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A ten fold reduction of nicotine yield in tobacco smoke does not spare the central cholinergic system in adolescent mice

Yael Abreu-Villaça[a,*], Monique Correa-Santos[a], Ana C. Dutra-Tavares[a], Danielle Paes-Branco[a], Andre Nunes-Freitas[a], Alex C. Manhães[a], Cláudio C. Filgueiras[a], Anderson Ribeiro-Carvalho[b]

[a] *Laboratório de Neurofisiologia, Departamento de Ciências Fisiológicas, Instituto de Biologia Roberto Alcantara Gomes, Universidade do Estado do Rio de Janeiro (UERJ), Av. Prof. Manuel de Abreu 444, 5 andar—Vila Isabel, Rio de Janeiro, RJ 20550-170, Brazil*

[b] *Departamento de Ciências, Faculdade de Formação de Professores da Universidade do Estado do Rio de Janeiro, Rua Dr. Francisco Portela 1470—Patronato, São Gonçalo, RJ 24435-005, Brazil*

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ABSTRACT

The tobacco industry has gradually decreased nicotine content in cigarette smoke but the impact of this reduction on health is still controversial. Since the central cholinergic system is the primary site of action of nicotine, here, we investigated the effects of exposure of adolescent mice to tobacco smoke containing either high or low levels of nicotine on the central cholinergic system and the effects associated with cessation of exposure. From postnatal day (PN) 30 to 45, male and female Swiss mice were exposed to tobacco smoke (whole body exposure, 8 h/day, 7 days/week) generated from 2R1F (HighNic group: 1.74 mg nicotine/cigarette) or 4A1 (LowNic group: 0.14 mg nicotine/cigarette) research cigarettes, whereas control mice were exposed to ambient air. Cholinergic biomarkers were assessed in the cerebral cortex and midbrain by the end of exposure (PN45), at short- (PN50) and long-term (PN75) deprivation. In the cortex, nicotinic cholinergic receptor upregulation was observed with either type of cigarette. In the midbrain, upregulation was detected only in HighNic mice and remained significant in females at short-term deprivation. The high-affinity choline transporter was reduced in the cortex: of HighNic mice by the end of exposure; of both HighNic and LowNic females at short-term deprivation; of LowNic mice at long-term deprivation. These decrements were separable from effects on choline acetyltransferase and acetylcholinesterase activities, suggesting cholinergic synaptic impairment. Here, we demonstrated central cholinergic alterations in an animal model of tobacco smoke exposure during adolescence. This system was sensitive even to tobacco smoke with very low nicotine content.

1. Introduction

Adolescence is the period of development when smoking typically begins and adolescents were shown to present a peculiar sensitivity to tobacco smoke (DiFranza, 2008; Riggs et al., 2007). Nicotine is an alkaloid present in all tobacco products at varying concentrations and is considered a major psychoactive component of tobacco smoke. Indeed, most information on the underlying biological mechanisms that promote tobacco use during adolescence as well as the effects of exposure originate from studies on rodent models of nicotine exposure. Over the last 15 years, experimental studies demonstrated that this component has immediate and long term effects on several neurotransmitter systems, on gene expression and, ultimately, on behavior, and that several effects are distinct from those resulting from exposure at adulthood (for review: Yuan et al., 2015).

The tobacco industry has gradually decreased nicotine content in cigarette smoke (Hoffmann and Hoffmann, 1997), but the impact of this reduction on health is still controversial (Dunsby and Bero, 2004). In this regard, tobacco smoke is a complex aerosol consisting of approximately of 5600 components (Perfetti and Rodgman, 2011) and, in rodent models, other constituents of tobacco smoke

Abbreviations: AChE, acetylcholinesterase; ChAT, choline acetyltransferase; CHT, high-affinity presynaptic choline transporter and activities; CT, control; HighNic, tobacco smoke with high nicotine levels; LowNic, tobacco smoke with very low nicotine levels; nAChRs, nicotinic acetylcholine receptors; PN, postnatal day.

