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Research article

# Adolescent nicotine alters dendritic morphology in the bed nucleus of the stria terminalis☆

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## HIGHLIGHTS

- Adolescent nicotine induces dendritic growth in BNST.
- Increases in branch points indicate that new branches are formed.
- Circuit remodeling including BNST may subserve lasting behavior changes.

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## ABSTRACT

Adolescent nicotine increases dendritic elaboration in several areas associated with the extended amygdala. It also increases anxiety-like behavior in adulthood. An unresolved question is whether adolescent nicotine alters dendritic structure in the bed nucleus of the stria terminalis (BNST), which may contribute to altered anxiety-like behavior. To investigate this possibility, adolescent male Sprague–Dawley rats were administered nicotine (0.5 mg/kg/day) 3 days a week for 2 consecutive weeks, starting at postnatal day P (32). 17 days following the end of dosing, brains were processed for Golgi–Cox staining, and neurons were digitally reconstructed in three dimensions. Animals previously treated with nicotine exhibited an increase in the total number of branches and total length of dendrites on BNST neurons. Sholl analysis revealed an increase in the number of intersections with concentric spheres, increased amount of dendritic material within concentric spheres, and an increase of dendritic branching within concentric spheres occurring between 20 and 300 μm from the soma in dendrites. Collectively, our results show that adolescent nicotine alters dendritic structure (by triggering new branch growth), and, by inference, connectivity of the BNST, which may contribute to alterations in behavior induced by adolescent nicotine.



## 1. Introduction

Adolescence represents a unique period of vulnerability to the neurological effects of drugs of abuse [1]. Dendritic remodeling in response to adolescent nicotine exposure has been demonstrated in several brain regions associated with nicotine dependence and anxiety-like behavior, including the nucleus accumbens (NAcc) shell [12,13,6] and the basolateral amygdala [2]. These observed changes came in the form of increased dendritic branching and were present several weeks following the final nicotine exposure. Importantly, alterations in dendrite morphology have the potential to greatly alter the amount or pattern of synaptic connectivity with these brain regions [3], and may mediate the neurobehavioral effects of chronic nicotine exposure.

Whether neurons in the BNST exhibit plasticity in response to adolescent nicotine exposure is currently unknown. In animal models, chronic adolescent nicotine increases anxiety-like behavior persisting into adulthood [16,15]. While nicotine is known to influence several brain regions involved in the development of anxiety-like behavior, the bed nucleus of the stria terminalis (BNST) has recently emerged as an area of particular interest, as studies have suggested a role for the BNST in coordinating activity of the autonomic, neuroendocrine, and somatic motor systems. Lesion studies have demonstrated a role for the BNST in physiological fear, food intake regulation, social behavior, and goal orientated behavior [4]. Nicotine exposure potently activates the BNST (e.g.,

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[14], and therefore places the BNST as a structure of interest in understanding the basis for psychological states such as anxiety and addiction. Therefore, the objective of the present study was to establish whether similar dendritic remodeling is present in the BNST in response to adolescent nicotine exposure.

## 2. Methods and methods

### 2.1. Animals

Animal subjects ($N = 17$) were male Sprague–Dawley rats (Harlan, Indianapolis, IN) that arrived at our facility on postnatal day (P) (21). Animals were group housed (4–5 per cage) with a 12-h light/dark cycle and ad libitum access to food/water. Nicotine administration took place during the light cycle. All housing and experimental procedures were carried out in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals in addition to requirements of the George Mason University IACUC.

### 2.2. Dosing

Rats were given 11 days to adjust to housing conditions prior to the start of drug administration on P32. Rats were randomly assigned to receive subcutaneous (SC) injections of either nicotine (0.5 mg/kg, free base; $n = 9$) or 0.9% saline ($n = 7$) control. This drug administration protocol was chosen because it accurately represents intermittent and low-intensity consumption patterns in human adolescent populations, and has previously been shown to alter dendrite morphology in other brain areas following nicotine exposure [2]. Rats were administered nicotine or saline via subcutaneous injections 3 times per week for two weeks from P32–P43. This age range is placed within a conservative estimate of adolescence in the rat as is reflected by sexual maturation and indices of brain development [9].

