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Research Report

Evidence for elevated nicotine-induced structural plasticity in nucleus accumbens of adolescent rats

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ABSTRACT

Male Long–Evans rats were administered nicotine bitartrate or sodium tartrate either during adolescence (p29–43) or adulthood (p80–94). Route of administration was via subcutaneously implanted osmotic pump (initial dose 2.0 mg/kg/day, free base). Five weeks following nicotine administration, brains were processed for Golgi–Cox staining. Medium spiny neurons from nucleus accumbens (NAc) shell were digitally reconstructed for morphometric analysis. Total dendritic length and branch number were greater in medium spiny neurons from animals pretreated with nicotine during adolescence. A branch order analysis indicated that increased branch number was specific to higher order branches. Mean branch lengths did not differ with respect to treatment as a function of branch order. Thus, nicotine-induced increases in total dendritic length were a function of greater numbers of branches, not increased segment length. In contrast, adult nicotine exposure did not significantly alter total dendritic length or branch number of medium spiny neurons. Total dendritic length and branch number of a second morphological type, the large aspiny neuron, did not differ following either adolescent or adult pretreatment. The age-dependent alteration of accumbal structure was associated with qualitatively different behavioral responses to drug challenge. These data provide evidence that drug-induced structural plasticity in nucleus accumbens is considerably more pronounced during adolescence.

1. Introduction

Considerable research has been directed at determining whether adolescence represents a unique period of vulnerability to drugs of abuse, including nicotine. These efforts have provided mounting evidence that the reinforcing (Adriani et al., 2002; Vastola et al., 2002; Belluzzi et al., 2004; Shram et al., 2006; Brielmaier et al., 2007) and psychomotor effects (Faraday et al., 2001, 2003; Schochet et al., 2004) of nicotine differ between adolescents and adults. There is also evidence that chronic nicotine exposure during adolescence produces a number of long-lasting neurobiological effects, including up-regulation of nicotinic cholinergic receptors (Trauth et al., 1999) and alteration of catecholaminergic systems (Trauth et al., 2001; Slotkin, 2002). It is possible that these changes relate to elevated plasticity of the mesolimbic reward pathway during the adolescent period, a phenomenon that has received little attention. Consistent with this notion is the finding that nicotine differentially affects expression of at least one plasticity-related gene (*arc*) in adolescents as

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compared with adults (Schochet et al., 2005). Increased *arc* expression was observed in prefrontal cortex, but not ventral striatum, although its baseline expression in ventral striatum was found to be greater in adolescents. There has been no demonstration of a corresponding increase in structural plasticity in these components of the mesolimbic dopamine pathway.

The nucleus accumbens (NAc) is a key element of the mesolimbic dopamine pathway involved in both the reinforcing and psychomotor effects of psychostimulants (Wise, 2002). Studies using adult rats have shown that psychostimulant administration induces dendritic remodeling of the principal cell type from NAc, the medium spiny neuron (MSN) (Robinson and Kolb, 2004). This structural plasticity is particularly pronounced with nicotine exposure (Brown and Kolb, 2001). Given that MSNs are projection neurons comprising the output path of the mesolimbic dopamine pathway, it can be expected that alteration of their morphology would substantially affect the efficacy of drug action. Indeed, nicotine-induced changes in MSN morphology have been shown to be accompanied by psychomotor alteration in response to drug challenge (Brown and Kolb, 2001; McDonald et al., 2005). Structural changes in MSNs include increased dendritic length and spine density, and it appears that alteration in dendritic length is a result of dendritic branching (Robinson and Kolb, 2004).

We have recently shown (McDonald et al., 2005) that chronic periadolescent nicotine administration produces increases in dendritic length of MSNs comparable to that seen with adults (Brown and Kolb, 2001), and that this phenomenon is associated with long-lasting psychomotor alteration. However, our study differed from that of Brown and Kolb (2001) with respect to the magnitude, rate and pattern of drug administration. Most notably, we administered the drug continuously via osmotic pump, while Brown and Kolb (2001) employed intermittent, acute, subcutaneous injections. Consequently, it was not possible to conclude whether adolescents and adults are differentially vulnerable to nicotine-induced dendritic remodeling. The primary goal of the current study was to determine whether continuous nicotine administration differentially affects dendritic morphology of MSNs from adolescent- and adult-pretreated animals.

