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Nicotine-induced dendritic remodeling in the insular cortex

Daniel G. Ehlinger[a,*], Hadley C. Bergstrom[b], Craig G. McDonald[a], Robert F. Smith[a]

[a] Department of Psychology, George Mason University, 4400 University Dr. 3F5, Fairfax, VA 22030, United States
[b] Department of Psychiatry, The Uniformed Services University, 4301 Jones Bridge Rd., Bethesda, MD 20814, United States

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ABSTRACT

The insular cortex has emerged as a novel target for nicotine addiction research. One unresolved question about the insular cortex is whether its neurons exhibit nicotine-induced dendritic remodeling similar to other brain regions implicated in nicotine addiction. To test this question, Long–Evans rats were administered nicotine via osmotic pump for two weeks. Thirty-seven days following the end of nicotine dosing, rats were sacrificed for Golgi-Cox staining and pyramidal neurons from the rostral agranular insular cortex were digitally reconstructed in three dimensions. Results from morphometric analyses revealed an increased complexity of dendrites in the insular cortex following nicotine. Increases were found for both total dendrite length and number of bifurcations. Sholl analyses revealed these changes depended on the distance from the soma, with the most prominent changes distributed at distal points along the dendritic tree. A follow-up comparison of length and bifurcation measurements from Sholl analyses suggested that new dendritic branches, rather than growth of existing dendrites, most likely contributed to overall changes in complexity. No change in dendrite morphology was found for apical dendrites. Together, these results show the insular cortex is a target for neuroplasticity following nicotine exposure.

1. Introduction

Nicotine remains one of the most heavily abused addictive substances, despite a current decline in the prevalence of smoking [18]. Among the known brain regions implicated in nicotine addiction, the insula has recently emerged as a novel target. The impetus for this new direction is based primarily on an influential study showing that former smokers with stroke damage localized to the insula reported a sudden and profound decrease in smoking and, importantly, the urge to smoke [17]. Subsequent experiments in rodent models have validated a role for the insula in nicotine dependence, showing that insula inactivation effectively abolished nicotine-cued learning behavior, including conditioning place preference [6], self-administration [8], and nicotine-cue approach [22]. Taken together, these findings place the insula within a neural circuit that underlies nicotine dependence.

Despite recent advances, one unresolved question about the insula is whether its neurons exhibit plasticity after chronic nicotine. Changes to dendritic morphology represent one persisting form of plasticity that is an important neural correlate of nicotine dependence [3]. Plasticity of dendritic morphology has been demonstrated in a number of brain regions implicated in nicotine addiction, including the nucleus accumbens shell (NAcc) [3,15,16], cingulate cortex [3], prelimbic cortex (PL) [1,21] and amygdala (lateral and basal subnuclei; BLA) [2]. To date no study has established whether neurons located in the insula exhibit structural alterations after nicotine. The objective of the present study was to reconstruct and analyze the dendritic morphology of neurons located in the insula following an extended drug free (37 days) period from chronic nicotine exposure.

2. Methods

2.1. Subjects

Animal subjects ($N = 18$) were adult-male Long–Evans hooded rats (Harlan, Indianapolis, IN) that arrived at our facility on postnatal day 70 (P70). Animals were group housed (4–5 per cage) with ad libitum access to food and water, with a 12-h light/dark cycle, throughout the study. All housing and experimental procedures were carried out in accordance with the National Institute of Health *Guide for the Care and Use of Laboratory Animals*, in addition to requirements of the George Mason University IACUC.

2.2. Dosing

Animals were randomly assigned to a nicotine treatment group ($n = 9$) or a sham control ($n = 9$). Nicotine bitartrate was continuously administered from P80 to P93 via subcutaneously implanted Alzet osmotic pumps (Model 1002; Durect Corp., Cupertino, CA).

* Corresponding author. Tel.: +1 703 993 1358.
E-mail address: dehlinge@gmu.edu (D.G. Ehlinger).

