunit was also found increase remarkably in the fetal brain by nicotine treatment in the present study. However, other nAChRs subunits like fetal $\alpha 3$, $\alpha 5$, $\beta 3$, and $\beta 4$ were not changed in both fetal forebrain and hindbrain. Thus, the results indicate that exposure to prenatal nicotine during

pregnancy can impact on the development of central cholinergic systems at sites of N-receptors, and this influence is at level of gene expression. Furthermore, the influence of prenatal nicotine on expression of the nAChRs in the fetal brain during in utero developmental stages could be different on various subunits.

Although major focus of the present study was on the effects of prenatal nicotine on gene expression of different nAChRs subunits in the fetal brain, we also paid attention to possible mechanisms linked to the gene responses following the prenatal treatment. Therefore, rat fetal blood oxidative status was tested. Notably, fetal blood PO₂ was significantly decreased by a high dose of nicotine administrated chronically and repeatedly. Furthermore, fetal hemoglobin was increased, probably as compensation to hypoxia, suggesting oxidative stress was present in utero. Hypoxia could be one of the mechanisms for changes of expression of nAChRs. Previous study reported that hypoxia in PC12 cells increased $\alpha 7$ nAChRs expression (Utsugisawa et al., 2000). Therefore, fetal hypoxia induced by prenatal nicotine may contribute to the changes of expression of central subunits of nAChRs associated with restricted brain growth.

Beside of potential contribution of in utero hypoxia, other mechanisms are also possible. For example, nicotine per se as toxic chemical may affect the development of nAChRs in the fetal brain. It is known that nicotine from maternal side can easily pass the placental barrier and be rapidly absorbed into the fetal bloodstream (Ankarberg et al., 2001; Dempsey and Benowitz, 2001). Considering a high dose of nicotine was used in the present study, the substance in the fetal circulation may act directly on the brain nAChRs. Notably, some α and β subunits were changed following prenatal nicotine, while the others not. In light of this, in utero hypoxia and/or nicotinic neurotoxin induced changes of nAChRs expression at gene level were various according to the receptor subunits. The finding from the present study offers new opportunity to determine which mechanism (nicotine-induced hypoxia or nicotine per se as neurotoxin) plays a major role in the effects observed here.

In conclusion, the present study showed that prenatal nicotine can change the development of both α and β subunits of nAChRs in the fetal brain at the gene level in association with restriction of fetal brain growth. Furthermore, we found that the influence of prenatal nicotine on the fetal brain nAChRs differs among subunits of the nAChRs in association with fetal oxidative stress in utero. Because the nAChRs are closely related to brain functions, an impaired development of those gene expressions may induce brain disorders.

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Fig. 1. The effect of subcutaneous injection of nicotine in maternal rats on fetal blood PCO₂, PO₂. Control: the control animals; early; maternal s.c. nicotine from GD 3 to 21; Middle: maternal s.c. nicotine from GD 8 to 21; later: maternal s.c. nicotine from GD 15 to 21. * vs. control group.

Fig. 2.

The effect of subcutaneous injection of nicotine in maternal rats on fetal blood hemoglobin. Control: the control animals; early; maternal s.c. nicotine from GD 3 to 21; middle: maternal s.c. nicotine from GD 8 to 21; later: maternal s.c. nicotine from GD 15 to 21. * vs. control group, * vs. middle group.

Fig. 3.

The effect of subcutaneous injection of nicotine in maternal rats on fetal body wet and dries weight. Control: the control animals; early; maternal s.c. nicotine from GD 3 to 21; middle: maternal s.c. nicotine from GD 8 to 21; later: maternal s.c. nicotine from GD 15 to 21. * vs. control group, * * vs. late group.

Fig. 4.

The effect of subcutaneous injection of nicotine in maternal rats on fetal brain wet and dries weight. Control: the control animals; early; maternal s.c. nicotine from GD 3 to 21; middle: maternal s.c. nicotine from GD 8 to 21; later: maternal s.c. nicotine from GD 15 to 21. * vs. control group.

Fig. 5. $\alpha 2$, 4, 7 mRNA, and $\beta 2$ mRNA expression compared the control group in fetal forebrain and hindbrain on nicotine treating from GD3 to GD21. * vs. control group.

Fig. 6. $\alpha 2$, 4, 7 mRNA, and $\beta 2$ mRNA expression in the fetal forebrain and hindbrain following maternal subcutaneously injection of nicotine from GD8 to GD21. * vs. control group.

Fig. 7.

$\alpha 2$, 4, 7 mRNA, and $\beta 2$ mRNA expression in the fetal forebrain and hindbrain following maternal subcutaneously injection of nicotine from GD15 to GD21. * vs. control group.

Table 1

Gene name	Accession	Primer	Product length (bp)
$\alpha 2$	NM133420	F: 5'-gcagcatcgatgtgacctttccc (position 783) R: 5'-tataggttccggtggcattgata (position 956)	174
$\alpha 3$	NM052805	F: 5'-tgttctacctgccctccgactg (position 765) R: 5'-cagcgaggtggaatggtct (position 879)	115
$\alpha 4$	NM024356	F: 5'-tccgctttggcttgccattgct (position 317) R: 5'-Tcccagcgagctttagtcgtg (position 206)	112
$\alpha 5$	NM017018	F: 5'-tggacgcaaccagcaactacaa (position 535) R: 5'-Caacctgggatccatcgatgtcca (position 652)	118
$\alpha 7$	NM012832	F: 5'-Cctatggagggtggctcactggac (position 515) R: 5'-gacatctgggtatggctctttgc (position 660)	145
$\beta 2$	NM019297	F: 5'-tcattcgtcgaaccactcttc (position 871) R: 5'-Caccacagtctgagggcagtag (position 975)	105
$\beta 3$	NM133597	F: 5'-Aggtgtagttggcatttgag (position 1768) R: 5'-tcagggatgagcagaggagtag (position 1851)	84
$\beta 4$	NM052806	F: 5'-gccgttctctgtgggtgttcgtg (position 1388) R: 5'-gagtccttgagggtgcgtggat (position 1484)	97
18S rRNA	M11188	F: 5'-cttagttggtggagcgattgtctg (position 1353) R: 5'-gttattgctcaatctcgggtggc (position 1500)	148