Involvement of α7 Nicotinic Acetylcholine Receptors in Gene Expression of Dopamine Biosynthetic Enzymes in Rat Brain

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ABSTRACT

Brain dopaminergic systems are critical in mediating the physiological responses to nicotine. The effects of several concentrations of nicotine (0.08, 0.17, or 0.33 mg/kg body weight) and involvement of α7 nicotinic acetylcholine receptors (nAChRs) in gene expression of key enzymes in dopamine biosynthesis were evaluated in the ventral tegmental area (VTA) and substantia nigra (SN), cell bodies of the mesocorticlimbic and nigrostriatal pathways. Nicotine elicited a dose-dependent elevation of mRNA for tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine biosynthesis in VTA and SN. The VTA was more sensitive to lower concentrations of nicotine with maximal response observed with the lowest dose of nicotine. Nicotine also elevated mRNA levels of TH’s essential cofactor tetrahydrobiopterin (BH4) in both dopaminergic locations. The changes in TH and GTPCH mRNAs were correlated. Pretreatment with the α7 nAChR antagonist methyllycaconitine prevented the nicotine-induced rise in TH or GTPCH mRNA in VTA and SN. Administration of α7 nAChR agonist 3-[2,4-dimethoxybenzilidene]anabaseine at 1 to 10 mg/kg or (E,E)-3-(cinnamylidene)anabaseine at 0.3 to 1 mg/kg increased TH mRNA in VTA and SN, but not in peripheral catecholaminergic cells. Thus, agonists of α7 nAChRs have therapeutic potential for increasing TH gene expression in dopaminergic regions without some of nicotine’s disadvantages, such as its harmful effects on the cardiovascular system. The findings indicate that nicotine may regulate dopamine biosynthesis by alterations in gene expression of TH and its cofactor. The α7 nAChRs are involved in mediating these effects of nicotine.

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ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor; TH, tyrosine hydroxylase; SN, substantia nigra; VTA, ventral tegmental area; BH4, tetrahydrobiopterin; MLA, methyllycaconitine; DMXB, 3-[2,4-dimethoxybenzilidene]anabaseine; 3CA, (E,E)-3-(cinnamylidene)anabaseine; GTPCH, GTP cyclohydrolase I.
Although TH is the rate-limiting step in biosynthesis of dopamine, as well as of other catecholamines, it is also dependent on levels of its essential cofactor, tetrahydrobiopterin (BH4) (Nagatsu and Ichinose, 1999). Intraventricular administration of BH4 increases the biosynthesis of dopamine in nigrostriatal neurons (Kettler et al., 1974). Moreover, patients with Parkinson's disease and dementia not only have reduced levels of dopamine but also reduced levels of BH4. These observations suggest that nicotine's effect on the dopaminergic system may be influenced by regulation of BH4 as well as of TH. The effect of nicotine on biosynthesis of BH4 has not been examined previously.

Nicotine mediates its actions through nAChRs, which exist as a variety of subtypes. Diverse nAChRs are formed as pentamers from different combinations of α- and β-type subunits (for review, see Leonard and Bertrand, 2001). Receptors containing α4β2 and α7 subunits are, respectively, the major high- and low-affinity nAChRs in the brain. Both types of receptors are present in the SN and VTA (Tsuneki et al., 2000). Previously, we found that the α7 nAChR subtype is required for the nicotine-elicted elevation of TH mRNA in PC12 cells. Moreover, α7 receptor agonists were at least as effective as nicotine in triggering these effects (Gueorguiev et al., 2000). The α7 subunits can form a homopentameric receptor, which is reported to desensitize rapidly compared with other nAChR subtypes. Their high Ca\(^{2+}\) conductance could enable α7 nAChRs to take part in synaptic mechanisms in which calcium acts as a second messenger (Pugh and Berg, 1994). Whether these receptors play a role in regulation of gene expression of dopamine biosynthetic enzymes in vivo is unclear. Information regarding their role in the rewarding properties of nicotine and the withdrawal syndrome are contradictory. Studies by Schilstrom et al. (1998), Nomikos et al. (1999), and Panagis et al. (2000) indicate involvement of α7 nAChRs in nicotine-induced dopamine release in the nucleus accumbens, and in locomotion, whereas studies by Grottick et al. (2000) suggest a negligible role for α7 nAChRs in nicotine-induced hyperlocomotion and reward.

