

***Chrna5* genotype determines the long-lasting effects of developmental *in vivo* nicotine exposure on prefrontal attention circuitry**

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Abstract

Maternal smoking during pregnancy repeatedly exposes the developing fetus to nicotine and is linked with attention deficits in offspring. Corticothalamic neurons within layer VI of the medial prefrontal cortex are potential targets in the disruption of attention circuitry by nicotine, a process termed teratogenesis. These prefrontal layer VI neurons would be likely targets because they are developmentally excited and morphologically sculpted by a population of nicotinic acetylcholine receptors (nAChRs) that are sensitive to activation and/or desensitization by nicotine. The maturational effects of these $\alpha 4\beta 2^*$ nAChRs and their susceptibility to desensitization are both profoundly altered by the incorporation of an $\alpha 5$ subunit, encoded by the *chrna5* gene. Here, we investigate nicotine teratogenesis in layer VI neurons of wildtype and $\alpha 5^{-/-}$ mice. *In vivo* chronic nicotine exposure during development significantly modified apical dendrite morphology and nAChR currents, compared with vehicle control. The direction of the changes was dependent on *chrna5* genotype. Surprisingly, neurons from wildtype mice treated with *in vivo* nicotine resembled those from $\alpha 5^{-/-}$ mice treated with vehicle, maintaining into adulthood a morphological phenotype characteristic of immature mice together with reduced nAChR currents. In $\alpha 5^{-/-}$ mice, however, developmental *in vivo* nicotine tended to normalize both adult morphology and nAChR currents. These findings suggest that *chrna5* genotype can determine the effect of developmental *in vivo* nicotine on the prefrontal cortex. In wildtype mice, the lasting alterations to the morphology and nAChR activation of prefrontal layer VI neurons are teratogenic changes consistent with the attention deficits observed following developmental nicotine exposure.

Keywords

Medial prefrontal cortex; Nicotinic acetylcholine receptors; *In vivo* developmental nicotine; Corticothalamic; Electrophysiology

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1. Introduction

It is estimated that between 10 and 14 percent of women in North America smoke cigarettes regularly during pregnancy (Osadchy et al., 2009; Tong et al., 2009). Exposure of the developing fetus to cigarette smoke is linked statistically with persistent neurobehavioral consequences including attention deficits that can manifest as Attention Deficit Hyperactivity Disorder (ADHD) (Pauly and Slotkin, 2008; Thakur et al., 2013). Recent experimentation in rodent models suggests that nicotine, a major psychoactive component of cigarette smoke, likely contributes directly to the pathogenesis of these attention deficits (Counotte et al., 2011; Schneider et al., 2011). However, the neurobiological mechanisms by which chronic developmental nicotine exposure disrupts developing attention circuits are not understood, significantly limiting the potential to develop appropriate therapeutic intervention for affected children.

Nicotinic acetylcholine receptors (nAChRs) located on corticothalamic pyramidal neurons within layer VI of the medial prefrontal cortex (mPFC) are uniquely positioned to mediate developmental effects of *in vivo* nicotine on prefrontal attention circuitry. Stimulation of $\alpha 4\beta 2^*$ nAChRs on these neurons by the neurotransmitter acetylcholine is critical for optimal attention performance in mature animals (Bailey et al., 2010; Guillem et al., 2011). Signaling at these nAChRs is tightly regulated during development (Bailey et al., 2012; Kassam et al., 2008; Winzer-Serhan and Leslie, 2005), and normal activation of these receptors appears to be required for the morphological maturation of mPFC layer VI neuron apical dendrites from an immature morphological pattern characterized by long apical dendrites that predominantly travel across the width of the mPFC to terminate within cortical layer I, to a mature morphological pattern wherein approximately only half of the apical dendrites terminate within cortical layer I (Bailey et al., 2012). We hypothesize that the larger currents and greater calcium influx of $\alpha 5$ subunit-containing $\alpha 4\beta 2^*$ nAChRs on mPFC layer VI neurons (Bailey et al., 2010; Tapia et al., 2007) contribute to the cellular mechanisms underlying apical dendritic retraction for a subset of this neuronal population during postnatal maturation (Bailey et al., 2012; Pugh and Berg, 1994). Chronic *in vivo* nicotine exposure may subvert this developmental role for nAChRs through its ability to desensitize these receptors (Cohen et al., 2005; Pidoplichko et al., 1997; Quick and Lester, 2002), potentially imparting long-term effects on prefrontal attention systems.