* Corresponding author at: Laboratório de Neurofisiologia, Departamento de Ciências Fisiológicas, Instituto de Biologia Roberto Alcantara Gomes, Centro Biomédico, Universidade do Estado do Rio de Janeiro, Av. Prof. Manuel de Abreu 444, 5 andar—Vila Isabel, Rio de Janeiro, RJ, 20550-170, Brazil.
E-mail address: yael.a.v@yahoo.com.br (Y. Abreu-Villaça).

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were suggested to either play independent roles or interfere with nicotine effects on the central nervous system (Belluzzi et al., 2005; Hall et al., 2014; Villégier et al., 2003, 2006, 2010). Tobacco smoke extracts were shown to be stronger reinforcers than nicotine alone (Costello et al., 2014) and, in PC12 cells (a neuronotypic cell line), these extracts promoted a more robust increase in neurite formation and decrease in cell number than nicotine (Slotkin et al., 2014). In parallel, epidemiologic studies have shown that while smokers prefer cigarettes that deliver nicotine, both regular and denicotinized cigarettes reduce subjective measures of tobacco craving and withdrawal (Pickworth et al., 1999), and that denicotinized cigarettes decrease usage of nicotine-containing cigarettes significantly more than nicotine gum (Johnson et al., 2004). These and other evidence suggest that, when compared to nicotine alone, tobacco smoke exposure as a preclinical model is a better approach to study the human condition of smoking and the impact of varying levels of nicotine in tobacco.

Despite that, only recently studies began to directly investigate the effects of tobacco smoke in animal models of adolescent exposure. Bruijnzeel et al. (2011) showed that exposure of adolescent rats to tobacco smoke resulted in reduced neurogenesis and increased gliogenesis in the dentate gyrus. Our group was the first to demonstrate that exposure of adolescent mice to tobacco smoke generated from cigarettes containing very low nicotine levels elicits significant short- and long-term effects on novelty seeking and anxiety-like behaviors, and that the effects are distinct from those identified in mice exposed to tobacco smoke containing high nicotine levels (Abreu-Villaça et al., 2015). As for the cholinergic system, tobacco smoke exposure during adolescence was shown to increase [3H]Choline uptake even in mice exposed to tobacco containing very low nicotine levels (Abreu-Villaça et al., 2010).

Nicotinic acetylcholine receptors (nAChRs), the primary target of nicotine, are widely distributed in the central nervous system (for review: Abreu-Villaça et al., 2011; Hurst et al., 2013). The upregulation of these receptors plays a major role in both the establishment and maintenance of nicotine addiction (Ortells and Barrantes, 2010). Interestingly, exposure to nicotine during adolescence was shown to elicit more profound and persistent upregulation of nAChRs as compared to adults, and suppression of cholinergic synaptic activity upon withdrawal (for review: Slotkin, 2008). Considering the unique features of the response of the adolescent brain to nicotine, it is somewhat surprising that the effects of tobacco smoke on nAChRs and on other biomarkers of the cholinergic system in animal models of adolescence exposure have received little attention. Here, we used an animal model to investigate the impact of a subchronic tobacco smoke exposure on the central cholinergic system of adolescent mice, as well as the short- and long-term effects associated with cessation of exposure. To further study potential neurochemical differences elicited by reduced nicotine concentrations in tobacco smoke, active smoking was emulated by exposing adolescent mice to tobacco smoke generated from cigarettes containing a high (1.74 mg/cigarette) or a very low level of nicotine (0.14 mg/cigarette). The basal forebrain and the midbrain are the two major cholinergic outputs of the central nervous system. Cholinergic neurons in the basal forebrain project to the entire cerebral cortex, hippocampus, and amygdala, while projections from the midbrain ascend to the basal forebrain and thalamus (Woolf, 1997). Here, we evaluated the cerebral cortex and the midbrain regions that are enriched in cholinergic innervation and that, in previous studies, showed significant alterations in cholinergic biomarkers during and after adolescent nicotine exposure (Abreu-Villaça et al., 2003; Ribeiro-Carvalho et al., 2008, 2009). Four cholinergic biomarkers were assessed: α4β2 nAChR binding with [3H]Cytisine, binding of [3H]hemicholinium-3 (HC-3) to the high-affinity presynaptic choline transporter (CHT), choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) activities.