Herein, we examine nicotine's effect on TH and GTPCH gene expression in the cell bodies of the nigrostriatal and mesocorticolimbic dopaminergic systems. The potential involvement of α7 nAChRs is evaluated, as well as the ability of specific nAChR agonists to elicit the effects of nicotine in central and peripheral catecholaminergic regions.

### Materials and Methods

**Animals, Drug Treatment, and Doses.** All animal experiments were approved by the Animal Care and Use Committee. Adult, pathogen-free, male Sprague-Dawley rats (250 to 300 g) were purchased from Taconic Farms (Germantown, NY) and housed four per cage. They were maintained under controlled conditions on a 12-h light/dark cycle at 23 ± 2°C. Animals were given food and water ad libitum.

The (−)-nicotine-ditartrate (Sigma/RBI, Natick, MA) was freshly dissolved in saline and used for injections. Several doses of nicotine (0.087, 0.175, or 0.35 mg of nicotine base per kilogram of body weight in saline) were administrated by five subcutaneous injections in the nape of the neck every 12 h. Animals were euthanized by decapitation 3 h after the last injection. All groups contained six to eight animals.

In some experiments, rats were injected intraperitoneally with methyllycaconitine (MLA, 4.2 mg/kg; Sigma-Aldrich, St. Louis, MO), which was shown to pass the blood-brain barrier (Turek et al., 1995). MLA was administered 15 min before the subcutaneous injections of nicotine (0.175 mg/kg) or saline. In other experiments, α7 nAChR agonists 3-[2,4-dimethoxybenzylidene]anabaseine (DMXB, also known as GST-21) and E,E-3-(cinnamylidene)anabaseine (3CA) (Meyer et al., 1997), gifts from Dr. Edwin M. Meyer (University of Florida, Gainesville, FL), were used. Several doses (1–10 mg/kg for DMXB and 0.3–1 mg/kg for 3CA in saline) were administered by intraperitoneal injections five times, 12 h apart.

Immediately after decapitation, blood was collected into EDTA-containing tubes on ice, centrifuged 20 min at 4000g, and plasma was kept at −70°C for determination of cotinine levels. Frontal sections 4.8 to 5.5 mm from the bregma were taken using a tissue slicer with digital micrometer (Stoeltin, Wood Dale, IL) and placed in ice-cold saline. The SN and VTA were punched and immediately frozen in liquid nitrogen.

**Isolation of RNA and Northern Blots.** The levels of mRNAs for TH or GTPCH were determined by Northern blot analyses as described previously (Serova et al., 1999b). Briefly, the brain punches from each animal were homogenized in RNA-Stat 60 (Tel-Test, Friends Woods, TX). Total RNA was then isolated and fractionated on 1.2% agarose gels. The RNA was transferred to Gene-Screen Plus membranes (PerkinElmer Life Sciences, Boston, MA), and hybridizations were subsequently performed with rat (\(^{32P}\))UTP-labeled GTPCH cRNA or with cDNA probes labeled with (\(^{32P}\))dCTP for rat TH and 18S rRNA as a control. Hybridization with cDNA probes was performed at 42°C with RNA probe at 68°C in ULTRAhyb solution (Ambion, Austin, TX). After washing, the blots were exposed to BioMax film (Eastman Kodak, Rochester, NY) within the linear range of the signal. Autoradiograms were scanned and analyzed by using Image-Pro Analysis software (Media Cybernetics, Silver Spring, MD). The values for TH and GTPCH mRNA were normalized to levels of 18S rRNA.

