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DEVELOPMENTAL REGULATION OF NICOTINIC ACETYLCHOLINE RECEPTORS WITHIN MIDBRAIN DOPAMINE NEURONS

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Abstract—We have combined anatomical and functional methodologies to provide a comprehensive analysis of the properties of nicotinic acetylcholine receptors (nAChRs) on developing dopamine (DA) neurons of Sprague–Dawley rats. Double-labeling *in situ* hybridization was used to examine the expression of nAChR subunit mRNAs within developing midbrain DA neurons. As brain maturation progressed there was a change in the pattern of subunit mRNA expression within DA neurons, such that α3 and α4 subunits declined and α6 mRNA increased. Although there were strong similarities in subunit mRNA expression in substantia nigra (SNc) and ventral tegmental area (VTA), there was higher expression of α4 mRNA in SNc than VTA at gestational day (G) 15, and of α5, α6 and β3 mRNAs during postnatal development. Using a superfusion neurotransmitter release paradigm to functionally characterize nicotine-stimulated release of [^{3}H]DA from striatal slices, the properties of the nAChRs on DA terminals were also found to change with age. Functional nAChRs were detected on striatal terminals at G18. There was a decrease in maximal release in the first postnatal week, followed by an increase in nicotine efficacy and potency during the second and third postnatal weeks. In the transition from adolescence (postnatal days (P) 30 and 40) to adulthood, there was a complex pattern of functional maturation of nAChRs in ventral, but not dorsal, striatum. In males, but not females, there were significant changes in both nicotine potency and efficacy during this developmental period. These findings suggest that nAChRs may play critical functional roles throughout DA neuronal maturation.

Key words: adolescent, fetal, nicotine, sex, striatum, maternal smoking.

Maternal smoking during pregnancy has been correlated with a number of adverse outcomes in the offspring (Lichtensteiger et al., 1988; Lichtensteiger and Schlumpf, 1993), including cognitive deficits that are often manifested in early childhood as attention deficit hyperactivity disorder (ADHD) (Millberger et al., 1996). Though the underlying neurobiological mechanism responsible for this disorder has been a matter of some controversy, alterations in development of central dopamine (DA) systems have been implicated (Lichtensteiger et al., 1988; Pliszka et al., 1996; Castellanos, 1997). In animal studies, maternal nicotine exposure produces sex-dependent alterations in the development of DA neurochemical markers (Fung, 1988, 1989; Lichtensteiger et al., 1988; Ribary and Lichtensteiger, 1989; Lichtensteiger and Schlumpf, 1993; Muneoka et al., 1997), and induces hyperactivity in the offspring, which is believed to result from alterations in mesolimbic and nigrostriatal DA systems (Schlumpf et al., 1988; Fung and Lau, 1989; Richardson and Tizabi, 1994; Tizabi et al., 1997; Ajarem and Ahmad, 1998). However, one point of intense controversy is whether the actions of nicotine are mediated directly on DA neurons or are the result of indirect effects such as hypoxia (Slotkin, 1998).

Adult midbrain DA neurons express a variety of nicotinic acetylcholine receptor (nAChR) subunit mRNAs, including α3–α7, β2 and β3 (Charpantier et al., 1998; Elliott et al., 1998; Sorenson et al., 1998; Klink et al., 2001; Azam et al., 2002). Nicotine has been shown to directly activate these neurons (Pidoplichko et al., 1997; Yin and French, 2000). Moreover, nicotine stimulates release of DA within the striatal target region by direct action on nicotinic receptors on DA terminals (Rapier et al., 1990; Grady et al., 1992; Clarke and Reuben, 1996; Wonnacott, 1997). Although this system has been extensively studied in the adult, not much is currently known about the properties of nAChRs within developing midbrain DA neurons.

