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Continuous Nicotine Administration Produces Selective, Age-Dependent Structural Alteration of Pyramidal Neurons From Prelimbic Cortex

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ABSTRACT Emerging evidence indicates that adolescence represents a developmental window of enhanced nicotine-induced neuroplasticity in rat forebrain. However, whether nicotine produces age-dependent structural alteration of neurons from medial prefrontal cortex remains to be determined. We characterized the dendritic morphology of layer V pyramidal neurons from prelimbic cortex following adolescent (P29-43) or adult (P80-94) nicotine pretreatment. Nicotine administration was via osmotic pump [initial dose 2.0 mg/(kg day), free base]. Five weeks after drug administration concluded, brains were processed for Golgi-Cox staining and pyramidal neurons digitally reconstructed for morphometric analysis. Overall, nicotine pretreatment produced increased basilar, but not apical, dendritic length of pyramidal cells, a finding consistent with previous work using adult animals. Given the compelling evidence for morphologically distinct functional subtypes of cortical pyramidal neurons, we endeavored to determine whether nicotine-induced dendritic alteration was specific to putative structural subtypes. Neurons were segregated into two groups based on the extent of dendritic arbor at the distal portion of the apical tree (i.e., the apical tuft). The size of the apical tuft was quantitatively determined using principal component analysis. Cells with small and elaborate apical tufts were classified as simple and complex, respectively. We found that adult nicotine pretreatment produced increased basilar dendritic length and branch number in simple but not complex pyramidal cells. In contrast, adolescent nicotine pretreatment produced a modest but significant increase in basilar dendritic length in complex but not simple cells. These data suggest that nicotine alters dendritic morphology of specific subpopulations of pyramidal neurons and that the subpopulation affected is dependent on the age of drug exposure. **Synapse 62:31–39, 2008.**

INTRODUCTION

Individuals who do not start using tobacco during adolescence rarely initiate use later in life (Chen and Kandel, 1995), suggesting that age of first experience(s) with nicotine is a relevant epigenetic factor in the origin of nicotine abuse. Supporting animal models of nicotine exposure suggest that both the behavioral response to and neurochemical effects of nicotine differ between adolescents and adults (Adriani et al., 2003). The age-dependent behavioral and neurochemical impact of nicotine most likely relates to the unique developmental profile of the adolescent brain (Spear, 2000).

The frontal cortex continues to develop well into adulthood, and it is the cortical region which exhibits the greatest plasticity between adolescence and adulthood (Sowell et al., 1999, 2001). Frontal cortex development is nonlinear with cortical thickness increasing to early childhood and declining between adolescence and young adulthood (Shaw et al., 2006). The prelimbic (PL) and infralimbic regions of the medial prefrontal cortex (mPFC) in particular undergo a relatively late-wave of neuronal loss between adolescence and

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adulthood (Markham et al., 2007). Reduction in cortical thickness coincides with continued synaptic remodeling (Lewis, 1997) and increased prefrontal functioning through adolescence (Casey et al., 2000). In addition, there is continued ingrowth of dopaminergic and serotonergic projections to both pyramidal cells and GABAergic interneurons of the mPFC (Benes et al., 2000; Cunningham et al., 2002; Kalsbeek et al., 1990). The ongoing maturation of the prefrontal cortex may render adolescents selectively vulnerable to environmental factors including nicotine's putative neurodevelopmental effects (Slotkin, 2002; Smith, 2003).

Layer V pyramidal neurons from the prelimbic region of the mPFC have been shown to exhibit neuroplasticity in response to acute, intermittent nicotine administration (Brown and Kolb, 2001). To date, no work has examined whether adolescent nicotine administration produces similar long-term structural alterations in prelimbic cortex. We have recently shown that continuous nicotine administration during the adolescent period produces structural changes in medium spiny neurons from nucleus accumbens (NAcc) (McDonald et al., 2005) that are comparable with those observed in adults (Brown and Kolb, 2001). However, nicotine exposure during adulthood did not produce increased dendritic length in accumbal medium spiny neurons (McDonald et al., 2007), suggesting that continuous nicotine administration produced dissimilar structural remodeling compared with acute, intermittent exposure. In the current study, our primary goal was to directly compare how adolescent and adult nicotine administration influenced dendritic morphology of pyramidal cells from Layer V of PL cortex. We hypothesized that nicotine would produce age-dependent dendritic alteration of Layer V pyramidal cells from PL cortex.