To test the hypothesis that developmental *in vivo* nicotine would change the apical dendrite maturation and nAChR currents of layer VI neurons, we chronically exposed mice to either *in vivo* nicotine or vehicle control during prenatal and early postnatal development, and examined layer VI neurons in young mice near the end of the treatment period and in adulthood. In these neurons, the nAChR $\alpha 5$ subunit (encoded in mouse by the gene *chrna5*) appears to normally associate with $\alpha 4\beta 2^*$ nAChRs, significantly influencing receptor function (Bailey et al., 2010; Kuryatov et al., 2008) and making these receptors resistant to upregulation by *in vivo* nicotine (Gahring and Rogers, 2010; Mao et al., 2008). In order to determine the contribution of the $\alpha 5$ subunit towards nicotine teratogenesis in this neuronal population, we performed these experiments in wildtype mice and also in mice genetically deleted for *chrna5* ($\alpha 5^{-/-}$). We also examined the electrophysiological responses of layer VI

neurons to stimulation of nAChRs as well as to desensitization by acute nicotine in brain slice.

In wildtype mice, developmental *in vivo* nicotine exposure allowed an immature pattern of long apical dendrites to persist into adulthood. In addition, it decreased acute nAChR responses compared with vehicle control both in young and adult mice. In short, developmental *in vivo* nicotine makes mPFC layer VI neurons of wildtype mice resemble the morphological and electrophysiological phenotypic patterns of those observed in $\alpha 5^{-/-}$ mice in previous studies (Bailey et al., 2010, 2012), as well as in the $\alpha 5^{-/-}$ mice treated with vehicle control in this current study. These findings suggest that developmental *in vivo* nicotine acts by desensitizing $\alpha 5$ subunit-containing nAChRs. By contrast, developmental *in vivo* nicotine exerts a different pattern of changes in layer VI neurons of $\alpha 5^{-/-}$ mice, in the opposite direction to its effects in wildtype mice. In summary, *in vivo* nicotine exposure during development has lasting consequences for the morphology and nAChR activation of medial prefrontal layer VI neurons, and the direction of these effects is determined by *chrna5* genotype.

2. Materials and methods

2.1. Experimental animals and developmental *in vivo* nicotine treatment

Genetically modified mice on a C57BL/6 background had all nAChR $\alpha 4$ subunits labeled with YFP (Nashmi et al., 2007) and were either homozygous wildtype for the nAChR $\alpha 5$ subunit or homozygous null for the nAChR $\alpha 5$ subunit ($\alpha 5^{-/-}$) (Salas et al., 2003). Mice were bred in separate homozygous lines that were less than five generations removed from shared parents that were heterozygous null for the nAChR $\alpha 5$ subunit ($\alpha 5^{+/-}$). The $\alpha 4$ -YFP staining was used to verify neuronal localization within layer VI, as previously described (Bailey et al., 2012). Timed-pregnant nulliparous females of each genotype were randomly assigned to receive *ad libitum* access to food and water containing either 200 $\mu\text{g}/\text{mL}$ nicotine hydrogen tartrate (calculated as free base) and 2% (wt/vol) saccharin sodium salt hydrate, pH 7.0, or equimolar tartaric acid and 2% (wt/vol) saccharin sodium salt hydrate, pH 7.0, throughout gestation and postnatal development until offspring weaning on postnatal day (P) 21. Solutions were prepared fresh twice per week. The concentration of nicotine was selected for *in vivo* treatment based on previous studies examining nicotine teratogenesis in C57BL/6 mice (Heath et al., 2010; Pauly et al., 2004) and because it has been shown to lead to levels of the metabolite cotinine in pregnant mice (Pauly et al., 2004; Sparks and Pauly, 1999) similar to those observed in pregnant women who are heavy smokers (Klebanoff et al., 1998; Kvalvik et al., 2012; Mercelina-Roumans et al., 1996). Mice were separated according to sex at weaning and housed in groups of two to four per cage at an ambient temperature of 22 °C with a 12-h light/dark cycle with lights on at 7:00 A.M. Neurons located within layer VI of the mPFC from male offspring were examined for nAChR currents and morphology while the mice were young (P14-20) and in adulthood (P115-150), as described below. Refer to Fig. 1 for a schematic illustrating the timeline of developmental drug treatment and the ages at which offspring were examined. Five offspring from each experimental group were examined for young mice and four offspring were examined from each experimental group in adulthood. Male mice were randomly sampled from at least