**Determination of Plasma Cotinine Concentrations.** Plasma was freshly prepared and kept at −70°C until the assay. Levels of cotinine, a principal blood and urinary nicotine metabolite, were measured by Double Antibody Nicotine Metabolite 125I RIA kit (EURO/DPC Ltd., Gwynedd, UK) according to the manufacturer's protocol. The standard curve was determined using cotinine, concentrations that ranged from 100 to 15,000 ng/ml. The interassay coefficients of variation were less than 5%. Plasma (0.025 ml) from saline- or nicotine-treated rats was combined with 0.1 ml of 125I-cotinine (0.175 ml of nicotine metabolite antiserum was 0.03 ml). Tubes were incubated at room temperature for 30 min. After centrifugation and precipitation, the 125I-labeled precipitates were counted in a gamma counter.

**Statistical Analysis.** The data were analyzed by one-way analysis of variance followed by Fisher's least significant difference test for comparison of the means (for more than two experimental groups) or Student's t test (for two experimental groups). Comparison between changes in TH and GTPCH mRNAs was determined by correlation analysis. Levels of p < 0.05 were considered significant.

### Results

**Effect of Different Doses of Nicotine Injections on TH and GTPCH Gene Expression.** Injections of 0.35 and 1.75 mg/kg nicotine were previously shown to elevate rat TH mRNA levels in VTA and SN (Serova et al., 1999a). Therefore, we studied whether lower concentrations of nicotine are also effective in triggering elevated mRNA levels. Rats were injected with several concentrations of nicotine (0.087, 0.175, and 0.35 mg/kg in saline), five times at 12-h intervals. Animals were euthanized 3 h after the last injection and the levels of cotinine, the major nicotine metabolite, were determined in plasma. The concentration of cotinine was 26 ± 3.2 ng/ml in animals treated with 0.087 or 0.175 mg/kg nicotine.
and was elevated to \(44 \pm 7.1\) ng/ml with 0.35 mg/kg nicotine. With injections of the highest dose of nicotine (0.35 mg/kg), plasma cotinine was \(320 \pm 46\) ng/ml.

RNA from SN and VTA from each individual animal was isolated, and relative levels of TH and GTPCH mRNAs were determined by Northern blots. Nicotine injections elicited a significant dose-dependent effect on TH mRNA levels in VTA \(F_{3,15} = 5.14, p \leq 0.012\) as well as in SN \(F_{3,15} = 3.37, p \leq 0.032\) (Fig. 1). In VTA, the maximal increase in TH mRNA was observed with the lowest concentration of nicotine. The extent of induction was smaller with higher nicotine concentrations. In contrast, in the SN, higher concentrations of nicotine (at least 0.175 mg/kg) were required to trigger significant elevations in TH mRNA. As in previous experiments (Serova et al., 1999a), 0.35 mg/kg nicotine increased TH mRNA levels in VTA and SN. The intermediate dose of nicotine (0.175 mg/kg) was also sufficient to elevate the mRNA levels in both locations, whereas the lowest dose (0.087 mg/kg) raised TH mRNA only in VTA.

Next, we examined whether nicotine alters mRNA for GTPCH, the rate-limiting enzyme for biosynthesis of BH4, an essential cofactor for TH (Fig. 2). In the VTA, nicotine injections elicited a dose-dependent rise of GTPCH mRNA levels \(F_{3,15} = 4.27, p \leq 0.024\). The levels of GTPCH mRNA in the VTA of animals injected with 0.087 and 0.175 mg/kg nicotine were significantly higher than in controls, whereas the highest concentration failed to significantly increase GTPCH mRNA. In SN, a significant, although modest, elevation in GTPCH mRNA was observed only with 0.175 mg/kg nicotine. High doses of nicotine (1.75 mg/kg) had no significant effect on GTPCH mRNA in either the VTA or SN (data not shown).

Correlation analysis was used to determine the relationship between the effects of nicotine on TH and GTPCH mRNA levels. A significant correlation \((p < 0.01)\) was found in both dopaminergic locations. In the VTA, the correlation coefficient was 0.73, and in the SN it was 0.48.