Pyramidal cells in prefrontal cortex are arguably the most structurally complex cells in the neocortex (Elston, 2003). In Layer V, a principal output layer of the cerebral cortex, pyramidal cells have been tentatively classified into heterogeneous subpopulations based on morphology, molecular profile, physiology, and pattern of connectivity (Le Be et al., 2007; Molnar and Cheung, 2006; Morishima and Kawaguchi, 2006; Tsiola et al., 2003; Voelker et al., 2004). Moreover, recent work provides compelling evidence that mPFC possesses distinct microcircuits comprising subtypes of pyramidal cells with unique morphological and physiological properties (Wang et al., 2006). To date, no study has examined the possibility that there are pyramidal cell subtypes that respond differentially to psychostimulant-induced neurotransmitter dysregulation. There is some evidence suggesting that this might occur, such as the finding that dopamine (DA) (D)1 and D2 receptor expression appears to be specific to subsets of pyramidal neurons in mPFC (Gaspar et al., 1995). Nicotine-induced dendritic remodeling of select pyramidal neurons can be expected to have unique functional consequences (Elston, 2003). Consequently, a second aim of this study was to determine whether there are morphological subclasses of pyramidal neuron that are selectively sensitive to structural alteration following nicotine exposure.

MATERIALS AND METHODS

Subjects

Male Long-Evans hooded rats were obtained at postnatal day (P) 22 and P55-60 (Harlan, Indianapolis, IN). Animals were group-housed with ad libitum access to food and water. A 12-h light/dark cycle (lights on at 7:00 AM) was maintained throughout the study. All experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Dosing

Treatment groups consisted of a nicotine dose group (adolescent, $n = 9$; adult, $n = 10$) and a sham control (adolescent, $n = 10$; adult, $n = 9$). Nicotine bitartrate (experimental) or saline with sodium tartrate (control) was continuously administered from P29 to P43 (adolescence) and P80-P93 (adult) via subcutaneously implanted Alzet osmotic pumps (Model 1002; Durect, Cupertino, CA). This age range is comparable with Spear's (2000) conservative estimate of the time span of adolescence in the rat. Prior to implant surgery, animals were anesthetized with Equithesin (3.5 mg/kg). Pumps contained nicotine bitartrate dissolved in 0.9% NaCl. Control animals received pumps with a comparable concentration of sodium tartrate dissolved in 0.9% NaCl. Initial and final dose rates (calculated as free base) in the adolescent cohort were 2.0 and 0.95 mg/(kg day), respectively. The dose rate remained constant in the adult cohort. The initial dose rate [2 mg/(kg day)] was chosen to provide plasma nicotine levels comparable with those seen in human smokers (Slotkin, 2002). Following a 30-day abstinent period, both nicotine and control groups were evaluated for locomotor response to nicotine challenge (McDonald et al., 2007). One week following nicotine challenge animals were deeply anesthetized with tribromoethanol and perfused intracardially with 0.9% NaCl. Brains were then removed and placed in Golgi-Cox solution for 14 days. Following Golgi-Cox immersion, brains were stored in a 30% sucrose solution until vibratome sectioning (200-μm sections). Sections were stained using the protocol of (Gibb and Kolb, 1998). The Golgi-Cox solution was prepared according to the recipe of Glaser and Van der Loos (1981).

Anatomy

PL pyramidal neurons were selected from Golgi-stained tissue based on their location relative to major landmarks (e.g., genu of the corpus collosum) and cortical depth (500–600 μm). We chose to focus on analysis of pyramidal neurons from Layer V of the PL cortex since previous work has shown plasticity in this region in response to nicotine administration (Brown and Kolb, 2001). To rule out artifactual morphological differences resulting from differential sampling across layers, soma-to-pial differences were compared across pretreatment group and age using one-way ANOVA (ns). Neurons were traced by an experimenter blind to drug treatment. Cells with a prominent, single apical tree extending from the apex of the soma toward the pial surface of the cortex, two or more basilar dendritic trees extending from the base of the soma, and dendritic spines were chosen for reconstruction. Neurons were digitized in three dimensions under a 60× objective using Neurolucida (Microbrightfield, Colchester, VT). Reconstructed neurons averaged 559.6 ± 4 μm soma-to-pial surface distance. Morphometric parameters included total length and total number of branches for both apical and basilar trees. The distribution of dendritic length was quantified using Sholl analysis (Sholl, 1981) (20-μm increments). Soma size was estimated by measuring from the apex of the soma to its base. Soma-to-terminal length for the apical tree was also determined. Quantification of morphometric parameters was performed with Neuroexplorer (Microbrightfield, Colchester, VT). Neurons were selected for tracing only if they were well impregnated and possessed unobstructed dendrites that could be followed without interruption. Based on these criteria, 2–5 neurons were reconstructed per animal. A total of 61 pyramidal neurons (control $n = 31$; nicotine $n = 30$) from the adolescent, and 57 pyramidal neurons (control $n = 28$; nicotine $n = 29$) from the adult group were reconstructed for analysis.