### 2.3. Neuroanatomical evaluation

Following a 17-day drug abstinent period, all animals were sacrificed for Golgi–Cox tissue staining on P60. Rats were deeply anesthetized with a ketamine/xylazine solution (80 mg/15 mg/kg; Sigma–Aldrich) and perfused intracardially with 0.9% NaCl solution. Brains were immediately extracted and placed in individual specimen jars containing Golgi–Cox solution, prepared according to the recipe of [7], for 14 days at room temperature. Following Golgi–Cox immersion, brains were stored in a 30% sucrose solution until vibratome sectioning (200 μm sections). Sections were developed using the protocol of [8]. Briefly, sections were first alkalinized in ammonium hydroxide, developed and fixed using Kodak Rapid Fix dehydrated through a series of ethanols, and cleared in a solution of 1/3 xylene, 1/3 100% alcohol, and 1/3 chloroform.

Golgi–Cox stained neurons were manually reconstructed in three dimensions using Neurolucida (Microbrightfield Biosciences, Williston, VT) interfaced with an Olympus BX51 light microscope with motorized stage. All neurons were reconstructed under 60X objective by an experimenter blind to treatment group. Neurons were selected only if they were well impregnated and possessed unobstructed dendrites that could be followed without interruption. A total of 83 neurons (nicotine, $n = 48$; saline, $n = 35$) from the BNST were reconstructed in this study. As a specific primary cell type in the BNST has not been identified by previous research [11], we conducted a principal components analysis (PCA) on the 83 reconstructed neurons to determine whether our reconstructions contained more than one cell type. For PCA, the included morphometric parameters obtained with Neuroexplorer (Microbrightfield Bioscience, Williston, VT) included number of bifurcations (nodes), number of terminal endings, total dendritic length, total volume of dendritic arbor, area of the soma, and maximum diameter of the soma. PCA revealed two components with Eigen values greater than 1.0. Component 1 explained 96% of the variance in tree totals, nodes, terminals, total length, total volume, area of the soma, and maximum diameter of the soma displayed by each neuron, while component 2 explained only 2% of the variance in the measures. Therefore, without a distinction of different cell types revealed by PCA, we classified and analyzed all neurons as a single cell-type.

Morphological measurements were obtained using NeuroExplorer software (Microbrightfield Biosciences, Williston, VT). To assess the overall magnitude of morphological change, morphometric parameters of total number of bifurcations and total dendritic length were obtained. To assess the radial distribution of dendritic material, 3-D Sholl analyses (20 μm increments) were implemented to assess the number of intersections, number of bifurcations, and total length of the dendritic material within concentric spheres.

### 2.4. Statistical analysis

Three to six neurons were reconstructed from each of the 17 subjects. Rather than treat each cell as an independent measure, a mean value per animal for each morphometric parameter (3–6 neurons per animal) was calculated for all statistical analyses. For all parameters, mixed-ANOVA with treatment (nicotine vs. saline) as the between-groups factor and radial distance from the soma as the within-groups factor was conducted. Violations of the assumption of sphericity were corrected using the Greenhouse–Geisser correction for the degrees of freedom, and are indicated by a superscripted "a" preceding an F value. Following significant interactions, independent sample $t$-tests on distinct radii from the soma were conducted. To avoid false positives due to multiple comparisons, differences were only considered significant if the $p$-value for three consecutive points were each $p < .05$. This limits the influence that any single radius has on the analysis [2,5]. All data are presented as group means ± SEM.

## 3. Results

Fig. 1 illustrates the area of BNST from which sections were taken (A), representative stained cells (B and C), and representative reconstructions (D and E). ANOVA with between-group factor (treatment) and repeated measure (radius) revealed a significant main effect of pretreatment on number of intersections ($F(1,14) = 11.503$; $p < .004$), total dendritic length ($F(1,14) = 12.195$; $p < .004$), and number of bifurcations ($F(1,14) = 24.823$; $p < .000$). Nicotine treatment produced a significant increase in each of the parameters (Fig. 2).

Sholl analyses suggest that the nicotine-induced increases in morphological parameters are specific to certain radial distances from the soma. ANOVA revealed a trend between treatment and radius on number of intersections ($^aF(3.048, 42.668) = 3.048$; $p < .056$). Follow-up $t$-tests revealed an increased number of intersections in nicotine treated animals compared to saline treated animals between 20 μm and 80 μm from the soma (Fig. 3A). ANOVA also revealed a significant interaction between treatment and radius on total dendritic length ($^aF(3.063, 42.88) = 3.951$; $p < .014$), and follow-up t-tests revealed increased total length in nicotine treated animals compared to saline treated animals between 40 μm and 100 μm from the soma (Fig. 3B). Finally, there was a significant interaction between treatment and radius on number of bifurcations ($^aF(4.428,61.99) = 3.076$; $p < .019$). Although follow-up $t$-tests failed to meet our criterion of significant differences at three consecutive radii, significant increases in number of bifurcations in