three litters per experimental group and age, with the exception of adult male wildtype vehicle control mice, which were sampled from two litters. All data for adult male wildtype vehicle control mice in this study were not statistically different from data generated previously in our laboratory for adult wildtype mice (Bailey et al., 2012). All efforts were made to minimize animal suffering and to limit the number of mice used. Experimental animals were cared for according to the principles and guidelines of the Canadian Council on Animal Care and the experimental protocol was approved by the University of Toronto Animal Care Committee.

2.2. Electrophysiology and neuron morphology

Detailed methods for electrophysiology and neuron morphological analysis have been described previously (Bailey et al., 2012). Briefly, 400 μm thick coronal slices of the mPFC were prepared from offspring at postnatal week 3 (P14-20; hereafter termed “young” mice) and in adulthood (P115-150; hereafter termed “adult” mice) using the appearance of white matter and the corpus callosum as anterior and posterior guides (Gabbott et al., 2005; Paxinos and Franklin, 2001). Slices were cut in 4°C oxygenated sucrose artificial cerebrospinal fluid (ACSF) (254 mM sucrose, 10 mM D-glucose, 24 mM NaHCO_3 , 2 mM CaCl_2 , 2 mM MgSO_4 , 3 mM KCl, 1.25 mM NaH_2PO_4 , pH 7.4) and then recovered for at least two hours in 30°C oxygenated ACSF (composition listed above except 128 mM NaCl was substituted for sucrose). Slices were placed with their posterior sides facing up and analyzed in a modified chamber (Warner Instruments, Hamden, CT, USA) mounted on the stage of an Olympus BX50WI microscope (Olympus, Richmond Hill, ON, Canada), with room temperature oxygenated ACSF flowing over them at a rate of 3–4 mL/min.

Analyzed mPFC neurons were located medial to the white matter of each brain slice, within layer VI of the prelimbic, infralimbic and anterior cingulate cortical regions (Paxinos and Franklin, 2001). The neuron sampling location did not influence any measure in this study. Whole-cell recording of these mPFC layer VI neurons was performed using electrodes containing 120 mM K-gluconate, 5 mM KCl, 2 mM MgCl_2 , 4 mM $\text{K}_2\text{-ATP}$, 400 μM $\text{Na}_2\text{-GTP}$, 10 mM $\text{Na}_2\text{-phosphocreatine}$, 10 mM HEPES buffer and 0.3% (wt/vol) Neurobiotin Tracer (Vector Laboratories, Burlington, ON, Canada) (adjusted to pH 7.3 with KOH). Recordings were made using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA), acquired at 20 kHz and lowpass filtered at 2 kHz using a Digidata 1440A data acquisition system (Molecular Devices), and corrected for the liquid junction potential. Voltage clamp experiments were performed at -75 mV in the presence of 200 nM atropine (to block muscarinic acetylcholine receptors). Each experimental group contained at least 16 neurons in brain slices from at least 4 mice, as illustrated in Table 1.

Upon the completion of electrophysiological recording, slices were fixed overnight in a solution containing 4% (wt/vol) paraformaldehyde in 0.1 M phosphate buffer (pH 7.5) and then probed for $\alpha 4\text{-YFP}^*$ nAChRs according to a previously-reported immunohistochemistry protocol (Bailey et al., 2012). Inclusion of streptavidin-conjugated Alexa Fluor 594 (Life Technologies Inc., Burlington, ON, Canada) in the last step of this protocol allowed for the indirect labeling of both Neurobiotin-filled neurons and $\alpha 4\text{-YFP}^*$ nAChRs using this single fluorophore. Multiphoton imaging of labeled neurons and $\alpha 4\text{-$

YFP* nAChRs was performed using a Ti:sapphire laser (Mai Tai, Spectra Physics, Santa Clara, CA, USA) tuned to wavelength 780 nm and an Olympus Fluoview FV1000 microscope with an Olympus XLPlan N 25x, 1.05 NA water-immersion objective. Neurons were traced and analyzed using NeuroLucida software (MicroBrightField, Williston, VT, USA). Pyramidal neurons included in subsequent morphology analyses had distinguishable apical dendrites and were fully contained within the fixed brain slice. Labeled neurons having apical dendrites that did not definitively terminate within the brain slice (18 of 100 neurons for young mice and 7 of 69 neurons for adult mice) were not included in the morphology analyses.