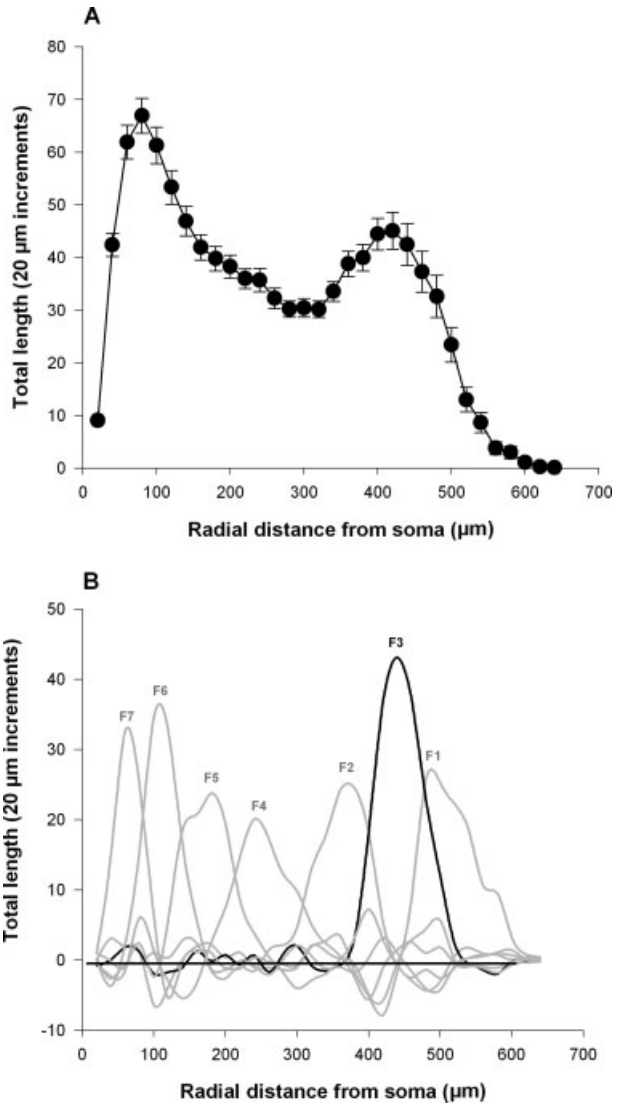

Fig. 1. (**A**) Sholl analysis of total apical length for all cells (n = 120). Circles represent the group mean ± the standard error of the mean (**B**) Length distributions of factor loadings. F3 indicates the factor that best fit the length distribution of the distal-most portion of the apical tree (A). These seven factors collectively account for 83.4% of the total variance.

Statistics

Data reduction and analysis

SPSS (v. 15) for Windows was used for all statistical analysis. The branching pattern of the distal apical dendrite is a defining feature for characterizing subtypes of cortical pyramidal cells (Molnar and Cheung, 2006). We used principal component analysis (PCA) to segregate our cell population based on the degree of "tuftedness" of the apical dendrite. For the PCA, we used a covariance association matrix, considering increments in radial distance from the soma as variables. Factors were subjected to Promax rotation to obtain simple structure (Dien et al., 2005). Factor scores were calculated for the factor (Factor 3) whose loading plot best matched the morphology of the distal portion of the grand-mean sholl plot (Fig. 1). The median factor score was used to segregate cells into two groups. Cells that fell below and above the median split were referred to as "simple" and "complex," respectively. Following assignment of cells into morphological categories, nicotine (n = 20 complex group; n = 10 simple group) and control (n = 11 complex group; n = 20 simple group) pretreated adolescents were compared with nicotine (n = 16 complex group; n = 14 simple group) and control (n = 13 complex group; n = 16 simple group) pretreated adults.

Group comparisons were made with univariate ANOVA. Each neuron was treated as an independent