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This method of drug administration was chosen to maintain a relatively stable elevation in blood serum levels of nicotine throughout the dosing period. Prior to pump implant surgery, animals were anesthetized using Equithesin (3.5 mg/kg). A small incision was made in the lower back for subcutaneous placement. Animals in the nicotine treatment group were implanted with pumps containing nicotine bitartrate dissolved in 0.9% NaCl, while control animals received pumps with a comparable amount of sodium tartrate dissolved in 0.9% NaCl to ensure a similar rate of infusion. The dose rate remained constant from P80 to P93 at 2 mg/kg/day, and was chosen because it represents plasma nicotine levels similar to those seen in human smokers [24]. After a 30-day abstinent period, both nicotine treatment and control animals were tested for locomotor sensitization following nicotine challenge [16]. Nicotine-induced locomotor sensitization was not observed in these animals. In addition, both nicotine and control groups were subject to an identical locomotor sensitization protocol, and it would not be expected for the nicotine challenge to interact with the prior dosing regimen and produce differences in dendritic morphology.

2.3. Neuroanatomy

One week following the behavioral sensitization experiment previously mentioned (37 days post-nicotine dosing), all animals were sacrificed for Golgi-Cox staining. Animals were deeply anesthetized with tribromethanol and perfused intracardially with 0.9% NaCl solution. Brains were immediately placed in the Golgi-Cox solution for 14 days, which was prepared according to the recipe of Glaser and Van der Loos [12]. Following Golgi-Cox immersion, brains were stored in a 30% sucrose solution until vibratome sectioning (200 μm sections). Sections were then stained using the protocol of Gibb and Kolb [11].

Golgi-Cox impregnated neurons were visualized using light microscopy and reconstructed in three dimensions using Neurolucida software (Microbrightfield Biosciences, Williston, VT). All neurons were traced by an experimenter blind to pretreatment group, and were reconstructed under a 60× objective.

We chose to focus morphological analyses of the rostral agranular insular cortex (rAIC). The rAIC integrates viscerosensory information [4], possesses significant cortico-cortical connections with the orbitofrontal, infralimbic, prelimbic, and cingulate cortices [9,14], and has direct projections to the NAcc, BLA, and ventral tegmental area (VTA) [10,14]. Each of these regions has been implicated in various aspects of nicotine dependence. Furthermore, the rAIC receives significant dopaminergic input from the VTA [14], similar to other regions where alterations in dendritic morphology following nicotine exposure have been reported. Pyramidal neurons were chosen for study, as they are the principal projection neurons of cortical structures including the rAIC. Pyramidal neurons were chosen based on the following criteria: (1) triangular shaped soma, (2) the presence of a prominent, single apical tree extending from the apex of the soma toward the cortical surface, (3) two or more basilar dendritic trees extending from the base of the soma with visible dendritic spines, (4) the presence of three or more branch orders. Only pyramidal neurons that were well impregnated and possessed unobstructed dendrites that could be followed without interruption were chosen for reconstruction. Pyramidal neurons were selected based on the boundaries of the agranular insular cortex as defined in Paxinos and Watson [20] and Jasmin et al. [14]. We used the rhinal fissure as an anatomical landmark. As the rostral portion of the agranular insular cortex was of interest in this study, agranular insular cortex morphological analysis was restricted to pyramidal neurons located between Bregma +3.2 mm and +2.5 mm [20]. As the rAIC receives significant dopaminergic input to layer V from the VTA [19], similar to other regions where alterations in dendritic morphology following nicotine exposure have been reported, pyramidal neurons were randomly sampled from a cortical depth of 600–900 μm from the pia surface to ensure the selection of neurons from layer V [14] (Fig. 1).

Morphometrics were obtained using NeuroExplorer software (Microbrightfield Biosciences, Williston, VT). Morphometric parameters were the total number of branches, and total dendrite length for both apical and basilar dendrites. These morphometric parameters were used to assess the overall magnitude of morphological change following chronic nicotine exposure. The distribution of dendritic length was quantified using Sholl analysis [23] (20 μm increments) with parameters of total length, intersections, and bifurcations (nodes) on basilar and apical dendritic trees. Whereas the distribution of total length reflects the total amount of dendritic material of the neuron at increasing distance from the soma, the distribution of intersections reflects the complexity of branching of the dendritic tree at increasing distance from the soma. The distribution of bifurcations reflects alterations in the branching pattern at increasing distance from the soma.