The present study combined anatomical and functional methodologies to provide an extensive ontogenetic analysis of mRNA and protein expression, as well as functional development of nAChRs, within midbrain DA neurons. This study was undertaken to 1) determine if there are functional nAChRs in the fetus that can directly mediate effects of prenatal nicotine exposure on the development of midbrain DA neurons, 2) examine properties of nAChRs during the brain growth spurt, another period of nicotine susceptibility, which spans approximately the first three postnatal weeks in rats and corresponds to third trimester to 2nd year of human development, 3) compare the functional properties of nAChRs on dorsal and ventral striatal DA terminals between adolescent and adult rats and 4) examine possible sex differences in nAChR properties from birth until adulthood. We have used a double-labeling technique to examine nAChR subunit transcript expression within DA neurons. Nicotine-stimulated [^{3}H]DA release from striatal slices was also used to examine the functional

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Abbreviations: α-BTX, α-bungarotoxin; DA, dihydroxyphenylethylamine dopamine; Dig, digoxygenin; G, gestational day; GB, Genius buffer; nAChR, nicotinic acetylcholine receptor; NIC, nicotine; P, postnatal day; PB, phosphate buffer; RT, room temperature; SNc, substantia nigra; SSC, standard saline citrate; TH, tyrosine hydroxylase; VTA, ventral tegmental area; ^{35}S-UTP, ^{35}S-uridine triphosphate.

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status of nAChRs on DA terminals throughout development.

EXPERIMENTAL PROCEDURES

Materials

The following materials were obtained from the indicated sources: bovine serum albumin, polyvinylpyrrolidone, poly-L-lysine, RNase A, (−)nicotine bitartarate, D(+)-glucose and Hepes (Sigma-Aldrich, St. Louis, MO, USA); pBluescript II SK+ (Stratagene, La Jolla, CA, USA); anti-digoxygenin (Dig)-AP Fab antibody, Dig-UTP, Genius system nonradioactive nucleic acid detection kit, restriction enzymes, T3, T7 polymerases, proteinase K and yeast tRNA (Boehringer Mannheim Biochemicals, Indianapolis, IN, USA); formamide (Fluka, Ronkonkoma, NY, USA); dextran sulfate (Pharmacia, Piscataway, NJ, USA); Hyperfilm, Bmax (Amersham, Arlington Heights, IL, USA); nuclear track emulsion (NTB2) (Kodak, Rochester, NY, USA); ^{35}S-uridine triphosphate (^{35}S-UTP) and [^{3}H]dihydroxyphenylethylamine (DA) (specific activity: 20–40 Ci/mmol) (New England Nuclear, Boston, MA, USA). Ecolume scintillation cocktail (MP Biomedical, Solon, OH, USA). All other chemicals were purchased from Fisher Scientific (Pittsburg, PA, USA).

Animals

Pregnant Sprague–Dawley rats (Harlan, San Diego, CA, USA) were maintained in a temperature- (21 °C) and humidity- (50%) controlled room on a 12-h light/dark cycle (lights on 07:00–19:00 h) with unlimited access to food and water. All experiments were carried out in accordance with the Institutional Animal Care and Use Committee at the University of California, Irvine, and were consistent with federal guidelines and conformed to international guidelines on the ethical use of animals. None of the animals suffered during the course of these experiments and every effort was made to minimize the number of animals to that needed to obtain consistent and statistically significant data. Some dams were killed by decapitation at gestational day (G) 15 and G18 and their fetuses harvested by cesarian section. Other dams were allowed to deliver, with the day of birth designated as postnatal day (P) 0. Litters were culled to 10 pups after birth to ensure adequate maternal care, and were weaned at P21 and group housed with littermates of the same sex. Only one pup of each sex per litter was assigned to an experimental group for anatomical studies. For neurotransmitter release, striata of several pups of a given sex from a single litter were pooled to ensure adequate tissue for the study.

Double-labeling *in situ* hybridization

Tissue preparation. Males and females, aged G15, G18, P1, P4, P7, P14, P21, P30, P40 and adult (P60 and older) were decapitated and brains immediately removed and frozen in −20 °C isopentane. The frozen brains were stored at −70 °C until use. Twenty-micron sections were cryostat cut and mounted onto slides, which were coated with gelatin and poly-L-lysine and kept at −20 °C. Sections were postfixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, for 1 h at 22 °C, then washed in PB, air dried, and stored desiccated at −20 °C until use.