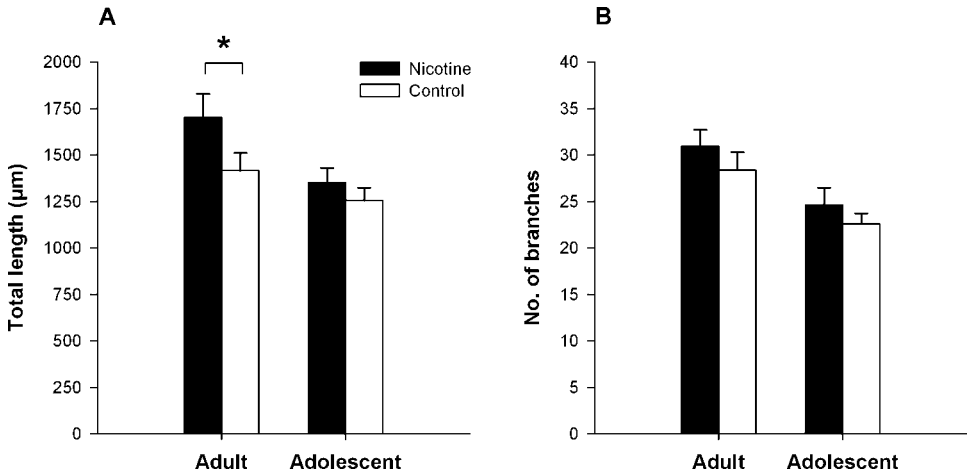

Fig. 2. Morphometric measurements of pyramidal neurons from the adolescent and adult-pretreated groups. (**A**) Nicotine increased total basilar length in the adult cohort. (**B**) Total number of branches per neuron. Bars represent the group mean ± standard error of the mean. *$P < 0.05$.

measure. Factors age (adolescent vs. adult), pretreatment (nicotine vs. control) and cell type (simple vs. complex) were the between-groups factors. The presence of an interaction was followed with an analysis of simple effects utilizing one-way ANOVA. Homogeneity of variance among groups was evaluated using Levine's test. Detection of heterogeneity of variance led to reanalysis using the Brown-Forsythe statistic. For ANOVA with repeated measures, radial distance from the soma was the within-group factor. Violations of the assumption of sphericity were corrected using the Greenhouse-Geisser correction for degrees of freedom. The presence of an interaction led to follow-up analyses using independent sample t tests with the appropriate Bonferroni correction. Significance was determined by $P < 0.05$ for all tests.

RESULTS

Statistical analysis of total basilar length showed a main effect of pretreatment $F(1, 108.1) = 3.95$; $P < 0.05$, with nicotine increasing total basilar length compared with control pretreatment (Fig. 2). There was also a main effect of age, with the older cohort possessing greater overall basilar length $F(1, 97.1) = 7.14$; $P < 0.01$ and branch number $F(1, 110.2) = 12.6$; $P < 0.001$, relative to the younger cohort. Importantly, total length and branch number of apical trees did not differ by pretreatment, a finding consistent with that of Brown and Kolb (2001).

Morphological classification

A comparison of cells falling above and below the median split revealed a significant effect of cell type on total length as a function of distance from the soma $F(1, 118) = 44.13$; $P < 0.001$. Follow-up analyses revealed that differences were confined to the points delineated by the highest factor loadings for factor 3 (Fig. 3). This finding is consistent with two morphological subtypes that differ based on the degree of "tuftedness" of the apical dendrite (Fig. 3). A sholl analysis considering sphere intersections, as opposed to total dendritic length, produced comparable results (data not shown). Simple cells tended to possess relatively small somas (20.5 ± 2.9) as compared with complex cells (21.5 ± 2.5), although the difference only approached significance $F(1, 118) = 3.59$; $P = 0.06$. In addition, the soma-to-terminal distance for the apical tree was greater in complex (520.3 ± 44.6) than in simple (425.0 ± 75.8) cells $F(1, 118) = 70.5$; $P < 0.001$. For the control cohorts, there was a trend towards greater total basilar length in complex (1455.5 ± 126.0) relative to simple (1291.1 ± 62.5) cells ($P = 0.08$). There was no interaction of pretreatment and age on total apical length or branch number with respect to cell type.

Effects of adult dosing

Representative digital reconstructions of PL pyramidal cells are presented in Figure 4. There was a significant interaction of pretreatment x age x cell type on total basilar length $F(1, 118) = 13.3$; $P < 0.001$ and branch number $F(1, 118) = 8.0$; $P < 0.01$. In the adult pretreatment group, both basilar dendritic length $F(1, 19.3) = 11.7$; $P < 0.001$ and branch number $F(1, 28) = 5.1$; $P < 0.05$ of simple cells was increased following nicotine treatment (Fig. 5). A sholl analysis showed an interaction of pretreatment x cell type $F(1, 53) = 5.17$; $P < 0.05$ with simple cells exhibiting enhanced total length in response to nicotine $F(1, 28) = 10.84$; $P < 0.001$. Follow-up comparisons revealed nicotine-induced increases at proximal distances of 60 $t(28) = 3.95$; $P < 0.01$ and 80 $t(28)$