2.3. Statistical analysis

All data are presented as mean \pm SEM. Contingency analysis of apical dendrite termination layer was performed using the Fisher's exact test. Morphology and electrophysiology data were analyzed by unpaired Student's *t*-test, one-way ANOVA or two-way ANOVA, as indicated. Data are reported as mean values for neurons within each experimental group. All significant effects on neuron morphology were also observed when neuron means per mouse were considered to be independent experimental units (see Supplemental Fig. 1). In addition, estimates of intra- and inter-litter variability did not differ from one another for either morphological or electrophysiological measures. Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA) and a level of $p < 0.05$ was required to indicate statistical significance.

3. Results

Our goal in pursuing this study was to assess the long-term consequence of developmental *in vivo* nicotine on the function and morphology of mPFC layer VI neurons and also to test whether changes arose during or subsequent to the *in vivo* nicotine exposure. Mice were treated throughout gestation and to weaning with either 200 $\mu\text{g}/\text{mL}$ nicotine tartrate or tartaric acid vehicle control in the drinking water, based on the concentration of nicotine required to produce similar concentrations of the metabolite cotinine in pregnant mice (Pauly et al., 2004; Sparks and Pauly, 1999) to that found in heavy smokers while pregnant (Klebanoff et al., 1998; Kvalvik et al., 2012; Mercelina-Roumans et al., 1996). Neurons were labeled for morphological reconstruction and measurements of electrophysiological properties such as nAChR currents were made in young mice or in adulthood. Finally, in order to understand better the effects of developmental *in vivo* nicotine on nAChRs, we took brain slices from young mice and applied acute nicotine to test desensitization of nAChR currents. Refer to Fig. 1 for a schematic illustration of the developmental drug treatment timeline and the ages at which male offspring were examined. Intrinsic electrophysiological properties for all neurons in this study are reported in Table 1 along with the numbers of mice and individual neurons analyzed.

3.1. Morphological changes exerted by developmental *in vivo* nicotine in wildtype mice

We first analyzed the long-term effects of developmental *in vivo* nicotine on mPFC layer VI neuron morphology. In adult wildtype mice that had been treated with vehicle control, half the layer VI neurons displayed a "long" morphology having apical dendrites that terminate

within cortical layer I, and half of the layer VI neurons displayed a “short” morphology having apical dendrites that terminate below cortical layer I. The mature pattern and proportion of long:short neurons observed here is similar to that seen in adult wildtype mice in an earlier study (Bailey et al., 2012). This initial, qualitative analysis found that developmental *in vivo* nicotine had a striking effect on the termination layer for layer VI neuron apical dendrites, shifting the proportion of “long” neurons from 50% (9 of 18 neurons) in vehicle control mice to 87% (13 of 15 neurons) in nicotine-treated mice (Fisher’s exact test, $p = 0.03$). Example tracings of layer VI neurons representative of these proportions are shown in Fig. 2A.

We then traced each neuron’s apical dendrite, and found that three-dimensional Sholl analysis confirms an effect of developmental *in vivo* nicotine to increase the complexity of distal apical dendrites (300 μm from the soma to the terminal) (Fig. 2B, two-way ANOVA, main effect of developmental *in vivo* nicotine, $F(1,620) = 14.01$, $p = 0.0002$). Examination of distal apical neuronal morphology in young mice during the last week of the developmental *in vivo* nicotine treatment showed that the increased complexity, or apparent lag in morphological maturity, in nicotine-treated mice was already present at this stage of postnatal development (data not shown, two-way ANOVA, main effect of developmental *in vivo* nicotine, $F(1,810) = 7.30$, $p = 0.007$). Interestingly, these increases in distal apical dendritic complexity were not accompanied by changes in overall apical dendrite tree length including all branches along the tree (for adult mice: vehicle control: $1885 \pm 134 \mu\text{m}$, $n = 18$, developmental *in vivo* nicotine: $1960 \pm 145 \mu\text{m}$, $n = 15$, Student’s *t*-test, $p = 0.7$; for young mice: vehicle control: $2206 \pm 83 \mu\text{m}$, $n = 16$, developmental *in vivo* nicotine: $2342 \pm 155 \mu\text{m}$, $n = 16$, $p = 0.4$).