**Role of \(\alpha7\) nAChRs in Regulation of TH and GTPCH mRNA Levels.** To examine which nicotine subtype is involved in the observed changes in TH and GTPCH mRNAs, MLA, a specific antagonist of the low-affinity \(\alpha7\) nAChR was used. It was injected (i.p.) 20 min before each injection of nicotine (0.175 mg/kg) or saline. MLA by itself did not significantly change TH and GTPCH mRNA levels in either VTA or SN. However, MLA prevented the effect of nicotine on TH and GTPCH mRNA levels in both these areas (Figs. 3 and 4). These findings suggest that activation of \(\alpha7\) nAChRs is required for nicotine-elicited elevation of TH and GTPCH mRNA levels in these dopaminergic locations.

To further delineate \(\alpha7\) nAChR involvement, DMXB and 3CA, two specific agonists, were used. Several doses of these

![Ventral Tegmental Area](image1)

**Fig. 1.** Effect of different doses of nicotine injections on TH mRNA levels in VTA and SN. Rats were injected with the indicated concentrations of nicotine (n) in saline, as described under Materials and Methods. RNA from brain tissue punches obtained from individual animals was analyzed separately by Northern blot. Representative Northern blots and summary data are shown for TH mRNA levels. Data are expressed as mean ± S.E., with levels of TH mRNA in the saline-treated control group taken as 1. *, \(p < 0.05\) versus control; †, \(p < 0.05\) versus 0.087 mg/kg nicotine.
drugs were administered (five i.p. injections, 12 h apart). Animals injected with 3CA displayed a significant rise in TH mRNA levels in both dopaminergic cell bodies of SN and VTA (Fig. 5). The SN responded to lower concentrations of DMXB than the VTA. DMXB at the doses of 1, 3, or 10 mg/kg increased TH mRNA levels in SN, whereas in VTA, 10 mg/kg (but not 1 mg/kg) DMXB triggered elevations of TH mRNA. Levels of TH mRNA under these conditions were also determined in peripheral catecholaminergic regions to evaluate potential side effects. In adrenal medulla and superior cervical ganglia, TH mRNA was not altered in rats treated with DMXB or 3CA, although nicotine itself elicited significant increases (Fig. 6).

**Discussion**

This study reveals, for the first time, that even low concentrations of nicotine are sufficient to elevate TH and GTPCH mRNA levels in both dopaminergic cell bodies of the mesocorticolimbic and nigrostriatal dopamine systems. Interestingly, the VTA is more sensitive than the SN to lower concentrations of nicotine. Thus, injections of 0.087 mg/kg nicotine significantly increased TH and GTPCH mRNA levels in VTA, but not in SN, of the same animals. This selective sensitivity to nicotine could be important for its rewarding properties. In the VTA, the lowest dose of nicotine was most effective. In contrast to their effect in the brain, these concentrations of nicotine are lower than required to increase gene expression of catecholamine-biosynthetic enzymes in adrenal medulla (Slotkin et al., 1976; Fossum et al., 1991; Hiremagalur and Sabban, 1995; Serova et al., 1999a). This might be partially due to the fact that nicotine administration results in higher effective concentrations in the brain compared with its concentration in periphery (for review, see Benowitz, 1990) or to lessened sensitivity to nicotine in the adrenal medulla.

The study reveals that the α7 nAChR subtype is involved in the nicotine-triggered elevation of mRNA for TH and GTPCH. These findings are based both on administration of MLA, the specific α7 nAChR antagonist, and two α7 nAChR agonists. MLA antagonized the ability of nicotine to induce TH or GTPCH mRNAs in both SN and VTA. In this regard, an equivalent dose of MLA, administered similarly, was found to inhibit nicotine’s effect on footshock stress-elicited mesoprefrontal DA metabolism and immobility response (George et al., 2000a,b). Because MLA was administered by intraperitoneal injections, the specific location of the α7 nAChRs involved is not yet clear. It remains to be determined whether administration of MLA directly to the region of the VTA would have a similar effect. However, α7 nAChRs have been found in the region of the VTA, specifically on glutama-
tergic inputs to the DA neurons of the VTA (Schilstrom et al., 1998; Panagis et al., 2000).