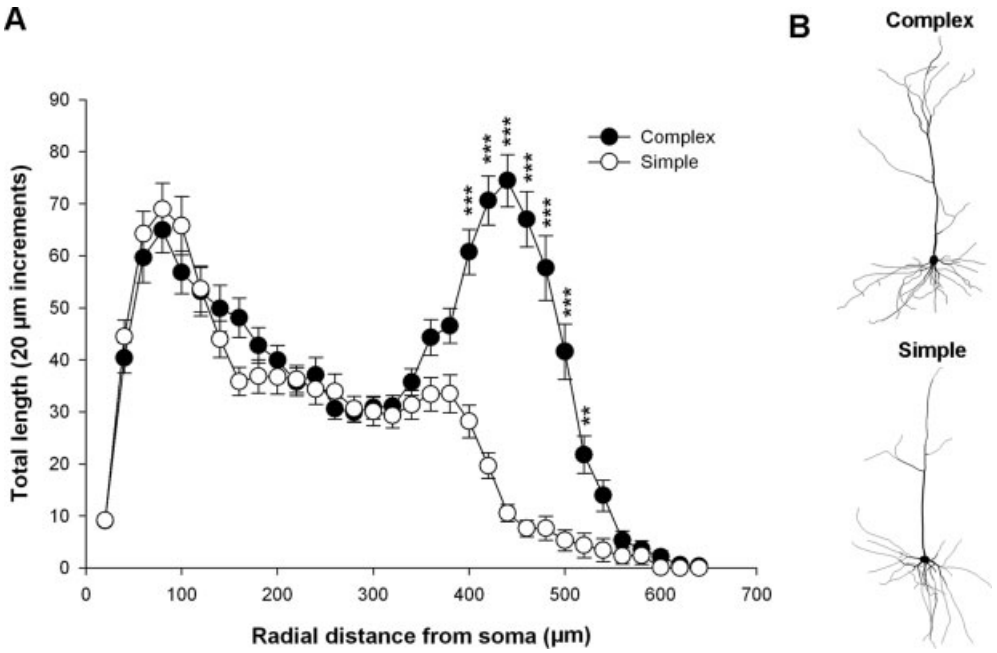

Fig. 3. (**A**) Segregation of putative cell subtypes based on median split of factor scores. Complex cells exhibited greater total dendritic length at the distal portion of the apical tree compared to simple cells. Circles represent the group mean ± the standard error of the mean (**B**) Representative digital reconstruction of a complex and simple neuron. ***$P < 0.001$; **$P < 0.01$.

= 4.94; $P < 0.001$ µm from the soma (Fig. 6). Notably, there was no effect of nicotine treatment on total basilar length and branch number of complex cells.

Effects of adolescent dosing

In the adolescent group, there was no effect of pretreatment on total basilar length or branching in simple cells. In complex cells, nicotine pretreatment increased overall basilar length $F(1, 29) = 4.2$; $P < 0.05$ compared with controls (Fig. 5).

DISCUSSION

Overall, chronic nicotine exposure increased basilar, but not apical, dendritic length of Layer V pyramidal neurons from PL cortex following a 5-week abstinence period. These data are in accord with Brown and Kolb (2001) who showed similar structural changes in Layer V pyramidal neurons following acute, intermittent exposure. In fact, the magnitude of the overall increase in dendritic length reported here for adults (16.7%) is very similar to that reported by Brown and Kolb (16.8%). Classification of pyramidal neurons utilizing PCA suggested the existence of morphological subtypes based on the extent of dendritic arbor at the distal portion of apical tree (Figs. 1 and 3). In the adult cohort, nicotine produced structural alteration only in pyramidal cells with relatively restricted

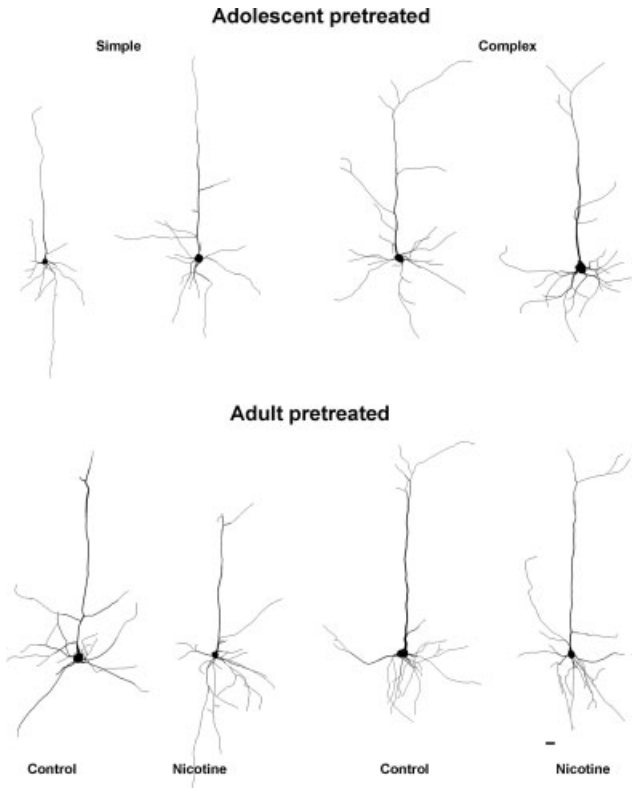

Fig. 4. Representative digital reconstructions of complex and simple prelimbic pyramidal neurons from adolescent and adult-pretreatment groups. Scale bar: 20 µm.