2. Results

2.1. Behavior

Statistical analysis of baseline activity (saline injection trial) revealed a main effect of dose ($F(1,34)=4.61$; $p<0.05$), with nicotine-pretreated animals showing lower activity levels than control animals. When each age group was examined separately, a significant dose effect was observed in only the adolescent pretreatment cohort ($t(17)=2.52$; $p<0.05$).

An analysis of difference scores revealed a main effect of age ($F(1,34)=8.47$; $p<0.01$) and a significant dose × age interaction ($F(1,34)=7.57$; $p<0.01$). A follow-up analysis indicated that experimental animals from the adult pretreatment cohort showed significantly reduced locomotor activity compared to controls in response to the nicotine challenge ($t(17)=2.10$; $p=0.05$). In contrast, nicotine-treated animals from the adolescent pretreatment cohort showed a trend ($p<0.06$) towards greater activity than controls in response to the challenge (Fig. 1).

2.2. Anatomy

Representative micrographs and examples of digital reconstructions of MSNs and large aspiny neurons (LAs) are presented in Fig. 2. Statistical analysis of dendritic length of MSNs showed a significant main effect of dose, with neurons from nicotine-treated animals having significantly greater total dendritic length than controls ($F(1,134)=7.71$; $p<0.01$). An analysis of branch number revealed that MSNs from nicotine-treated animals also possessed a greater number of branches than neurons from controls ($F(1,134)=10.96$; $p=0.001$). There was also a significant main effect of age, with MSNs from the adult pretreatment cohort possessing more branches than MSNs from the adolescent pretreatment cohort ($F(1,134)=4.60$; $p<0.05$).

When each age group was examined separately, it was found that total dendritic length ($t(65)=2.46$; $p<0.05$) and branch number ($t(65)=2.99$; $p<0.01$) were significantly greater in MSNs from animals pretreated with nicotine during adolescence (Figs. 3A, B). In contrast, there was no significant increase in total length or branch number for MSNs from animals pretreated during adulthood (Fig. 4).

A branch order analysis of MSNs from the adolescent pretreatment cohort revealed significant differences in the numbers of third ($t(65)=2.74$; $p<0.05$) and fourth ($t(65)=3.04$; $p<0.05$) order branches, although mean branch lengths did not differ (Figs. 3C, D). Given that mean branch lengths did not differ with respect to treatment, it can be concluded that the observed increase in total dendritic length was a function of greater branch number.

Statistical analysis of LAs revealed no main effects with respect to total dendritic length; nor was there an age × dose interaction (Fig. 5). Analysis of total branch number revealed

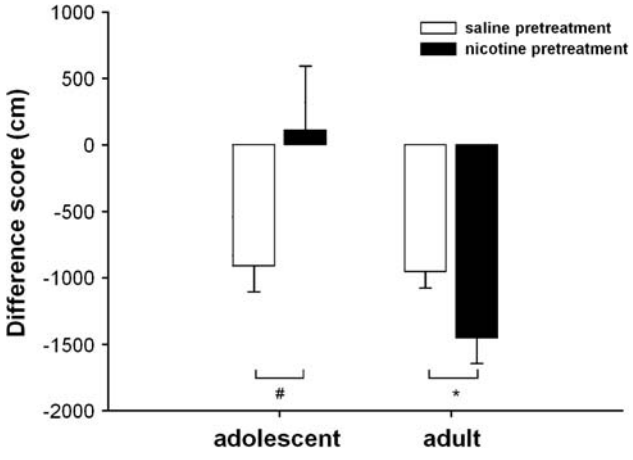

Fig. 1 – Response to nicotine challenge. Data are presented as difference scores, where distance traveled on day 1 (saline injection) was subtracted from distanced traveled on day 2 (nicotine challenge). #$p<0.06$ versus saline pretreated; *$p=0.05$ versus saline pretreated.