3.2. Electrophysiological changes exerted by developmental *in vivo* nicotine in wildtype mice

Since nAChR activation has been implicated in limiting neuronal process length (Lipton et al., 1988; Pugh and Berg, 1994), we had tested whether developmental *in vivo* nicotine interferes with nAChR signaling in layer VI neurons by measuring whole-cell current responses elicited by 1 mM ACh (15 s application, in the presence of 200 nM atropine) in brain slices from both young and adult mice. Previous work has shown that such nAChR currents are strongly developmentally regulated (Alves et al., 2010; Bailey et al., 2012; Kassam et al., 2008). In wildtype mice, we found not only a normal effect of age (Fig. 2C, two-way ANOVA, main effect of age, $F(1,77) = 8.11$, $p = 0.006$), but also a significant effect of developmental *in vivo* nicotine exposure to decrease nAChR signaling (main effect of developmental *in vivo* nicotine, $F(1,77) = 4.43$, $p = 0.04$). Representative nAChR current recordings from each experimental group in wildtype mice are shown in Fig. 2D.

3.3. Direction of morphological changes exerted by developmental *in vivo* nicotine depend on $\alpha 5$ subunit genotype

Previous work found that layer VI neurons in adult $\alpha 5^{-/-}$ mice are disproportionately of the “long” variety (Bailey et al., 2012), an effect that we have replicated in the initial qualitative analysis of the vehicle control $\alpha 5^{-/-}$ mice in this study (71% “long”; 10/14 neurons). Developmental *in vivo* nicotine did not change the long/short ratio significantly (60%

“long”, 9/15 neurons; Fisher’s exact test, $p = 0.7$). Representative tracings of neurons from $\alpha 5^{-/-}$ mice are shown in Fig. 3A.

Quantitative, three-dimensional Sholl analysis in adult wild-type mice (from Fig. 2B) and adult $\alpha 5^{-/-}$ mice (from Fig. 3B), which were both treated with vehicle control, confirmed previous findings (Bailey et al., 2012) that *chma5* deletion increases layer VI neuron apical dendrite complexity (two-way ANOVA, main effect of genotype, $F(1,928) = 3.97$, $p = 0.047$) but was most apparent in the distal region of 300 μm to the terminal (main effect of genotype, $F(1,609) = 7.08$, $p = 0.008$). Of note, Sholl analysis within adult $\alpha 5^{-/-}$ mice revealed a significant, persisting effect of developmental *in vivo* nicotine to restore the $\alpha 5^{-/-}$ layer VI neurons toward the mature wildtype pattern of less complicated apical dendrites, by decreasing the complexity of their distal apical dendrites (300 μm from the soma to the terminal) compared with the vehicle control $\alpha 5^{-/-}$ mice (Fig. 3B, main effect of developmental *in vivo* nicotine, $F(1,546) = 6.73$, $p = 0.01$). This effect was only observed in adult mice and not during the last week of the developmental nicotine treatment (data not shown, two-way ANOVA, main effect of developmental *in vivo* nicotine, $F(1,713) = 0.04$, $p = 0.8$). Again, the change in distal apical dendrite complexity observed for adult $\alpha 5^{-/-}$ mice was not accompanied by a change in the overall length of the apical dendrite tree (for adult mice: vehicle control: $2065 \pm 119 \mu\text{m}$, $n = 13$, developmental *in vivo* nicotine: $1877 \pm 90 \mu\text{m}$, $n = 15$, Student’s *t*-test, $p = 0.2$; for young mice: vehicle control: $2240 \pm 129 \mu\text{m}$, $n = 15$, developmental *in vivo* nicotine: $2198 \pm 98 \mu\text{m}$, $n = 18$, $p = 0.8$). It is important to emphasize that developmental *in vivo* nicotine yielded the opposite changes to adult distal apical dendrite morphology in wildtype and $\alpha 5^{-/-}$ mice.

