Neuroscience 170 (2010) 503–513

PERSISTENT GENE EXPRESSION CHANGES IN VENTRAL TEGMENTAL AREA OF ADOLESCENT BUT NOT ADULT RATS IN RESPONSE TO CHRONIC NICOTINE

M. B. DOURA, T. V. LUU, N. H. LEE AND D. C. PERRY*

Department of Pharmacology and Physiology, George Washington University, Washington, DC 20037, USA

Abstract—Because adolescent brains are undergoing extensive developmental changes, they may be uniquely sensitive to effects of addictive drugs like nicotine. We exposed adolescent and adult rats to nicotine infusion for two weeks, and then used whole genome microarray analysis to determine effects on gene expression in the ventral tegmental area. We examined brains immediately after two weeks of nicotine or saline, and also four weeks after termination of nicotine exposure. After identifying genes with a significant age× treatment interaction, we employed template matching to find specific patterns of expression across age and treatment. Of those genes that were transiently regulated (up- or down-regulated immediately following the end of nicotine treatment, but back to saline baseline 30 days later), two-thirds were specific to adult animals, while only 30% were specific to adolescents and 4% were shared across the two ages. In contrast, significant genes that were persistently regulated (altered following nicotine treatment and still altered 30 days later) were more likely (59%) to be adolescent, with only 32% in adults and 8% shared. The greatest number of significant genes was late-regulated (no change immediately after nicotine, but regulated 30 days later). Again, most were in adolescents (54%), compared to adults (10%) or shared (36%). Pathway analysis revealed that adolescent-specific genes were over-represented in several biological functions and canonical pathways, including nervous system development and function and long-term potentiation. Furthermore, adolescent-specific genes formed extensive interaction networks, unlike those specific for adults or shared. This age-specific expression pattern may relate to the heightened vulnerability of adolescents to the effects of addictive drugs. In particular, the propensity of adolescents to show persistent alterations in gene expression corresponds to the persistence of drug dependence among smokers who began their habit as adolescents. These findings support a model whereby adolescent brains are uniquely vulnerable to long-term changes in gene expression in the brain's reward pathway caused by early exposure to nicotine.

Key words: nicotine addiction, adolescent drug abuse, long-term potentiation, nervous system development, nicotinic receptors.

*Corresponding author. Tel: +1-202-994-3544; fax: +1-202-994-2870.
E-mail address: phmdcp@gwumc.edu (D. C. Perry).

Abbreviations: Acb, nucleus accumbens; CPP, conditioned place preference; DA, dopamine; FDR, false discovery rate; GCOS, Gene Chip Operating Software; IPA, Ingenuity Pathway Analysis; LTP, long-term potentiation; NMDA, N-methyl D-aspartic acid; PN, post-natal day; RT-PCR, reverse transcriptase polymerase chain reaction; SD, sprague–dawley; VTA, ventral tegmental area.

Recreational drug use is commonly initiated during adolescence, often beginning with tobacco products. Nicotine has been shown to have a number of adverse affects on the brain, particularly during early development. Epidemiological studies have demonstrated that adolescent smokers proceed to dependence quicker, are more likely to be dependent as adults, have higher average consumption, and exhibit increased co-morbidity with other drug use and more psychological problems compared to adults (Chen and Millar, 1998; Chambers et al., 2003; Adriani et al., 2006). Behavioral studies have shown distinct responses to nicotine in animals treated during adolescence relative to their adult counterparts. Pre-exposure to nicotine during, but not following, adolescence sensitizes rats to nicotine's effects on locomotor response and conditioned place preference (CPP), and increases self-administration of nicotine (Adriani et al., 2006). Pre-exposure to nicotine during adolescence also sensitizes rats to the rewarding effects of other drugs, particularly psychostimulants such as cocaine (Collins and Izenwasser, 2004; McMillen et al., 2005; McQuown et al., 2007). These data strongly suggest an increased vulnerability to effects of nicotine during adolescence.