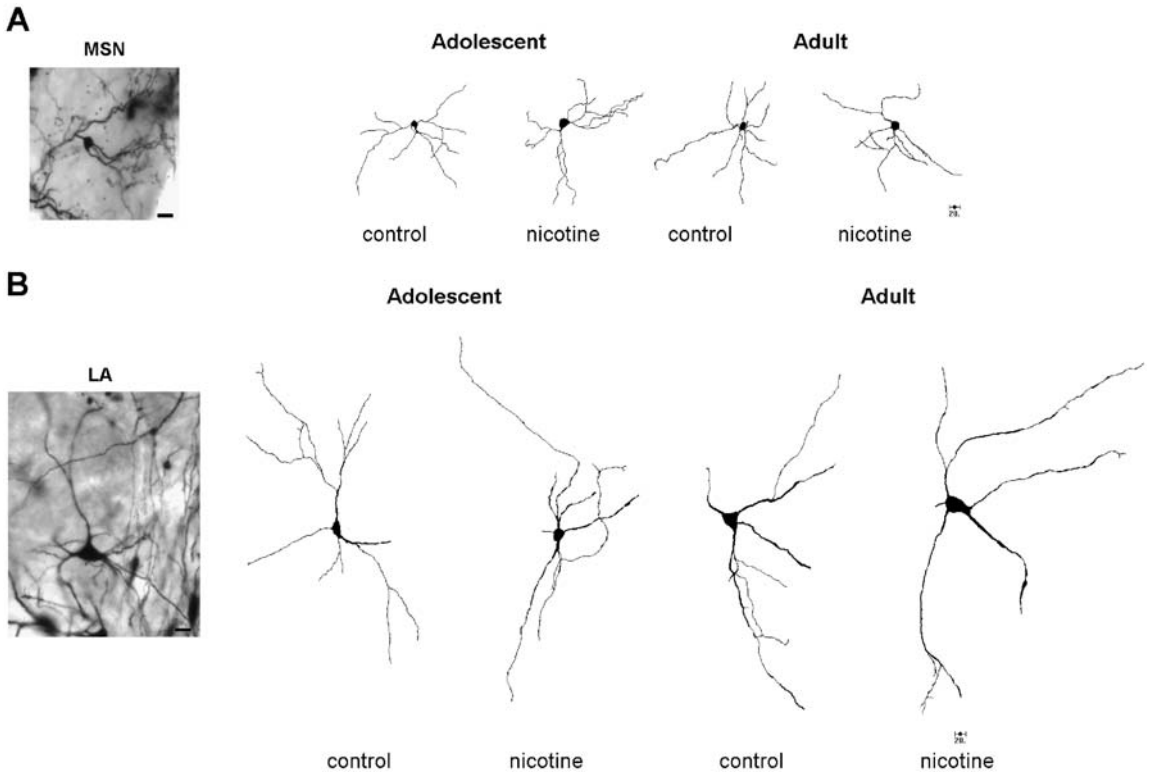

Fig. 2 – Representative micrographs and digital reconstructions of medium spiny (A) and large aspiny neurons (B). Scale bars: 20 μm.

only a main effect of age, with neurons from the adult pretreatment cohort possessing fewer branches than those from the adolescent pretreatment cohort ($F(1,98)=5.41$; $p<0.05$).

3. Discussion

Continuous nicotine administration during adolescence, but not adulthood, produced a significant increase in total dendritic length and branch number of MSNs from NAc shell. This phenomenon was evident 5 weeks following drug administration. The pattern of dendritic alteration induced by nicotine exposure was similar to that observed for cortical pyramidal neurons following environmental enrichment (Greenough and Volkmar, 1973) in that alterations were specific to higher order branches. In contrast, changes in total dendritic length or branch number were not observed in LAs of either cohort. Taken together these data provide evidence of both age-dependent and cell-specific nicotine-induced plasticity of accumbal neural circuitry.