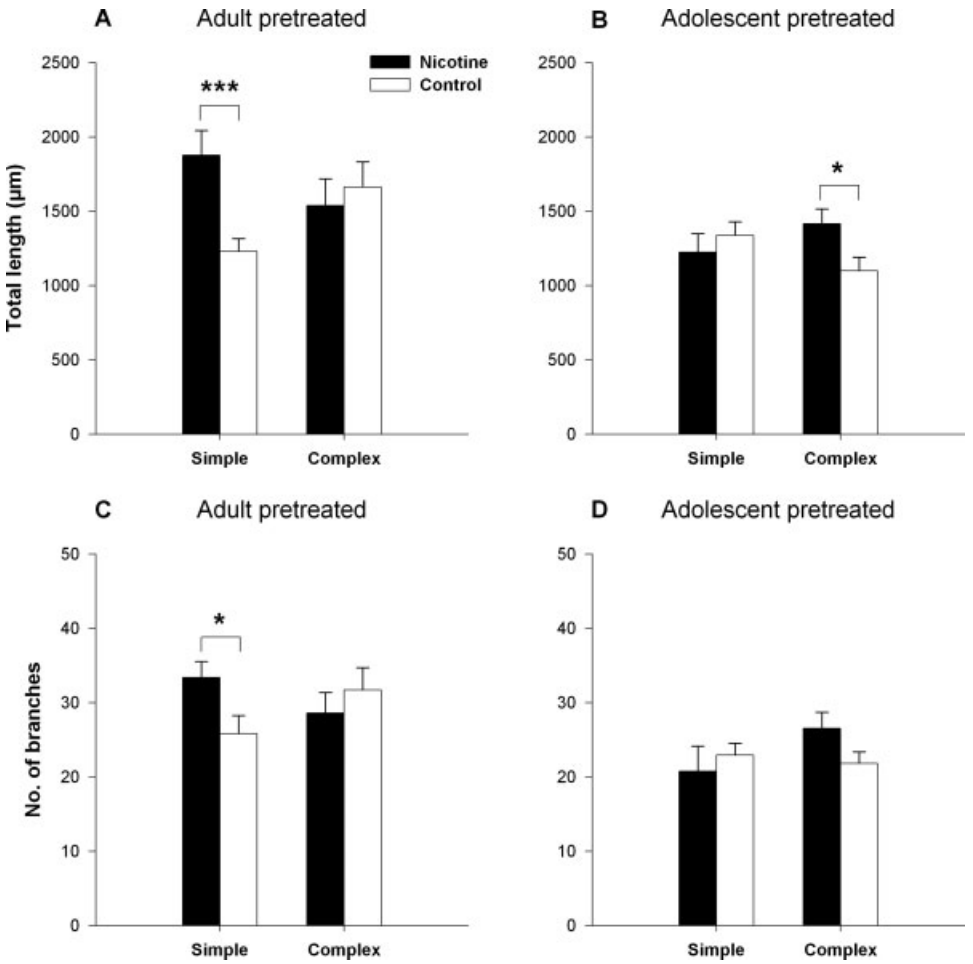

Fig. 5. Morphometric measurements of total basilar length and branch number from complex and simple prelimbic pyramidal neurons of the adolescent and adult pretreated groups. (Panels **A** and **B**) Nicotine increased total basilar length in simple cells from the adult (A) pretreated group. Nicotine increased total basilar length in complex cells from the adolescent (B) pretreated group. (Panels **C** and **D**) Nicotine increased number of branches in simple cells (C) from the adult group. Nicotine did not alter branching in simple or complex cells from the adolescent (D) pretreated group. Bars represent the group mean ± the standard error of the mean. $^{***}P < 0.001$; $^{*}P < 0.05$.

branching at the distal portion of the apical tree (simple cells). Specifically, nicotine exposure during adulthood increased basilar dendritic length and branch number in simple neurons (Fig. 5). Enhanced dendritic length was restricted to the proximal portion of the basilar trees (Fig. 6). In contrast to the adult cohort, nicotine treatment during adolescence produced an increase in total basilar length in cells with more elaborate apical tufts (complex cells) (Fig. 5). Collectively, these data suggest that nicotine produces selective, age-dependent structural alteration of prelimbic pyramidal cells.

