Neuroscience 179 (2011) 1–8

AGE-DEPENDENT EFFECTS OF INITIAL EXPOSURE TO NICOTINE ON SEROTONIN NEURONS

S. J. BANG[a,b] AND K. G. COMMONS[a,b]*

[a]*Department of Anesthesiology, Perioperative, and Pain Medicine, Children's Hospital Boston, 300 Longwood Avenue, Boston, MA 02115, USA*

[b]*Department of Anaesthesia, Harvard Medical School, Boston, MA 02115, USA*

Abstract—Adolescence is a critical vulnerable period during which exposure to nicotine greatly enhances the possibility to develop drug addiction. Growing evidence suggests that serotonergic (5-HT) neurotransmission may contribute to the initiation and maintenance of addictive behavior. As the dorsal raphe (DR) and median raphe (MnR) nuclei are the primary 5-HT source to the forebrain, the current study tested the hypothesis that there are age-dependent effects of acute nicotine administration on activation of 5-HT neurons within these regions. Both adolescent (Postnatal day 30) and adult (Postnatal day 70) male Sprague–Dawley rats received subcutaneous injection of either saline or nicotine (0.2, 0.4, or 0.8 mg/kg). Subsequently, the number of 5-HT cells that were double-labeled for Fos and tryptophan hydroxylase was counted in seven subregions within the DR and the entire MnR. The results show that acute nicotine injection induces Fos expression in 5-HT neurons in a region-specific manner. In addition, adolescents show broader regional activations at either a lower (0.2 mg/kg) and a higher (0.8 mg/kg) dose of nicotine, displaying a unique U-shape response curve across doses. In contrast, 5-HT cells with activated Fos expression were restricted to fewer regions in adults, and the patterns of expression were more consistent across doses. The results reveal dose-dependent effects of nicotine during adolescence with apparent sensitization at different ends of the dosage spectrum examined compared to adults. These data indicate that initial exposure to nicotine may have unique effects in adolescence on the ascending 5-HT system, with the potential for consequences on the affective-motivational qualities of the drug and the subsequent propensity for repeated use.

Key words: raphe, adolescence, serotonin, addiction.

Nicotine addiction begins in adolescence more often than in any other ages (Adriani et al., 2003, 2004; Schramm-Sapyta et al., 2009). Smoking at this developmental stage is associated with a greater tendency to develop drug dependence (Taioli and Wynder, 1991; Breslau and Peterson, 1996; Chen and Millar, 1998; Kandel and Chen, 2000). In animal models of nicotine addiction, several behavioral differences between adolescents and adults have been reported. Adolescent rodents are more prone to acquire place preference than adult rats with the same dose of nicotine (Vastola et al., 2002; Belluzzi et al., 2004; Torres et al., 2008; Shram and Lê, 2010) and are more motivated to self-administer nicotine after a period of abstinence (Adriani et al., 2002; Levin et al., 2006). In addition, adolescents are more resistant to the aversive effects of nicotine than adults (Wilmouth and Spear, 2004; Shram et al., 2006). However, the neurobiological mechanism underlying the developmental vulnerability to nicotine addiction is unclear.

A growing body of evidence suggests that modulation of the central serotonergic (5-HT) system contributes to the effects of nicotine. Studies have shown that acute injections of nicotine increase the levels of extracellular 5-HT or promotes [^{3}H]5-HT uptake in several regions including the cingulate and frontal cortices, hippocampus, and nucleus accumbens shell (Toth et al., 1992; Ribeiro et al., 1993; Summers et al., 1996; Awtry and Werling, 2003). In addition, serotonin receptor antagonists block the effects of nicotine on place preference (Carboni et al., 1988) and anxiety state (Cheeta et al., 2000, 2001). Chronic exposure to nicotine also produces adaptive changes in the 5-HT system, altering 5-HT transporter levels and 5-HT receptor signaling in several areas of the adolescent brain (Xu et al., 2001, 2002; Collins et al., 2004).