The finding that MSNs from the adult pretreatment cohort were not altered by nicotine stands in contrast to the findings of Brown and Kolb (2001). The disparity between studies most likely reflects the fact that in the present study nicotine was administered continuously throughout the dosing period, while Brown and Kolb employed intermittent subcutaneous injections. It has been shown that the extent of neurobehavioral alteration is dependent on the rate of drug administration, with rapid administration being substantially more efficacious (Samaha and Robinson, 2005). Accordingly, we suggest that continuous administration was not sufficient to produce significant alteration of dendritic morphology in adult animals. That continuous nicotine administration produced increased MSN dendritic length in only the adolescent pretreatment cohort can be taken as evidence of greater nicotine-induced structural plasticity during this period of development. This phenomenon may be attributable, in part, to elevated activity of plasticity-related genes such as *arc* and *c-fos* in the adolescent brain (Schochet et al., 2005). The former is dendritically targeted and has been implicated in activity-dependent synaptic alteration (Kelly and Deadwyler, 2003).

We note that, with the adolescent pretreatment cohort, the nicotine dose progressively decreased as a function of animal weight gain. It could be argued that this phenomenon lends further support to our evidence of increased drug-induced plasticity during adolescence. However, it is also possible that the progressive reduction in dose may have led to withdrawal over the latter portion of the dosing period, and that this might have contributed to the observed structural changes. Although this possibility cannot be entirely excluded, we note that adolescent animals exhibit substantially lower somatic and motivational signs of nicotine withdrawal than do adults (O'Dell et al., 2006, 2007). This finding also argues against any potential influence of the withdrawal period following dosing.

Although not the focus of our study, we did detect age-dependent differences in MSN and LA dendritic morphology. MSNs from the adult pretreatment cohort possessed a greater number of dendritic branches, while LAs from this cohort