There are contradictory studies regarding the importance of \(\alpha 7\) nAChRs in nicotine's effect on brain dopaminergic systems. Several studies indicate that \(\alpha 7\) nAChRs of the VTA mediate the rewarding effects, withdrawal syndrome, and reinforcing actions of nicotine. Nicotine-induced dopamine output in the nucleus accumbens was blocked by pretreatment with MLA in the VTA, indicating a role of \(\alpha 7\) nAChRs in this mechanism (Schilstrom et al., 1998). The \(\alpha 7\) nACh receptors in the VTA are reportedly involved in mediating the reinforcing actions not only of nicotine but also of cocaine (Panagis et al., 2000). Injection of MLA into the VTA of rats treated chronically with nicotine reduced their locomotion (Nomikos et al., 1999). In contrast, a study by Grottick et al. (2000) showed that agonists of the \(\alpha 7\) nAChRs, AR-R-17779 and DMAC [(\(-\))spiro[1-azabicyclo[2.2.2]octane-3,5’-oxazolidin]-2’-one and [4-[(1E,3E)-3-(5,6-dihydro-4H-[2,3’]bipyridyl-3-ylidene)-propenyl]phenyl]dimethyl-amine, respectively], failed to stimulate locomotion activity in both nicotine-nontolerant and sensitized rats, and MLA pretreatment did not reduce nicotine-triggered hyperlocomotion. The discrepancy between these studies on the involvement of \(\alpha 7\) nAChRs in nicotine-triggered locomotion and reinforcing behavior may be due to differences in the way MLA was administered or to the strain of rats.

The involvement of the \(\alpha 7\) nAChR was confirmed with the administration of \(\alpha 7\) nAChR agonists. DMXB and 3CA were able to elevate TH mRNA levels in VTA and SN. In contrast to the greater sensitivity of VTA to low concentrations of nicotine, the SN was more sensitive than the VTA to low concentrations of DMXB. Thus, mechanisms other than \(\alpha 7\) nAChR mediated might be involved in nicotine's action on TH in the VTA. The lower sensitivity of the VTA to the \(\alpha 7\) nAChR agonists, and the absence of an effect in the periphery are encouraging. Thus, \(\alpha 7\) nAChR agonists may be able to increase TH gene expression and dopamine synthesis, for example, in patients with Parkinson's disease or schizophrenia, without eliciting its addictive or pressor side effects.

The locus of the \(\alpha 7\) nAChR subunit gene has been genetically linked to schizophrenia (for review, see Leonard and Bertrand, 2001). Schizophrenics display a very high incidence of smoking, whereas nicotine is proposed to normalize a sensory gating deficit found in schizophrenics. In this regard, DMXB normalized an auditory evoked-potential deficit in mice with absence of sensory inhibition (Stevens et al., 1998).

The \(\alpha 7\) nAChRs are also implicated in modulating synaptic neurotransmission and in regulating neuronal growth, differentiation, survival, and memory (Meyer et al., 1997; Broide and Leslie, 1999; Jonnala and Buccafusco, 2001). Administration of DMXB enhanced a variety of cognitive behaviors in mice, monkeys, rats, and rabbits (Meyer et al., 1997; Kem, 2000). It is neuroprotective in cultured cells and in vivo to a variety of insults (Meyer et al., 1997, 1998; Li et al., 1999). Based on these findings, DMXB has shown promise as a possible therapeutic target for Alzheimer's disease (for review, see Kem, 2000).