2.4. Statistics

Three to six neurons were reconstructed from each of the 18 subjects, and all subjects were included in statistical analyses. In total, $N = 80$ neurons were reconstructed from nicotine ($n = 41$) and control ($n = 39$) subjects. For each subject, a mean value of each morphometric parameter based on three to six neurons per subject was calculated. Therefore, statistical analyses were based on the mean values per animal, rather than treating each cell as an independent measure. For the analysis of total length of dendrites and total number of branches, ANOVA with treatment (nicotine vs. control) as the between-groups factor was conducted.

Sholl analyses were conducted on the mean values per animal as described for the above morphometric parameters. For the analysis of total length, intersections, and bifurcations of dendrites, 2-way ANOVA with treatment (nicotine vs. control) as the between-groups factor and radial distance from the soma (radius) as the within-groups factor was conducted. Violation of the assumption of sphericity for repeated measures was corrected using the Greenhouse–Geisser correction for degrees of freedom. Following significant interactions, independent samples *t*-tests were conducted at distinct radii from the soma. To avoid spurious values due to multiple comparisons or an overly conservative post hoc test, differences were only considered significant if the *p*-values for three consecutive points were <.05. This technique ensures that a large number of radii are statistically significant, limits the influence of any single radius on the analysis, and ensures that the alterations in distinct-radii occur consecutively over a sizeable portion of the dendritic tree [2].

All data are presented as group means ± SEM. A superscripted letter "a" proceeding an *F* value indicates Greenhouse–Geisser-corrected value for degrees of freedom.

3. Results

Representative examples of digital reconstructions of rAIC pyramidal neurons from control and nicotine-treated animals are presented in Fig. 1C. Chronic nicotine exposure resulted in a significant increase in both the total length ($F_{1,16} = 5.92$; $p = .027$) and total number of bifurcations ($F_{1,16} = 5.43$, $p = .033$) of basilar dendrites of nicotine treated animals compared to control (Fig. 2A and B). No differences in total length or number of bifurcations were observed between groups for apical dendrites.

Sholl-analyses reveal that nicotine-induced alterations of basilar dendrite morphology depended on the radial distance from the soma. Chronic nicotine exposure resulted in a significant

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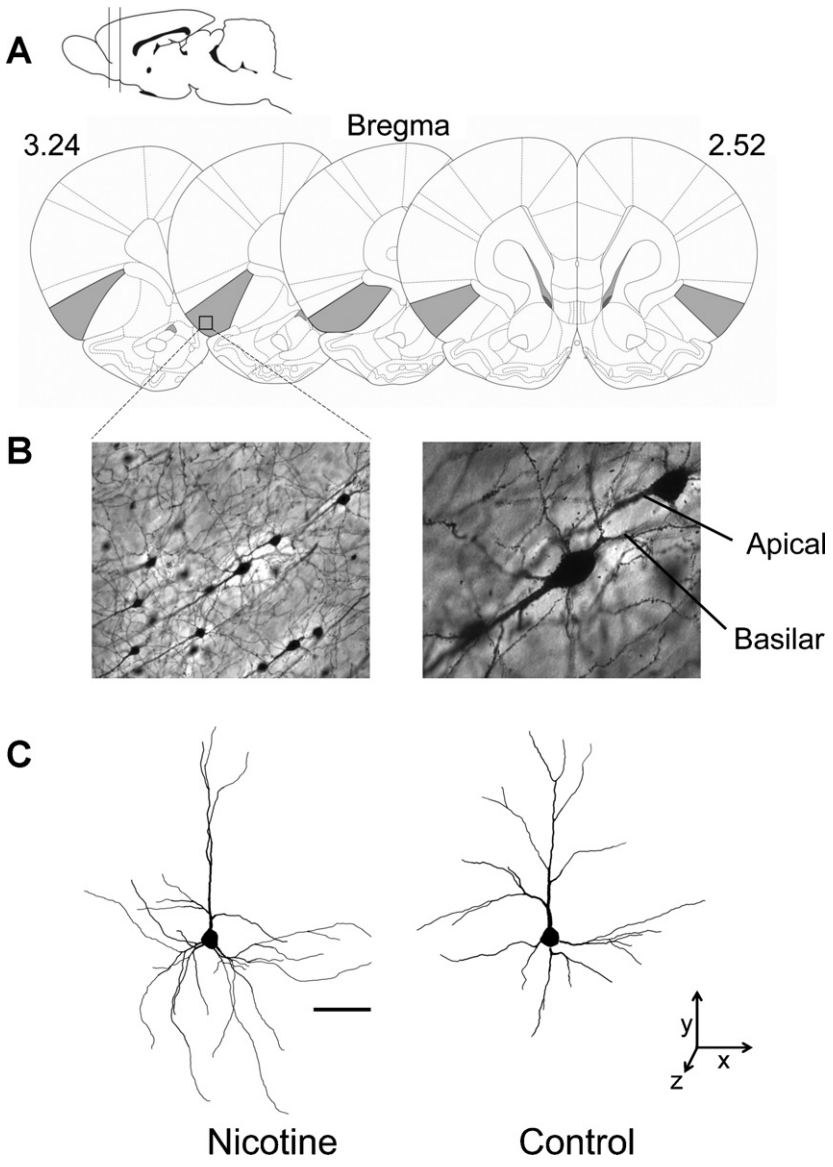