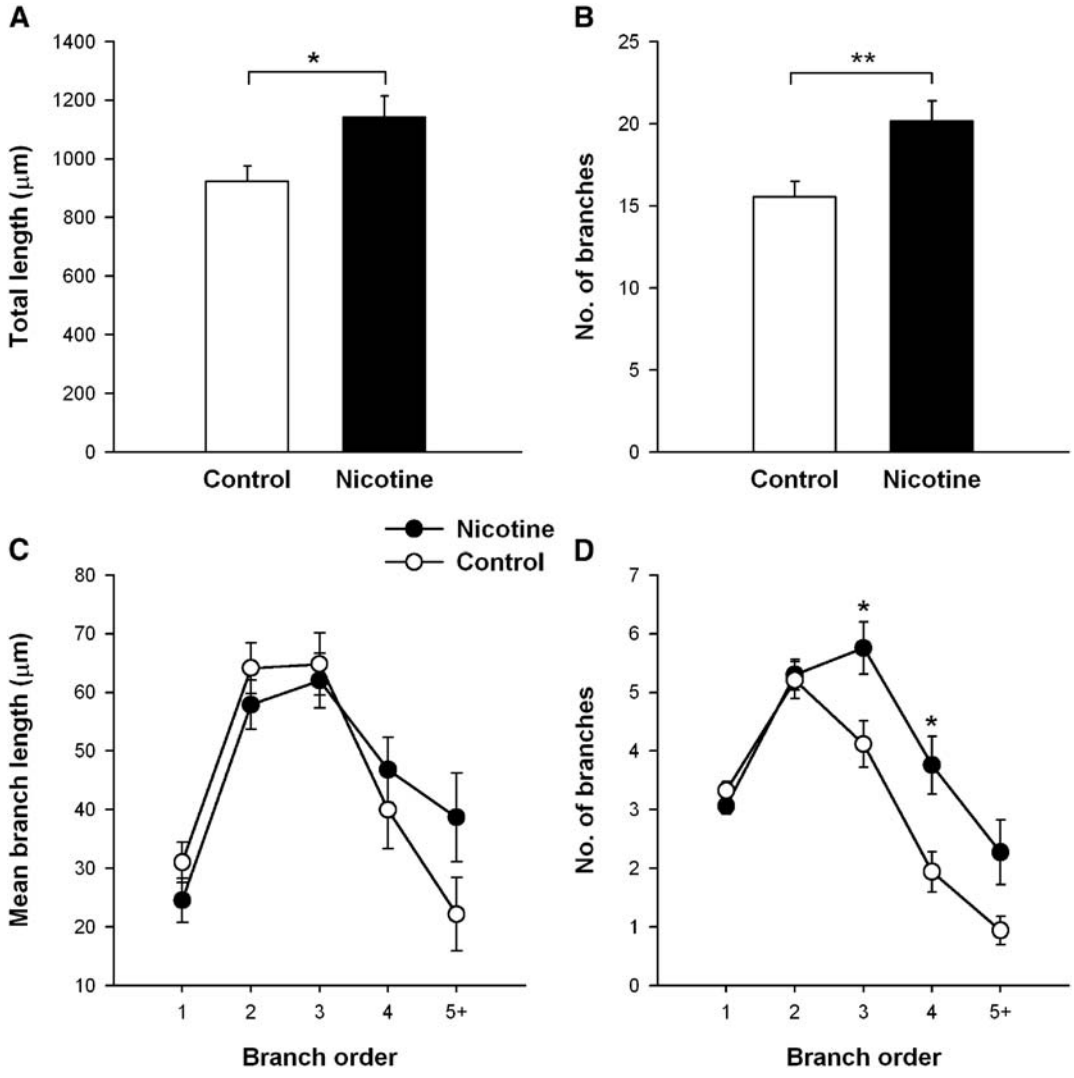

Fig. 3 – Morphometric measurements of MSNs from the adolescent-pretreated cohort. (A) Total dendritic length per neuron. (B) Total number of branches per neuron. (C) Mean branch lengths as a function of branch order. (D) Total number of branches as a function of branch order. $^{*}p<0.05$, $^{**}p<0.01$.

possessed fewer branches. Though the animals from each cohort were adults at the time of sacrifice, they did differ in age by 51 days, suggesting that there was continued synaptic reorganization within NAc shell over this time frame (p78–p129). Evidence of continued dendritic alteration throughout adulthood is certainly not without precedent (Cupp and Uemura, 1980; Page et al., 2002).

Age-dependent differences in nicotine's effect on MSN dendritic structure were accompanied by differing behavioral responses to nicotine challenge. We found that the response to challenge differed qualitatively, with adolescent-pretreated animals showing greater activity relative to controls and adult-pretreated animals showing reduced activity relative to controls. It is conceivable that different neural substrates, such as medial prefrontal cortex or NAc core, are affected when nicotine is continuously administered in adulthood as opposed to adolescence. Unfortunately, in the present study, NAc core did not stain sufficiently well to permit investigation of possible structure–function relationships with respect to this region. An alternative possibility is that the structural changes reported here are not necessarily functionally related to altered psychomotor response to drug challenge (Robinson and Kolb, 2004).

Increases in dendritic length/branching can be expected to substantially increase connectivity with presynaptic inputs from areas such as cingulate cortex and ventral tegmental area, as well as with other cell classes within NAc. Theoretical studies suggest that these morphological alterations have the potential to alter not only the weight of connections between previously connected neurons, but also allow for new connections between previously unconnected neurons (Chklovskii et al., 2004). The prediction that the extent of dendritic arbor should be associated with increased synaptic connectivity is consistent with empirical evidence that MSNs with greater dendritic length are more sensitive to dopamine-dependent modulation of glutamatergic signaling (Hernandez-Echeagaray et al., 2004). Although the current approach did not provide direct evidence of altered connectivity between neurons, the structural changes observed here are certainly in accord with a maladaptive rewiring of the mesolimbic dopamine pathway.