3.4. Electrophysiological changes exerted by developmental *in vivo* nicotine depend on $\alpha 5$ genotype

As with wildtype mice, we also observed a normal effect of age to decrease the magnitude of nAChR currents in $\alpha 5^{-/-}$ mice in this study (Fig. 3C, main effect of age, $F(1,72) = 12.91$, $p = 0.0006$). However, in this genotype group we found the opposite effect of treatment, that developmental *in vivo* nicotine significantly *increased* nAChR currents (Fig. 3C, main effect of developmental *in vivo* nicotine, $F(1,72) = 9.39$, $p = 0.003$). It is important to note that this effect of developmental *in vivo* nicotine in $\alpha 5^{-/-}$ mice was again in the opposite direction of that observed in wildtype mice. Moreover, these remarkably different effects of developmental *in vivo* nicotine across genotypes were observed both in young mice, still receiving *in vivo* nicotine treatment, and in adult mice, who had not received *in vivo* nicotine in months. Example nAChR current recordings from each experimental group in $\alpha 5^{-/-}$ mice are shown in Fig. 3D.

3.5. Genotype independent effects of developmental *in vivo* nicotine exposure

The genotype-specific effects of developmental *in vivo* nicotine observed in this study are limited to the apical dendrite morphology and to nAChR currents. We observed no long-term effects of developmental *in vivo* nicotine within either genotype on basal dendrite morphology (see Supplemental Fig. 2). Sholl analysis revealed no effect of developmental *in vivo* nicotine on basal dendrite complexity for adult mice of either wildtype (two-way ANOVA, main effect of developmental *in vivo* nicotine, $p = 0.1$) or $\alpha 5^{-/-}$ ($p = 0.7$)

genotypes. Moreover, there were no long-term effects of developmental *in vivo* nicotine exposure within either genotype on total length of basal dendrites, average length of basal dendrite trees, distance of the farthest basal dendrite terminal from the soma, or average distance of basal dendrite terminals from the soma (Student's *t*-test, all $p > 0.05$).

We did identify a genotype-independent effect of developmental *in vivo* nicotine to decrease average dendrite diameter along the length of all analyzed neurons from adult mice that was significant in apical dendrites (two-way ANOVA, main effect of developmental *in vivo* nicotine, $F(1,56) = 14.94$, $p = 0.0003$) and showed a strong trend towards significance in basal dendrites (two-way ANOVA, main effect of developmental *in vivo* nicotine, $F(1,61) = 3.64$, $p = 0.06$). Average apical dendrite diameters were: wildtype control: $1.25 \pm 0.02 \mu\text{m}$ ($n = 18$); wildtype developmental *in vivo* nicotine: $1.17 \pm 0.03 \mu\text{m}$ ($n = 15$); $\alpha 5^{-/-}$ control: $1.25 \pm 0.04 \mu\text{m}$ ($n = 12$); $\alpha 5^{-/-}$ developmental *in vivo* nicotine: $1.13 \pm 0.01 \mu\text{m}$ ($n = 15$). This effect of developmental *in vivo* nicotine exposure to decrease apical dendrite diameter was also found when we examined a single point on the proximal apical dendrite: $20 \mu\text{m}$ from the soma (two-way ANOVA, main effect of developmental *in vivo* nicotine, $F(1,56) = 12.17$, $p = 0.001$). These proximal apical dendrite diameters were: wild-type control: $1.94 \pm 0.10 \mu\text{m}$ ($n = 18$); wildtype developmental *in vivo* nicotine: $1.51 \pm 0.16 \mu\text{m}$ ($n = 15$); $\alpha 5^{-/-}$ control: $1.96 \pm 0.16 \mu\text{m}$ ($n = 12$); $\alpha 5^{-/-}$ developmental *in vivo* nicotine: $1.51 \pm 0.08 \mu\text{m}$ ($n = 15$). Developmental *in vivo* nicotine exposure did not influence the length of apical dendrite tufts for “long” neurons terminating within cortical layer I (two-way ANOVA, main effect of developmental *in vivo* nicotine, $F(1,28) = 0.34$, $p = 0.6$). Tuft lengths were: wildtype control: $104.3 \pm 21.9 \mu\text{m}$ ($n = 6$); wildtype developmental *in vivo* nicotine: $113.1 \pm 16.3 \mu\text{m}$ ($n = 12$); $\alpha 5^{-/-}$ control: $151.7 \pm 34.1 \mu\text{m}$ ($n = 8$); $\alpha 5^{-/-}$ developmental *in vivo* nicotine: $112.2 \pm 30.6 \mu\text{m}$ ($n = 6$). In addition, developmental *in vivo* nicotine did not influence the termination layer for apical dendrites of the “short” cells that terminated below cortical layer I, which on average extended to the mid-layer of cortex ($53 \pm 3\%$ of the total cortical width).