Fig. 1. rAIC and representative neuron reconstructions. (A) Stereotaxic rat brain atlas showing the Bregma coordinates of the rAIC from which neurons were reconstructed. Pyramidal neurons were selected from within rAIC boundaries (grey shaded). (B) Photomicrographs depict Golgi-Cox stained rAIC pyramidal neurons at 20× (left) and 60× (right) magnification. (C) Representative three-dimensional neuron reconstructions from nicotine and control rats. These nicotine and control neurons are representative of basilar dendritic lengths near their respective group means. Scale bar represents 100 μm. Atlas images modified from Paxinos v.6.

increase in the total length of basilar dendrites at consecutive radii between 60 μm and 140 μm from the soma compared to control ($^{a}F_{2.69,43.03} = 3.638$; $p = .024$) (Fig. 3A). Similarly, chronic nicotine exposure resulted in a significant increase in the number of intersections of basilar dendrites with consecutive radii between 80 μm and 140 μm from the soma compared to control ($^{a}F_{2.86,45.68} = 3.819$, $p = .017$) (Fig. 3B). Together, these data suggest that chronic nicotine treatment increases the complexity of

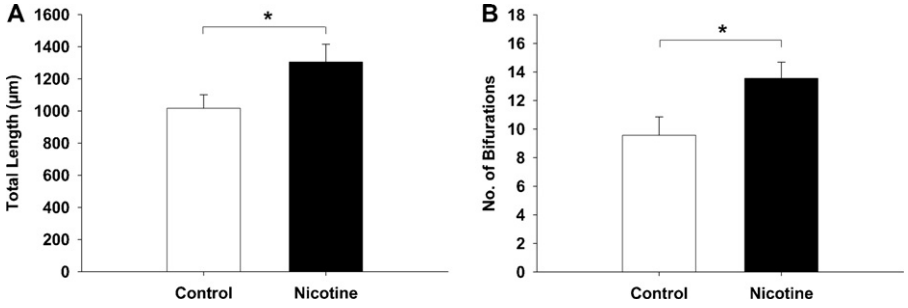

Fig. 2. Nicotine increases total length and number of bifurcations of basilar dendrites. (A) Nicotine pretreated rats show a 31% increase in the total length of basilar dendrites compared to control. (B) Nicotine pretreated rats show a 40% increase in the total number of bifurcations on basilar dendrites compared to control. *$p < .05$.