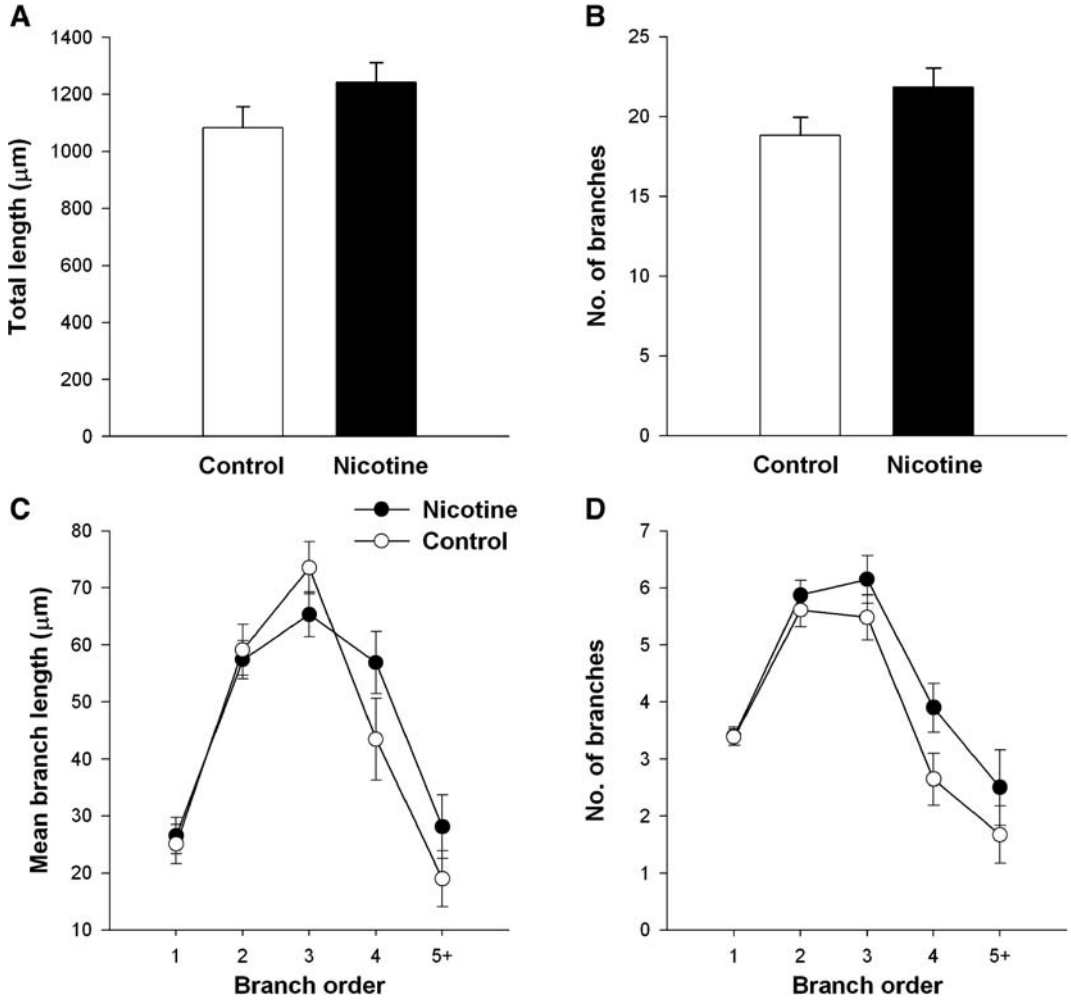

Fig. 4 – Morphometric measurements of MSNs from the adult-pretreated cohort. (A) Total dendritic length per neuron. (B) Total number of branches per neuron. (C) Mean branch lengths as a function of branch order. (D) Total number of branches as a function of branch order.

Our finding that MSNs showed greater structural plasticity than LAs suggests that these cell classes might exhibit differential sensitivity to presynaptic dopamine release. This contention is supported by the finding that dopaminergic afferents preferentially make contact with MSNs rather than LAs (Kawaguchi et al., 1995). There is also evidence that these cell classes differ with respect to their response to glutamate. Specifically, LAs have been shown to be significantly less prone to amino acid excitotoxicity (Di Figlia, 1990), suggesting relatively modest sensitivity to glutamatergic inputs. The anatomical data presented here are consistent with the notion that LAs are affected to a lesser extent by nicotine's modulatory influence on dopaminergic and/or glutamatergic synaptic transmission. However, as a final caveat, we note that the LAs described here may represent a functionally heterogeneous group of neurons.

In conclusion, the present study has shown that continuous nicotine administration during adolescence produces a selective alteration of accumbal neural circuitry, and that structural plasticity of MSNs is more pronounced when exposure occurs during this critical developmental period as compared to adulthood. Our findings are in accord with recent molecular evidence (Schochet et al., 2005) that the mesolimbic dopamine system of adolescents exhibits elevated nicotine-induced neuroplasticity.

4. Experimental procedure

Animal subjects were male Long–Evans hooded rats (Harlan, Indianapolis, IN). Animals were group housed with *ad libitum* access to food and water. A 12-h light/dark cycle (lights on at 7:00AM) was maintained throughout the study.