cRNA probe preparation. The cDNAs (length in base pairs) were kindly provided by Dr. J. Boulter (UCLA, Los Angeles, CA, USA). α3 (1858) And β3 (1780) were subcloned in pBluescript (pBS) II SK+, between *Eco*RI and *Hind*III and *Kpn*I and *Eco*RI sites, respectively. The remaining clones are as indicated: α4–1 (2110), cloned in pSP64 in the *Hind*III site, α5 (1607), α6 (1760), cloned in pBS SK(−) in the *Eco*RI site, α7 (2100), β2 (2196), cloned in pSP65 in the *Eco*RI site, and β4 (2522), cloned in pBS SK(−) in the *Eco*RI site. Plasmids were linearized with the appropriate restriction enzyme and ^{35}S-labeled riboprobes were synthesized in antisense and sense orientations by using ^{35}S-UTP, according to the method of Simmons et al. (1989). All probes were further subjected to alkaline hydrolysis to yield products with an average size of 600 bases according to Cox et al. (1984). Nonradioactive Dig-labeled cRNA probe for tyrosine hydroxylase (TH; 230 base pairs) was synthesized by using Dig-labeled UTP and appropriate transcription enzyme. The concentrations of the Dig-labeled probes were determined by dot blotting. The specificity of the cRNA probes used in the present study was extensively examined in Azam et al. (2002).

TH Dig/nAChR subunit mRNA colocalization. Tissue sections were processed for double labeling *in situ* hybridization as described previously (Winzer-Serhan and Leslie, 1997). Briefly, sections were pretreated with proteinase K (1 μg/ml) for 10 min at 22 °C, acetylated, dehydrated through graded ethanol (50, 70, 95 and 100%) and air-dried. Sections were then incubated for 18 h at 60 °C with 1:1 dilution of Dig-labeled antisense TH riboprobe (0.1 μg/ml): ^{35}S-labeled sense or antisense nAChR subunit probes (2×10^{7} cpm/ml) in hybridization solution (50% formamide, 10% dextran sulfate, 0.02% Ficoll, 0.02% polyvinyl pyrolidone, 0.02% bovine serum albumin, 500 μg/ml tRNA, 10 mM dithiothreitol, 0.3 M NaCl, 10 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0). After hybridization, sections were incubated with RNase A (20 μg/ml) for 30 min at 37 °C, followed by two 5-min and two 10-min high stringency washes of decreasing salinity (2×–0.5× standard saline citrate (SSC) buffer) at 22 °C and a 30 min wash in 0.1× SSC at 65 °C. After the hot wash, the slides were incubated in Genius buffer (GB) 1 (100 mM Tris–HCl, 150 mM NaCl, pH 7.5) for 1 min, followed by a 30-min wash in 5% non-fat dry milk in GB1+0.25% Triton-X (GB2) at 22 °C. The anti-Dig alkaline phosphatase conjugated Fab antibody (sheep), prepared as 1:5000 dilution in GB2, was applied by drop technique and slides incubated for 3 h at 37 °C. The slides were washed three times for 1, 5 and 10 min in GB1+0.25% Triton-X. Color reagent, composed of 50 μl NBT and 37.5 μl BCIP in 10 ml GB3 (100 mM Tris–HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) was applied and slides incubated overnight at 22 °C. The next day, the slides were washed twice in GB4 (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and once in double deionized water, dehydrated with brief dips in graded ethanol (50, 70, 95 and 100%), air dried and apposed to β-max film for an appropriate period of time. Following film development, slides were coated with 3% parlodion in isoamylacetate and dipped in liquid NTB2 emulsion on the reference date of the ^{35}S-UTP. After the appropriate exposure period, slides were developed in Kodak developer D-19, fixed, coverslipped and analyzed.

Data analysis. For each subunit, data from male and female rats were pooled to yield an $n=2$ for developmental ages G15–P10. For α5, α6, α7 and β3 subunits, an additional two animals per sex were prepared for ages P14, P21, P30, P40 and adult. Since there were no major sex differences in subunit expression at any of these ages, all data for males and females were pooled. For each animal, at least two sections per subunit were analyzed and averaged. The data presented for each subunit and each age are means±S.E.M. from all animals (males and females).

The hybridization signal for each nAChR subunit riboprobe was quantified on the autoradiographic films using a video-based computerized image analysis system (MCID, Image Research Inc., St. Catharines, Ontario, Canada). The total optical density of the hybridization signal was measured in the substantia nigra (SNc) and the ventral tegmental area (VTA), as identified by digoxigenin-labeled TH cells on the corresponding emulsion-dipped slide, as well as identifiable landmarks, such as interpeduncular nucleus and/or medial terminal nucleus of accessory optical tract (which visibly separated SNc from the VTA in some sections) (Paxinos and Watson, 1986), to delimit the VTA. The