Few studies have characterized pyramidal cell subpopulations in mPFC. It is possible that the morphological subtypes suggested by our analysis fall into pyramidal cell categories identified in other cortical regions such as frontal cortex (Morishima and Kawaguchi, 2006) and primary visual cortex (Tsiola et al., 2003). As with the present work, size of the apical tuft, soma-to-apical terminal distance, and soma size were defining structural features used to distinguish cell subtypes (Molnar and Cheung, 2006; Tsiola et al., 2003). However, it is important to note structural similarity across different cortical regions is not likely to equate to functional similarity, given that connectivity would differ substantially.

One of the few studies providing evidence of pyramidal cell subtypes in mPFC is that of Wang et al. (2006), who identified a physiological subtype with unique dendritic morphology. This subtype possessed an early apical bifurcation giving rise to dual apical trees. Although we encountered cells with comparable morphology in the present study, they comprised a small proportion of our total sample, which precluded a separate analysis of this population. However, we note that our analysis unanimously placed neurons with early bifurcating apical trees into the complex grouping.

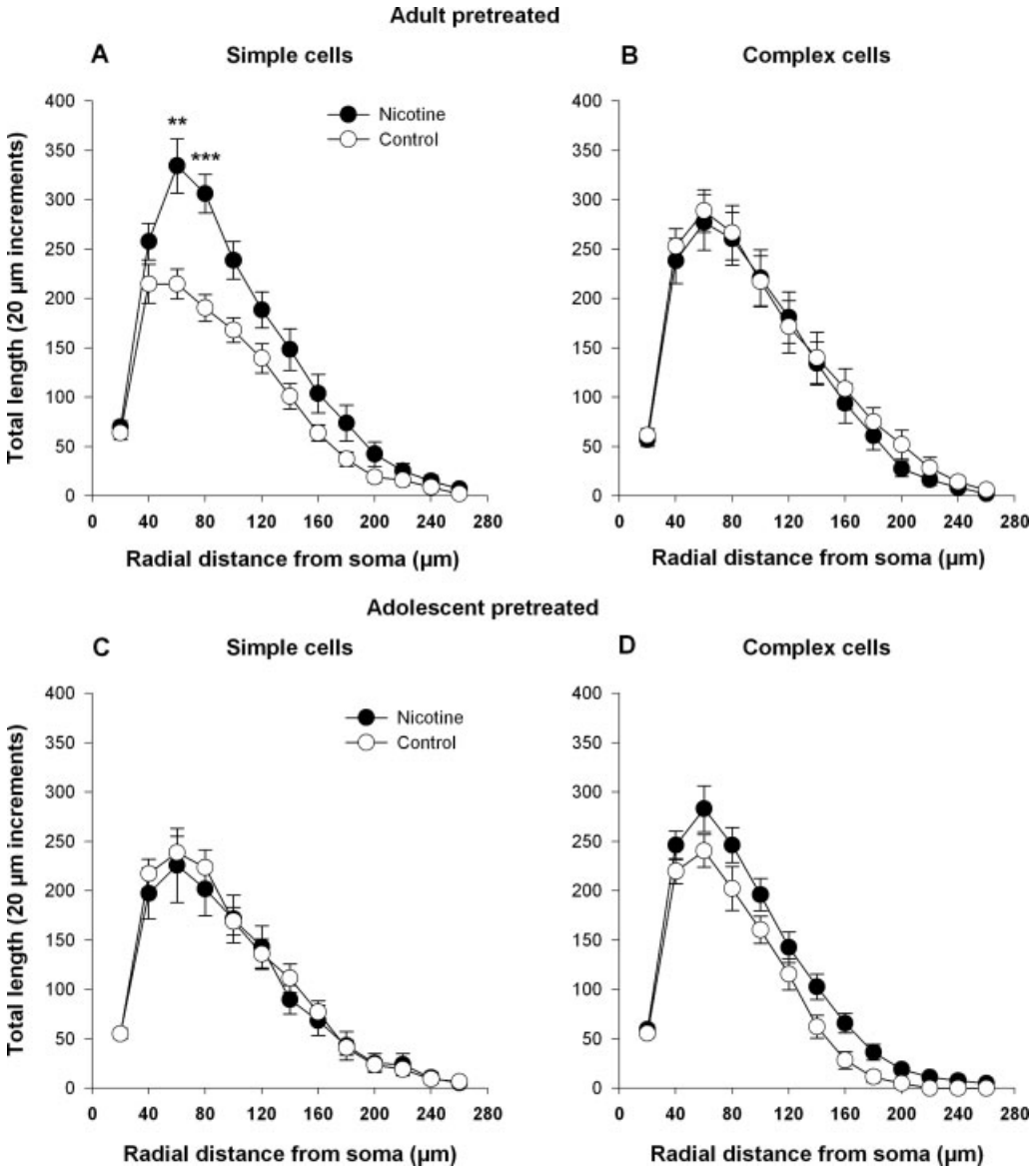

Fig. 6. Sholl analysis of total basilar length from simple and complex pyramidal neurons of the adult and adolescent pretreated groups. (Panels **A** and **B**) Adult nicotine pretreatment enhanced total basilar length at proximal points (60 and 80 µm) from the soma in simple (A) but not complex (B) neurons. (Panels **C** and **D**) Adolescent nicotine pretreatment did not alter total basilar length of simple (A) or complex (B) neurons. Circles represent the group mean ± the standard error of the mean. $^{***}P < 0.001$; $^{**}P < 0.01$.