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 was continuously administered either during adolescence (post-natal day (p) 29 to p43 or adulthood (p80 to p94) via subcutaneously implanted Alzet osmotic pumps (Model 1002; Durect Corp., Cupertino, CA). There is no method for drug administration which perfectly mimics human consumption. Daily subcutaneous injections produce transient increases in nicotine blood serum which do not reflect the pattern seen with humans. Consequently, we chose to employ mini-pumps in the current study as human smokers tend to maintain

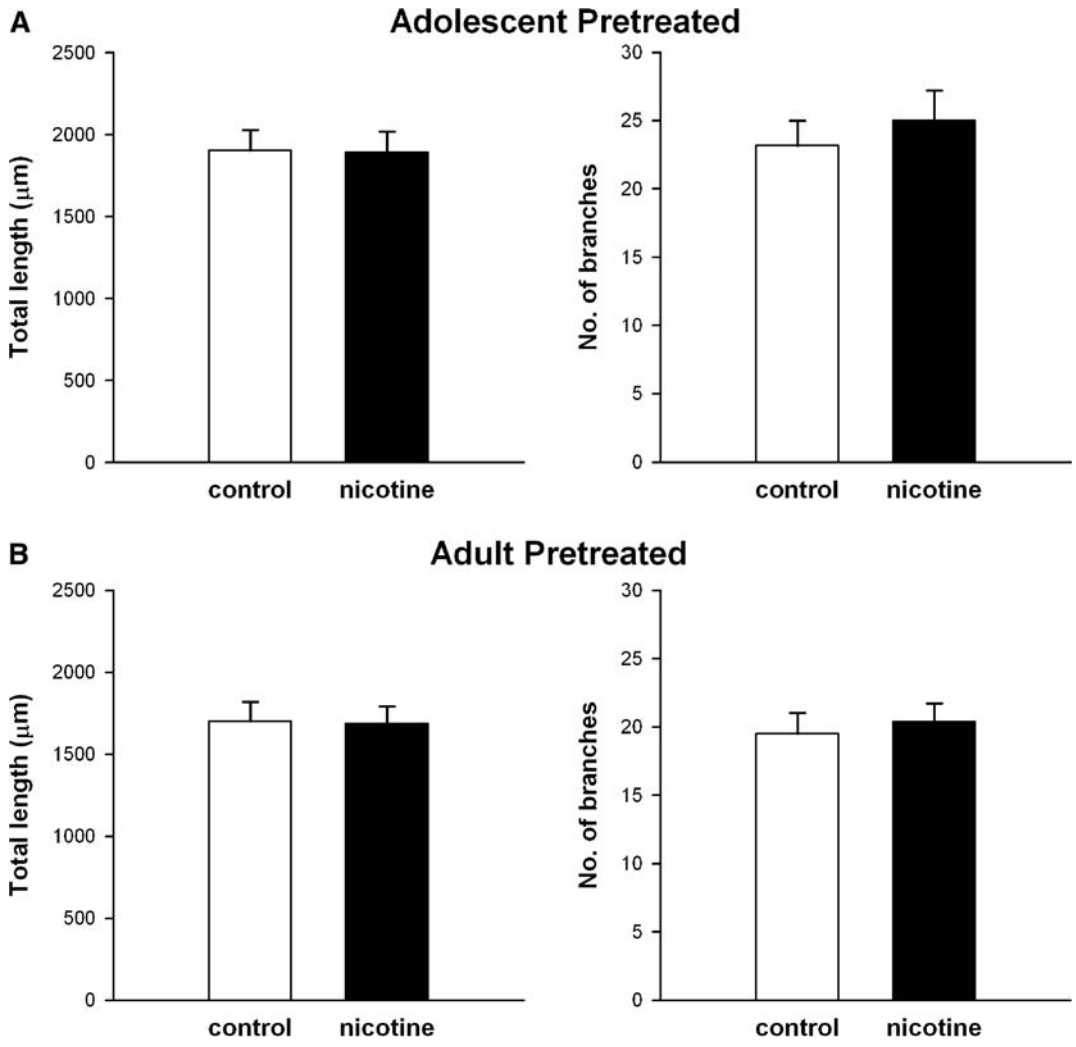

Fig. 5 – Total dendritic length and branch number for LAs from the adolescent (A)- and adult-pretreated cohorts (B).

elevated blood serum levels throughout the day. Moreover, previous work has provided data regarding blood serum levels produced by mini-pump infusion (Slotkin, 2002). The caveat with this approach is that nicotine is continuously infused throughout the dosing period.