As shown in Table 1, we identified an effect of developmental *in vivo* nicotine to decrease action potential spike amplitude in adulthood significantly (two-way ANOVA, main effect of developmental *in vivo* nicotine, $F(1,64) = 7.63$, $p = 0.008$). However, we identified no additional effects of developmental *in vivo* nicotine on other intrinsic electrophysiological properties at either age, including resting membrane potential, input resistance, and spontaneous excitatory postsynaptic potential (sEPSC) frequency and amplitude.

3.6. Interactions between $\alpha 5$ subunit genotype and developmental *in vivo* nicotine exposure

Chrna5 genotype appears to alter the long-term consequences of developmental *in vivo* nicotine on mPFC layer VI neurons. We tested this hypothesis by comparing the morphological and electrophysiological effects of developmental *in vivo* nicotine across genotypes, as illustrated for adult mice in Fig. 4. To permit this comparison, distal apical dendrite complexity was measured by summing the total number of Sholl intersections per neuron within the region of $300 \mu\text{m}$ from the soma to the dendrite terminal. Two-way analysis in adult mice demonstrated significant interactions between the effects of *chrna5* genotype and developmental *in vivo* nicotine to alter apical dendrite complexity (Fig. 4A,

interaction of effects, $F(1,57) = 4.63$, $p = 0.04$), and nAChR currents (Fig. 4B, interaction of effects, $F(1,64) = 6.91$, $p = 0.01$). Specifically, we observed that the direction of effects following developmental *in vivo* nicotine is dependent on the presence of the nAChR $\alpha 5$ subunit. Similar analysis in young mice found a significant interaction between the effects of $\alpha 5$ subunit genotype and developmental *in vivo* nicotine on nAChR currents (data not shown, interaction of effects, $F(1,85) = 6.98$, $p = 0.01$). However, since apical dendrite complexity is altered by developmental *in vivo* nicotine only in wildtype mice at this age (as detailed above), there did not appear to be a morphological interaction in the young mice (data not shown, interaction of effects, $F(1,60) = 0.51$, $p = 0.5$).

3.7. Potential mechanism of *in vivo* nicotine teratogenesis: receptor desensitization

In wildtype mice, developmental *in vivo* nicotine appeared to have a stronger effect on apical dendrite morphology than it does on the nAChR currents. Specifically, we found that developmental *in vivo* nicotine led to an aberrant pattern of apical dendritic morphology that resembles that of $\alpha 5^{-/-}$ mice, yet the nAChR currents were not reduced to the level seen in $\alpha 5^{-/-}$ mice. One potential explanation for this discrepancy is that our acute brain slice recording conditions would permit substantial washout of residual *in vivo* nicotine. So, instead of seeing the young nAChR currents acutely desensitized in the presence of nicotine, we saw the young nAChR currents after some degree of recovery. We therefore sought to assess the ability of acute nicotine in brain slice to desensitize $\alpha 4\beta 2^*$ nAChRs in layer VI neurons of young wildtype and $\alpha 5^{-/-}$ mice. These desensitization experiments are most relevant to understanding nicotine's effects in the young mice because they are the ones receiving nicotine or vehicle control *in vivo*.

To perform this experiment, we measured the nAChR currents in response to 1 mM ACh (15 s application in the presence of 200 nM atropine, as above) before and after slices were exposed to 300 nM nicotine for ten minutes (plus a ten minute washout period to allow holding currents to return to baseline). This concentration of acute nicotine in brain slice is consistent with that observed in the blood of smokers (Henningfield et al., 1993; Rose et al., 2010). Example young nAChR current recordings before and after acute nicotine in brain slice for neurons from each experimental group are shown in Fig. 5A.