There is some evidence that DA (D)1 and D2 receptor are localized to different classes of pyramidal cells in the prelimbic region (Gaspar et al., 1995; Vincent et al., 1995). DA release originating from the ventral tegmental area (VTA) is enhanced in prefrontal cortex after repeated nicotine administration (Nisell et al., 1996). Given that D1 and D2 receptor subtypes may be localized to different pyramidal cell subpopulations, it is reasonable to speculate that nicotine-mediated DA release may differentially activate subpopulations of pyramidal neurons. The extent to which selective DA-mediated activation of pyramidal cells may alter dendritic structure remains to be determined.

It has been well-established that prefrontal cortex (Sowell et al., 1999) undergoes late synaptic refinement, although the nature of the development of pyramidal microcircuits through the adolescent period is unknown. In adult rodents, structural plasticity in the prelimbic region has been demonstrated after amphetamine (Robinson and Kolb, 1997), cocaine (Robinson and Kolb, 1999), and Delta(9)-THC (Kolb et al., 2006). In addition, low-dose nicotine has been shown to interact with stressors to elevate plasticity-related immediate early genes arc, NGFI-B, and c-Fos in the prefrontal cortex (Schiltz et al., 2007). Collectively, these data demonstrate the capacity for neuronal plasticity in the adult prefrontal cortex. Acute nico-

tine administration has been shown to increase expression of arc and c-fos mRNA in mPFC to a greater extent in adolescents than in adults (Schochet et al., 2005), suggesting a higher degree of plasticity in the adolescent mPFC. The extent to which immediate early genes arc and c-fos are directly related to long-term structural alteration in response to nicotine is not understood.

Recent electrophysiological evidence suggests that DA-glutamatergic interactions in pyramidal cells (Tseng and O'Donnell, 2005; Tseng et al., in press) and DA-dependent functioning of interneurons from mPFC (Tseng and O'Donnell, 2007) continue to mature throughout adolescence. These data are consistent with evidence that D1 (Leslie et al., 1991; Tarazi et al., 1999) and NMDA receptor subunit expression (Monyer et al., 1994; Williams et al., 1993) continue to be modified during this period. In support of a relatively late development trajectory for connectivity with other brain regions, it has been shown that afferent input to mPFC from ventral tegmental DA neurons and amygdala continues to increase throughout adolescence (Cunningham et al., 2002; Kalsbeek et al., 1990). Collectively, these data underscore the relative underdevelopment of neurotransmitter systems in the adolescent mPFC, and may account for age-related differences in nicotine-induced structural plasticity.

Nicotine enhances the release of DA (Nisell et al., 1996), acetylcholine (Arnold et al., 2003), and glutamate (Lambe et al., 2003) in prefrontal cortex, as well as activating interneurons (Couey et al., 2007) that contact pyramidal cells. The pattern of innervation of specific subpopulations of pyramidal cells remains to be determined; however, it is known that prefrontal subpopulations of pyramidal cells have different efferent targets (Morishima and Kawaguchi, 2006). Increases in total dendritic length following nicotine exposure can be expected to increase connectivity within a local circuit as the potential for synaptic connectivity has been shown to covary with respect to this parameter (Chklovskii et al., 2004). Thus, nicotine-induced dendritic remodeling has the potential to strengthen and possibly even rewire intracortical circuits (Chklovskii et al., 2004). Our data provide evidence of elevated synaptic connectivity in putative subclasses of pyramidal cells, suggesting that nicotine has a selective effect on PL microcircuits. This may have specific functional consequences given the finding that the morphological subclasses of pyramidal cells that are believed to comprise prefrontal microcircuits possess unique physiological properties (Wang et al., 2006).

In conclusion, the present study extends previous work (Brown and Kolb, 2001), showing that continuous nicotine administration during adulthood and adolescence produce long-term structural alteration of Layer V pyramidal neurons from PL cortex. We also provide, to our knowledge, the first evidence that psychostimulant-induced structural alteration can be specific to morphological subtypes. Our findings suggest that subpopulations of PL pyramidal cells continue development through adolescence, as evidenced by their age-dependent structural response to nicotine exposure. That nicotine appears to alter a different subpopulation of PL pyramidal cells during adolescence as compared to adulthood suggests that PL cortical function may be differentially affected depending on the age of drug exposure.

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