Our adolescent dosing period was comparable to Spear's (2000) conservative estimate, based on physiological and behavioral criteria, of the adolescent period 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. The initial dose rate (2 mg/kg/day; free base) was chosen to provide plasma nicotine levels comparable to those seen in human smokers (Slotkin, 2002). The final dose rates for adolescent- and adult-pretreated animals were 0.95 and 2.0 mg/kg/day, respectively.

After a 30-day abstinent period, both nicotine and control animals were given a 1 mL/kg IP injection of 0.9% NaCl and then immediately placed in the open field chamber (42 × 42 × 30 cm). Distance traveled was recorded over a 10-min period using the Videotrack tracking system (Viewpoint Life Sciences Inc., Montreal, Que). Average values were determined in five minute blocks. Approximately 24 h later, all animals were given a 0.18 mg/kg (free base) IP injection of nicotine bitartrate and directly placed into the open field chamber for evaluation of locomotor activity. Because all animals were challenged with nicotine, locomotor response to the challenge was evaluated relative to baseline activity. Accordingly, the response to the challenge is expressed as a difference score, where distance traveled on day 1 (saline injection) was subtracted from distance traveled on day 2 (nicotine challenge). In piloting our protocol, we found that there is no significant habituation to the open field chamber across 2 days of saline injections. A significant reduction in activity following nicotine challenge can therefore be attributed to the drug's locomotor depressant effect. Baseline locomotor activity and difference scores were evaluated separately using two-way ANOVA with age and dose as factors. Follow-up analyses were made using independent samples *t*-tests. Experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

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).

Medium spiny neurons from the NAc shell were selected and traced by an experimenter blind to drug treatment. Cells with a soma diameter of 10–20 μm and densely spined dendrites were considered to be MSNs. A second morphological type, the large aspiny neuron (LA), was also reconstructed to provide an indication of whether structural plasticity was cell-specific. These cells were comparable in morphology to striatal tonically active cholinergic interneurons (Tepper and Bolam, 2004). In the current study, they were most frequently observed at the ventral margin of NAc shell bordering ventral pallidum. Cells which possessed a soma diameter greater than 20 μm and aspiny dendrites were classified as LAs. In a few cases, LAs possessed a small number of dendritic segments that were sparsely spined. Neurons were digitized in *x*, *y* and *z* coordinates under a 60× objective using Neurolucida (Microbrightfield, Colchester, VT). We chose to focus on analysis of neurons from the NAc shell because staining was comparatively poor in the core. Previous work has shown that the shell is highly plastic in response to psychostimulant administration (Robinson and Kolb, 2004). Morphometric parameters examined were: total dendrite length, total branch number, mean dendrite length as a function of branch order and number of branches as a function of branch order. Quantitation was carried out using Neuroexplorer (Microbrightfield, Colchester, VT). Branch order was assigned using the centrifugal method. Neurons were selected for tracing only if they were well impregnated, with unobstructed dendrites that could be followed without interruption. Based on these criteria, 1–5 neurons were reconstructed per animal. A total of 67 medium spiny neurons (control $n=34$; nicotine $n=33$) and 53 large aspiny neurons (control $n=26$; nicotine $n=27$) were reconstructed from the adolescent cohort. For the adult cohort, 71 medium spiny neurons (control $n=31$; nicotine $n=40$) and 49 large aspiny neurons (control $n=23$; nicotine $n=26$) were reconstructed. Each neuron was treated as an independent measure. Statistical comparisons of morphometric parameters were made using two-way ANOVA with dose and age as factors. Follow-up analyses were carried out using independent sample *t*-tests. When multiple comparisons were made the appropriate Bonferroni correction was applied.

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