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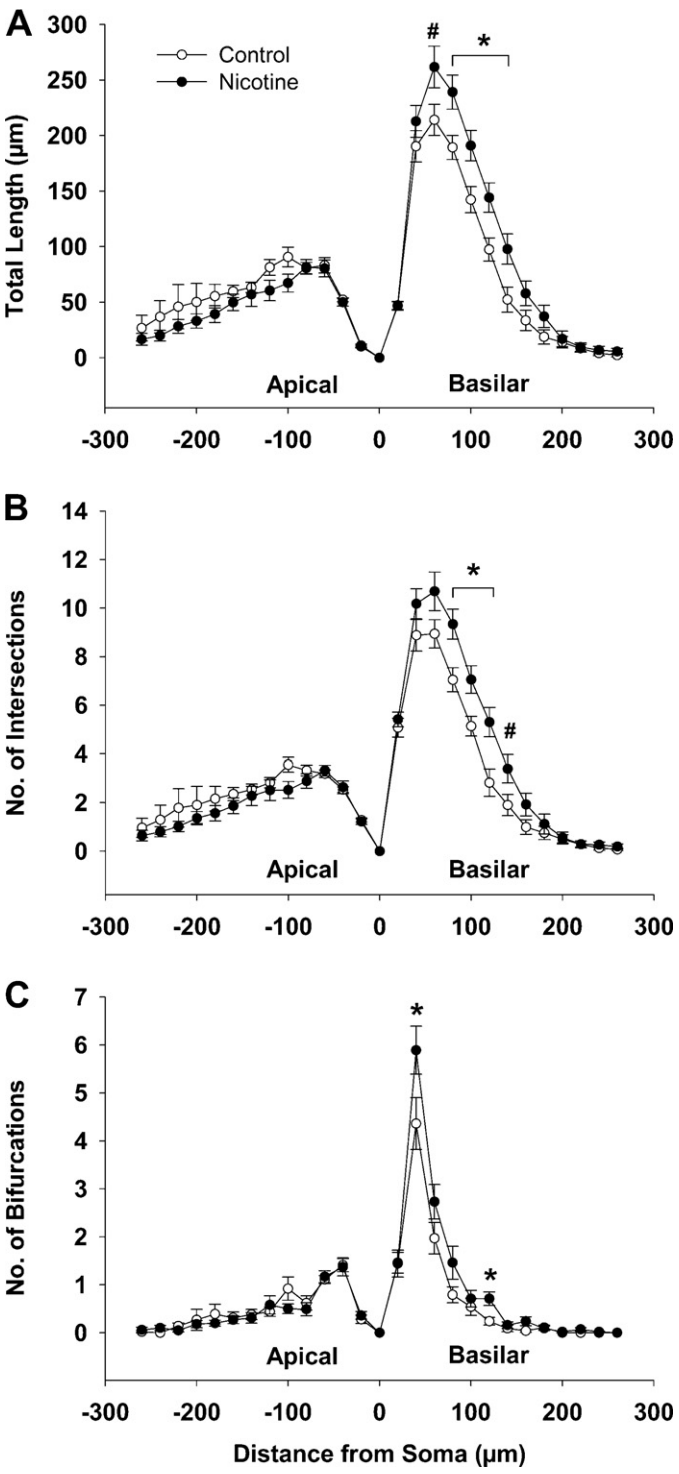

Fig. 3. Sholl-analysis of basilar dendrites. (A) Nicotine-induced increases in dendritic length were confined to basilar dendrites between 60 and 140 μm from the soma. (B) Nicotine-induced increases in the number of intersections of dendrites with concentric spheres surrounding the soma were confined to basilar dendrites between 80 and 140 μm from the soma. (C) Nicotine-induced increases in the number of bifurcations were observed at 40 and 120 μm from the soma. $^{*}p < .05$; $^{\#}p < .07$.

dendritic morphology of basilar dendrites at increasing distance from the soma. No differences in total length or intersections by radial distance were observed between groups for apical dendrites.

Sholl-analyses suggested a trend toward an increase in the number of bifurcations at distinct radial distance from the soma in nicotine treated animals compared to control ($^{a}F_{2.66,42.66} = 2.625$, $p = .07$) with significant increases in the number of bifurcations at 40 μm and 120 μm (Fig. 3C). These distances from the soma are consistent with the observed locations of increased total length and intersections. Furthermore, as both total length and the number of bifurcations are increased at similar distances from the soma, it can be suggested that the observed increase in total dendritic length is a product of an increased number of branches, rather than an overall growth of existing branches. Similar to the other morphometric parameters, no differences in number of bifurcations by radial distance were observed between groups for apical dendrites.