2. Methods

2.1. Animals and treatment

All experiments were carried out with the approval of the Animal Care and Use Committee of the Universidade do Estado do Rio de Janeiro (CEA/014/2011). Swiss mice were bred and maintained in our animal facility at 21–22 °C on a 12-h light/dark cycle (lights on at 1a.m.) with *ad libitum* access to food and water. To avoid the influence of litter size in the prenatal nutritional status, we only considered litters of 8–12 pups at birth. On the second postnatal day (PN2), litters were culled to 8 mice and, by PN21, animals were weaned and separated by sex.

To maximize potential differences between experimental groups, we chose two types of reference research cigarettes: one with the highest and one with the lowest level of nicotine concentration available at the Kentucky Reference Cigarette Program (http://www2.ca.uky.edu/refcig/). From PN30 to PN45, Swiss male and female adolescent mice were exposed to tobacco smoke generated from type 2R1F (HighNic group: nicotine = 1.74 mg/cigt) or type 4A1 (LowNic group: nicotine = 0.14 mg/cigt). Accordingly, the difference in nicotine yield between HighNic and LowNic groups is more than 10 fold (Abreu-Villaça et al., 2015), while other components of tobacco present little difference (HighNic group: total particulate matter = 28.6 mg/cigt; tar = 23.4 mg/cigt; carbon monoxide = 22.0 mg/cigt; LowNic group: total particulate matter = 29.7 mg/cigt; Tar = 26.8 mg/cigt; carbon monoxide = 17.2 mg/cigt). Whole body exposure to cigarette smoke was carried out for 8 h/day, from 9a.m. to 5p.m., 7 days/week in a chamber connected to an automatic cigarette-smoking machine (Teague Enterprises, Davis, CA, USA). A smoke mixture containing 89% sidestream smoke (smoke released from the burning end of a cigarette) and 11% mainstream smoke (smoke from the puff stream), as a surrogate for active smoking (Abreu-Villaça et al., 2013a; Slotkin et al., 2001), was generated by the smoking machine in a staggered manner at the rate of a single 35 ml puff of 2 s duration each minute for the entire period of 8 h. Control mice (CT) were exposed to ambient air in a chamber identical to the one used for smoke exposure. Mice had *ad libitum* access to food and water during exposure.

A previous study from our group (Abreu-Villaça et al., 2015) quantified total suspended particulate concentration and cotinine serum levels using the same research cigarettes and protocols of exposure that were used here. As described elsewhere (Abreu-Villaça et al., 2015), during tobacco smoke exposure, there were 12 periods of total suspended particulate collection (2 consecutive days) for each type of cigarette. The average particulate concentrations were $38.4 \pm 3.9\ mg/m^3$ and $39.2 \pm 2.7\ mg/m^3$ (mean ± E) for the 2R1F and the 4A1 cigarettes respectively, levels comparable to those active smokers are exposed to. Cotinine (nicotine metabolite) serum levels, quantified at the last day of exposure, were 109.1 ± 24.0 ng/ml in HighNic mice. LowNic and CT mice presented cotinine levels below the detection limit of the technique (<8 ng/ml) and there were no significant differences between males and females (Abreu-Villaça et al., 2015). Based on evidence that cotinine values are proportional to those of nicotine (Trauth et al., 2000b), these data suggest that mice from the LowNic group absorbed very low levels of nicotine.

Two hundred and forty-eight mice (124♂ and 124♀) from thirty-nine litters were distributed across the three treatment groups (HighNic = 40♂, 40♀, LowNic = 43♂, 41♀ and CT = 41♂, 43♀) and were decapitated at the following time-points: 1) by the end of