The aim of the present study was to determine if acute nicotine has age-dependent effects on 5-HT neurons in the dorsal raphe (DR) and the median raphe (MnR), which are the primary source of forebrain 5-HT. Acute nicotine administration was used to model initial exposure, since the subjective experience of the first exposure to nicotine may influence the propensity for repeated use and subsequently the development of addictive behavior. As forebrain afferents of the DR and MnR are known to display a high level of topographic organization (Vertes, 1991; Van Bockstaele et al., 1993; Vertes et al., 1999; for a review; see Michelsen et al., 2007), we examined the topography of nicotine's effect by assaying the protein appearance of the immediate-early gene product Fos within 5-HT neurons in specific subregions within the DR and MnR. Our working hypothesis is that the responses of 5-HT neurons to nicotine is enhanced in adolescents compared to adults and may be topographically organized. We postulate that the age difference in region-specific effects of nicotine on 5-HT

*Correspondence to: K. G. Commons, Children's Hospital Boston, 300 Longwood Avenue, Enders 311, Boston, MA 02115, USA. Tel: +1-617-909-2220; fax: +1-617-730-0967.
E-mail address: kathryn.commons@childrens.harvard.edu (K. G. Commons).

Abbreviations: DR, dorsal raphe; DR-CD, caudal dorsal pole of dorsal raphe; DR-CV, caudal ventral pole of dorsal raphe; DR-LW, lateral wings of dorsal raphe; DR-MD, mid dorsal pole of dorsal raphe; DR-MV, mid ventral pole of dorsal raphe; DR-RD, rostral dorsal pole of dorsal raphe; DR-RV, rostral ventral pole of dorsal raphe; MnR, median raphe; PND, postnatal day; TPOH, tryptophan hydroxylase.

0306-4522/11 $ - see front matter
doi:10.1016/j.neuroscience.2011.01.032

release could be relevant for understanding the nature of a biological vulnerability to initiation of nicotine addiction during adolescence.

EXPERIMENTAL PROCEDURES

Subjects

A total of 87 male Sprague–Dawley rats were used in this study. Forty rats were adolescents at postnatal days (PND) 26 (80–120 g) and 47 rats were adults at PND66 (320–360 g) upon arrival. Rats were housed two per cage, maintained on a 12-h light/dark schedule, and given access to food and water *ad libitum*. Care and use of animals was approved by the Institutional Care and Use Committee at Children's Hospital and was consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Drugs

Nicotine was administered at 0.2, 0.4, or 0.8 mg/kg, expressed as the base, freshly prepared by dissolving nicotine hydrogen tartrate (Sigma-Aldrich, St. Louis, USA) in 0.9% normal saline (BD) and adjusting the pH to 7.0. Solutions were prepared such that injected volume was 1 ml/kg body weight. The doses we used were selected to span a behaviorally effective range by comparison with the literature (Matta et al., 2007). A single acute dose of nicotine was used to model the effects of initial drug exposure in order to identify biologically distinct responses at different ages.

Procedures

All subjects were handled for 4 days before experiments. Subjects were injected subcutaneously with either nicotine or normal saline. Three different doses of nicotine (0.2, 0.4, and 0.8 mg/kg) were injected into 17, 14, 16 subjects, respectively, and the rest received saline injection. Subjects were returned to their home cage and remained there for 15 min for the brain nicotine level to be at its peak (Turner, 1975). All experiments started between 9:00 and 10:00 AM, 2-3 h after the initiation of the light cycle.

Immunohistochemistry

Two hours after administration with either nicotine or saline, animals were deeply anesthetized with an overdose of sodium pentobarbital (100 mg/kg, i.p.; 50 mg/mL, Lundbeck Inc., Deerfield, IL 60015, USA). Transcardial perfusion was made with 4% paraformaldehyde in normal saline. Brains were removed and stored in 4% paraformaldehye for 24 h, and equilibrated in 30% sucrose in 0.1 M phosphate buffer for at least 2 days. Then, each brain was frozen, serially sectioned in the coronal plane (40 μm) using a freezing microtome, and processed while floating.