In contrast to DMXB, 3CA has been less well studied. It was found to be even more effective than DMXB in eliciting a delayed but sustained rise in intracellular calcium in PC12 cells, and triggered marked elevations in TH and DBH mRNA levels (Gueorguiev et al., 2000). This 3-cinnamylidene anabaseine compound with mixed agonist/antagonist properties binds \(\alpha 7\) nAChRs efficaciously, activates \(\alpha 7\) nAChRs in a Xenopus oocyte expression system, and has low inhibitory activity. The concentrations of 3CA used in this study were
also found to improve passive avoidance behavior in nucleus basalis-lesioned rats (Meyer et al., 1998).

Although the drugs used in the present study target α7, it should be noted that DMXB has partial agonist and antagonist properties on 5-hydroxytryptamine3 receptors (Machu et al., 2001) and is also an antagonist of α4β2 receptors (Kem, 2000). Therefore, further studies are required to ascertain whether these actions are also involved in the DMXB-triggered changes in TH gene expression in the dopaminergic neurons observed in this study. In this regard, studies by Tsuneki et al. (2000) demonstrated that in mice nigral dopaminergic neurons, nicotine can elicit Ca2+ mobilization via activation of two distinct nAChRs, those containing β2 or α7 subunits. Therefore, nAChRs, other than the α7 subtype may also contribute to nicotine-triggered elevation of TH and GTPCH gene expression in the VTA and SN. However, the involvement of the α7 nAChR subtype is supported by the
ability of MLA to block the nicotine-triggered increase in TH and GTPCH mRNA levels.

The results of this study indicate that low concentrations of nicotine induced not only TH but also GTPCH gene expression in the SN and VTA and required the α7 nAChR subtype. The nicotine-triggered changes in TH and GTPCH mRNA levels were significantly correlated. These findings suggest that there may be a common mechanism for regulation of expression of these two genes by nicotine. Previous studies have found that in TH and GTPCH mRNA levels are often, but not always concomitantly regulated by physiological treatments, such as stress or administration of estradiol (Se rova et al., 1999b; Serova and Sabban, 2002). Both genes are responsive in cell culture to increases in cAMP and glucocor ticoids (Zhu et al., 1994; Serova et al., 1997).

Although BH4 was not measured in this study, intracellular concentrations of BH4 are determined mainly by its de generation, with GTPCH catalyzing the formation of BH4 from GTP (Nagatsu and Ichinose, 1999). In this regard, nicotine administration at a dose of 0.5 mg/kg was found to significantly increase BH4 levels in the striatum and the hypothalamus (Tsai and Lee, 1995). It has been shown that dopamine neurons contain exceedingly low levels of BH4 (Hirayama and Kapatos, 1998). Moreover, the number of TH molecules in brain dopaminergic nerve terminals is highly dependent on the intracellular concentration of BH4 (Sumi-Ichinose et al., 2001). This suggests that nicotine may activate biosynthesis of dopamine (and other catecholamines) not only by way of increased gene expression of TH but also through elevation of its essential cofactor.

The nicotine-elicited elevation of GTPCH mRNA levels found in this study, and the likely subsequent increase of BH4, may lead not only to activation of dopamine biosynthesis in cell bodies but also to protection of dopaminergic neurons. Recently, it has been shown that BH4, within dopaminergic neurons, is necessary and sufficient for maintaining lower reactive oxygen species (Nakamura et al., 2001). These multiple functions of BH4 may play a crucial role in biochemical and behavior recovery in 6-hydroxydopamine-lesioned rats (Parkinson’s animal model) with administration of adenosin-associate vectors expressing TH, aromatic-l-amino acid decarboxylase, and GTPCH. These rats display greater dopamine production and improvement in rotation behavior than rats that carry only TH and aromatic-l-amino acid decarboxylase viral vector (Shen et al., 2000). The findings of this study indicate that the neuroprotective effects of nicotine may also involve its regulation of biotyperin biosynthesis.

Thus, our results reveal that nicotine use of α7 nAChRs may regulate brain dopaminergic systems by alterations in gene expression of TH and GTPCH. Agonists of α7 nAChRs have therapeutic potential for increasing dopamine biosynthesis in the central nervous system without side effects on the sympathoadrenal system.

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References


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