Adolescence is characterized by extensive physiological and psychological changes, and recent studies have shown the human brain continues to mature into the early 20s (Sowell et al., 2003). This is characterized by extensive growth during early adolescence, followed by a decrease in grey matter, a gradual loss of synapses, and a strengthening of the remaining synapses. Neurotransmitter receptor populations peak during adolescence and decline thereafter. This has been shown for GABA, serotonin, norepinephrine, dopamine, and acetylcholine receptors (Lujan et al., 2005; Crews et al., 2007). These changes occur throughout the brain and coincide with changes in complex social behaviors characterized by increased impulsivity, risk-taking, and sensation-seeking (Sowell et al., 2003). Exposure to nicotine during this period may have systemic affects which persist into adulthood and contribute to the observed increase in consumption of tobacco and other drugs of abuse, and the subsequent difficulty of quitting in those individuals that began smoking during adolescence.

We have previously shown that chronic nicotine exposure during adolescence differentially regulates nicotinic acetylcholinergic receptor subtype number in adolescent compared to adult male Sprague–Dawley (SD) rats (Doura et al., 2008). The goal of the current study was to identify the short-term and long-term changes in gene expression unique to, and shared between, adolescent and adult male SD rats exposed to chronic nicotine. We examined whole

0306-4522/10 $ - see front matter © 2010 IBRO. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuroscience.2010.06.071

genome expression in the ventral tegmental area (VTA) from nicotine- and saline-treated rats of both age groups. The VTA is the cell body region for the mesocorticolimbic dopamine reward pathway, and thus it is likely that gene expression in these cells may play important roles in mediating the addictive properties of drugs such as nicotine. We report striking differences in gene expression in response to chronic nicotine in a number of potentially relevant networks and pathways, including nervous system development and function, circadian rhythms, and long-term potentiation (LTP).

EXPERIMENTAL PROCEDURES

Treatment

Osmotic minipumps (Alzet model 2002; Durect Corporation, Cupertino, CA, USA) were filled with sterile saline or with nicotine hydrogen tartrate dissolved in saline, at concentrations designed to achieve a dose of 6 mg/kg/day, calculated as nicotine free base (37 μmol/kg). Minipumps were implanted into male Sprague–Dawley rats (Hilltop Lab Animals, Scottdale, PA, USA) of two ages, postnatal day (PN) 28–30 or PN 60–70; six animals were used for each treatment group. The period from PN 28–40 in rats is typically labeled periadolescence, that from PN 40–52 middle adolescence, and PN 52–60 late adolescence; "puberty" generally occurs during the last days of periadolescence (Spear, 2004). Thus, our treatment was performed at the early periadolescent and adult stages. Rats were anesthetized with isoflurane and the minipumps inserted into a subcutaneous pocket via a small incision made over the shoulders. While under anesthesia, animals were administered buprenorphine (0.1 mg/kg, s.c.) for post-operative pain. The wound was closed with clips and the area swabbed with antiseptic. After recovery from anesthetic (10–30 min), animals were returned to individual cages. Fourteen days after minipump implantation (PN 42–44 for adolescents, PN 74–84 for adults), some animals were lightly anesthetized with isoflurane and sacrificed by decapitation. The remaining animals were left untreated for 30 days (minipumps empty 14 to 17 days after implant, so animals were nicotine-free for from 27 to 29 days), during which time the adolescents reach full adulthood. The remaining animals were then sacrificed as described. Altogether, there were three treatment groups, comprised of saline, nicotine, and nicotine+30 days (nicotine withdrawal), for both the adolescent and adult animals. All measures were taken to minimize the numbers of rats used and their suffering. Animal use and procedures were approved by the George Washington University Medical Center Institutional Animal Care and Use Committee.