4. Discussion

Here, we report dendritic remodeling in the rostral agranular insular cortex (rAIC) following nicotine exposure. Dendrite plasticity was specific to the basilar dendritic tree, with the most prominent changes located at distal points (40 μm-140 μm) from the soma. We determined that increased branching, rather than growth of existing dendritic segments, was the most likely contributor to the overall change in dendritic length (31% increase). All structural modifications were observed 37 days following the end of nicotine dosing, indicating a form of plasticity in the rAIC that is present long after cessation from chronic nicotine exposure.

Although the present study suggests that dendritic remodeling in the rAIC is the result of nicotine exposure, the time-course of dendritic remodeling is currently unknown. It is possible that nicotine exposure is directly responsible for the structural plasticity observed in this study. Alternatively, it is also possible that the removal of nicotine following continuous exposure was responsible for the observed alterations in dendritic morphology. Morphometric analysis of dendrite structure at multiple time points following chronic exposure will be required to answer this important question.

The reported structural alterations in the rAIC following nicotine exposure were specific for the basilar dendritic tree, a finding in agreement with the pattern of structural modification reported previously in medial prefrontal cortex (mPFC) [1,3] and BLA [2]. No previous studies have addressed the specificity of structural alterations of dendrite morphology, nor the specificity of input, onto basilar vs. apical dendrites following drug exposure. However, we observe that most basilar dendrites in rAIC remain confined to the cortical layer in which the soma is located. As layer V of insular cortex receives the heaviest dopaminergic input from the VTA [10,14], it is possible that altered dopaminergic input during or following nicotine exposure contributes to the observed specificity of structural alterations layer V basilar dendrites.

The influence of nicotine on dendritic morphology is widespread in the brain, with examples of structural remodeling in both cortical and subcortical circuits. However, not all cortical regions exhibit structural plasticity in response to nicotine. For example, nicotine has not been shown to significantly alter pyramidal neuron dendrites in either the parietal [3] or infralimbic [2] cortices. It has also been observed, with the same dosing regimen used in the present study, that nicotine was insufficient to alter the dendritic morphology of medium-spiny neurons located in the NAcc shell [16], although alterations were found when nicotine was administered during the adolescent development period [15,16]. As prefrontal cortex and amygdala development is known to extend well into adulthood [2,7], it is possible that the rAIC might also exhibit age-dependent plasticity in response to nicotine.

Alterations in the complexity of dendrites located in the rAIC could be expected to modify the pattern of synaptic connectivity with other regions that have reciprocal connectivity with it, such as the nucleus accumbens, VTA, basolateral amygdala, and medial prefrontal cortex [4,9,10,14], which have all been implicated in various aspects of nicotine dependence. It has also been suggested

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that morphological alterations, such as the kind reported in this study, may alter both the weight and formation of new synaptic connections between previously unconnected neurons [5]. Together, these findings suggest the insula as a site for the integration of input from multiple regions involved in the formation and maintenance of nicotine dependence, and that neuron plasticity in the insula following nicotine exposure has the potential to significantly alter circuitry mediating nicotine dependence.

It is known that disruptions of insula functioning alter behavior related to nicotine dependence, including withdrawal and craving [13,17], conditioned place preference [6], self-administration [8] and nicotine-cue approach [22]. The insula is also an important site for the integration of viscerosensory information [4]. Dendritic remodeling in the insula following chronic nicotine exposure might be expected to modify the contribution or reward-value of viscerosensory (interoceptive) and environmental (exteroceptive) cues that are critical to the development of nicotine dependence [13]. Future research should aim to understand the alterations in behavior that accompany structural modifications of dendrite morphology in the insula, as well as the other mentioned brain regions, and how these alterations influence nicotine dependence.

In conclusion, we provide evidence for the insula as an important target for neuroplasticity following nicotine exposure. It is now clear that structural modifications of dendrites occur in multiple brain regions associated with the mesolimbic and mesocortical dopamine systems following nicotine exposure. It will be important to understand what role these alterations of dendrite morphology play in the development of nicotine dependence, as well as their influence on both normal and abnormal behavior.

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