Primary antisera were diluted in 0.1 M phosphate-buffered saline with 0.3% Triton X-100, 0.04% bovine serum albumin, and 0.1% sodium azide, and incubated with the tissue overnight at room temperature. Fos immunoreactivity was detected by incubating sections with a rabbit polyclonal anti-Fos antibody (1:10,000; PC38, EMD Biosciences, San Diego, CA, USA). 5-HT neurons were detected using an antiserum raised against tryptophan hydroxylase (TPOH), the synthetic enzyme for serotonin, raised in sheep (1:1000; AB1541, Chemicon International, Temecula, CA, USA). Sections were then incubated with a mixture of CY3-conjugated donkey anti-rabbit IgG (1:200; Jackson ImmunoResearch, West Grove, PA, USA) and AlexaFluor 488-conjugated anti-sheep IgG (1:200; Invitrogen Molecular Probes, Carlsbad, CA, USA) secondary antibodies for 90 min at room temperature. Sections were then rinsed with 0.1 M PBS, mounted onto glass slides, and coverslipped with 90% glycerol.

Data analysis

Cells that were double-labeled with Fos and TPOH were counted in seven subregions within the DR and the entire MnR (Fig. 1). In DR, three rostrocaudal divisions were made: caudal area was from −8.54 to −9.26 mm, middle area was from −7.73 to −8.45 mm, and rostral area was from−6.92 to −7.64 mm relative to Bregma (Paxinos and Watson, 2005). These three levels were then divided at the midpoint between the base of the aqueduct and the ventral extent of TPOH-containing cells into dorsal and ventral subregions. The lateral wings of the DR and the MnR are the remaining two groups that were both sampled in sections comprising mid-levels of the DR. Abbreviations for each region is as follows: DR-RD, rostral dorsal DR; DR-RV, rostral ventral DR; DR-LW, lateral wings of DR; DR-MD, mid dorsal DR; DR-MV, mid ventral DR; MnR, median raphe; DR-CD, caudal dorsal DR; DR-CV, caudal ventral DR.

For each rat, every fourth section was analyzed, representing approximately three sections per regions of interest. For each area, a minimum of six rats (average number of rats/group=7) contributed to the mean number of cells containing Fos and TPOH in each group. For sampling, the relevant area was illuminated for each fluorophore and digitally photographed using conventional fluorescence microscope and a 10× objective. Representative images were photographed using a 20× objective for preparation of the figure (see Fig. 2). Double-labeled cells were manually enumerated by visualization of the individual and merged images of each fluorophore. To be considered positive for Fos, Fos immunolabeling had to completely fill the nucleus with an intensity of labeling that was easily distinguished from background levels (Fig. 2). The mean number of double-labeled cells per section was determined for each rat within a group. Group means and standard error of the means were calculated for each subregion.

Each area was statistically analyzed with multifactorial analysis of variances (ANOVA) with repeated measures (SPSS 14.0; Chicago, IL, USA) with dose [0 mg/kg (saline-treated), 0.2 mg/kg, 0.4 mg/kg, and 0.8 mg/kg], and age (adults vs. adolescents) as between-subject factors and brain region as a within-subject factor. Significant interaction effects were followed by either simple interaction effects or simple main effects at each level of the involved factors. Post hoc comparisons using Bonferroni-corrected post hoc comparisons were followed with a threshold for significance of $P<0.05$ where appropriate.

RESULTS

Effects of acute nicotine administration on Fos expression in TPOH-containing cells (Fig. 2) were examined in adults versus adolescents at a dose of 0.2 (Fig. 3), 0.4 (Fig. 4), and 0.8 mg/kg (Fig. 5) in the eight regions of interest (Fig. 1): DR-RD, DR-RV, DR-LW, DR-MD, DR-MV, MnR, DR-CD, and DR-CV. The morphology of the raphe nuclei and abundance of 5-HT cells in the region of interests appeared comparable between the two age groups as shown in Fig. 1. Fig. 2 depicts representative images of DR-RD in which nicotine enhanced Fos expression (red) in TPOH-containing 5-HT cells (green) in both age groups at a dose of 0.2 mg/kg. The labeling of both TPOH and Fos were easily detectable and quantifiable. While there were few 5-HT cells that were double-labeled with Fos (arrow) in saline-injected rats (Fig. 2B, D), double-labeled 5-HT cells were commonly found in both adults (Fig. 2A) and adolescents (Fig. 2C) after nicotine injection.

ANOVA revealed significant interaction effects between dose and brain region ($F(21,553)=6.93$; $P<0.005$) as well as significant triple interactions among age, dose,