Tissue preparation

Rats were anesthetized with isoflurane and decapitated; brains were quickly removed and frozen on dry ice. Brain slices were made with a cold stainless steel rat brain slicer matrix (Zivic Instruments, Pittsburgh, PA, USA) with 1 mm coronal slice section intervals. Brain slices were immediately placed in cold saline and four punches (two from each side) were taken from the VTA of each animal using a 500 micron tissue biopsy punch (Zivic Instruments, Pittsburgh, PA, USA). Identification of tissue was done by reference to a rat brain atlas (Paxinos and Watson, 2007); coronal sections were used from −4.6 mm to −5.0 mm from bregma. Tissue punches were stored in RNAlater (Qiagen, Valencia, CA, USA) at −80 °C.

Total RNA isolation and gene expression profiling

Each VTA sample (representing an independent biological replicate) consisted of a pool from two animals (a total of eight punches). Total RNA was isolated and purified using Trizol reagent (InVitrogen, Carlsbad, CA, USA) and the RNeasy micro kit (Qiagen), respectively, according to the manufacturers' instructions. The purified RNA was amplified using the WT-Ovation Pico RNA Amplification System (Nugen, San Carlos, CA, USA) according to the manufacturer's instructions. cRNA target synthesis, hybridization onto the Rat 230 2.0 GeneChip, and posthybridization staining and scanning were performed using standard protocols as recommended by the manufacturer (Affymetrix, Santa Clara, CA, USA). Chip data were scaled using GeneChip Operating Software (GCOS; Affymetrix) version 1.4 and expression values were $\log_2$-transformed. In total, 18 hybridizations were performed representing three independent replicates for each of three treatment groups in both adolescent and adult animals.

Statistical and pathway analyses

GCOS scaled $\log_2$-transformed expression data were analyzed using the Partek Genomic Suite (Partek, St. Louis, MO, USA). We performed a one-way ANOVA followed by a 10% false discovery rate (FDR) to identify genes with a significant treatment effect in common between both age groups (Shared genes). To identify age-specific genes with a significant treatment effect (Age-specific genes), we performed a two-way ANOVA (age and treatment as the main factors) with 10% FDR and identified genes with a significant age×treatment interaction. The resulting significant genes were subjected to Template Matching/Feature Selection (Pavlidis and Noble, 2001) executed in MultiExperiment Viewer (Dana-Farber Cancer Institute, Boston, MA, USA; www.tm4.org/mev) in order to identify genes exhibiting specified patterns of expression. This search is based on a Pearson Correlation and significance was determined at $P<0.05$, allowing us to identify transient, persistent, and late response gene expression profiles that were unique to or shared between the two age groups. Further bioinformatics analysis was conducted on the significant genes to identify functional significance with respect to gene ontology, molecular networks and canonical pathways by means of Ingenuity Pathways Analysis (IPA 6.5 software; Ingenuity Systems, Redwood City, CA, USA) and GeneSpring GX 11 (Agilent Technologies, Santa Clara, CA, USA). Multigene interaction networks were produced using Pathway Studio 6 (Ariadne Genomics, Rockville, MD, USA).

Quantitative real-time reverse transcriptase PCR

To validate expression data for selected genes, we performed quantitative real-time RT-PCR (qRT-PCR) using SYBR green on an ABI Prism 7700 Detection System (Applied Biosystems, Foster City, CA, USA). Total RNA was reverse transcribed using random primers from TaqMan Reverse Transcription Reagents (Applied Biosystems) per manufacturer's instructions. PCR primers were chosen for specificity by NCBI BLAST of the rat genome and amplicon specificity was verified by first-derivative melting curve analysis with software provided by Perkin Elmer and Applied Biosystems. Quantitation and normalization of relative gene expression was accomplished using the comparative threshold cycle method previously described (Joe et al., 2005). The house-keeping genes GLI1 and RPL36AL exhibited no differential expression in our array analysis and were used for normalization of target genes. Table 1 shows genes identified as differentially expressed across age and treatment groups and chosen for qRT-PCR validation.

RESULTS

Whole genome expression was measured in the VTA of adolescent and adult male SD rats exposed to saline, chronic nicotine for 14 days, or 30 days withdrawal following chronic nicotine. A two-way ANOVA with 10% FDR