Percent desensitization of nAChR currents by acute nicotine was calculated for each neuron: $[\text{ACh current before acute nicotine} - \text{ACh current after acute nicotine}] / [\text{ACh current before acute nicotine}] \times 100$. Analysis of acute nicotine-induced receptor desensitization data in young mice found significant effects of genotype (Fig. 5B, two way ANOVA, main effect of genotype, $F(1,46) = 35.51$, $p < 0.0001$), where wildtype layer VI neurons appear less sensitive to desensitization by this paradigm than $\alpha 5^{-/-}$ neurons. This general pattern is similar to our previous findings in adult mice (Bailey et al., 2010) and to what we see in adulthood in this study (data not shown, main effect of genotype, $F(1,27) = 55.34$, $p < 0.0001$). Interestingly, developmental *in vivo* nicotine does not appear to alter the degree of acute nicotine desensitization in brain slice at either age (young: main effect of developmental *in vivo* nicotine, $F(1,46) = 0.05$, $p = 0.8$, adult: main effect of developmental *in vivo* nicotine, $F(1,27) = 1.57$, $p = 0.2$). In wildtype mice, however, the ability of acute nicotine exposure to desensitize receptors to nAChR currents was significantly greater in

young mice compared to adult mice (data not shown, main effect of age, $F(1,36) = 10.25$, $p = 0.003$). This greater sensitivity to desensitization in young wildtype mice underscores the vulnerability of the developing brain to nicotine.

We further explored the postulate that nAChR desensitization from developmental *in vivo* nicotine results in wildtype nAChR currents that resemble those from $\alpha 5^{-/-}$ mice. This desensitization would occur predominantly at night since mice show strong diurnal variation in water consumption, with the majority of drinking in the dark cycle and minimal consumption in the light cycle (Dole et al., 1983). As such, layer VI neurons in mice exposed to developmental nicotine would likely experience high nicotine and have acutely desensitized nAChR currents at night, and would likely experience low nicotine and have recovered nAChR currents during the day. Accordingly for the developmental nicotine groups only, we have calculated hypothetical “*in vivo* averages” for the nAChR currents in young mice, which combine the nAChR currents for each neuron before and after desensitization with acute nicotine in brain slice in an attempt to model the daily average nAChR current over 24 h. These averages were then compared with the normal, non-desensitized nAChR currents measured in the vehicle control groups at this age. Results of this analysis for young mice are shown in Fig. 5C and indeed suggest that the contribution of acute nicotine desensitization in developing wildtype mice causes their $\alpha 4\beta 2^*$ nAChRs to function in a manner similar to those of $\alpha 5^{-/-}$ mice *in vivo*. One-way ANOVA identified a significant difference amongst the experimental groups ($p = 0.0007$). Moreover, the Tukey’s post-hoc test for multiple comparisons found that nAChR currents from vehicle control wildtype mice were significantly different from each of the other three experimental groups (each comparison $p < 0.05$), whereas these other groups were not significantly different from each other (each comparison $p > 0.05$).

3.8. Summary of results

The changes in apical dendrite morphology and nAChR currents that result from developmental *in vivo* nicotine in wildtype and $\alpha 5^{-/-}$ mice are summarized in Table 2. This synopsis suggests that our model of “*in vivo* average” nAChR currents described above is consistent with the striking ability of developmental *in vivo* nicotine to make wildtype layer VI neurons resemble those of $\alpha 5^{-/-}$ mice morphologically. Since layer VI nAChR currents would only experience periods of strong desensitization until the end of *in vivo* nicotine treatment at P21, our findings raise the possibility that mechanisms underlying postnatal apical dendrite maturation can be manipulated prior to this age. By contrast, a different pattern of results arise from $\alpha 5^{-/-}$ mice. In these mice, developmental *in vivo* nicotine appears capable of inducing a substantial upregulation of nAChR currents which is unmasked by washout of acute nicotine. Like young wildtype mice exposed to vehicle control, young $\alpha 5^{-/-}$ mice have a predominantly immature apical dendritic phenotype. Despite strong desensitization of nAChRs in young $\alpha 5^{-/-}$ mice by acute nicotine, these mice would likely have periods of normalized, high nAChR currents during the day, which may be capable of triggering the apical dendrite maturation that results in their normalized pattern of apical dendrite morphology by adulthood.

4. Discussion

We have found that developmental *in vivo* nicotine in mice leads to persistent alterations in morphology and nAChR currents in mPFC layer VI neurons, a population of neurons that have been implicated in the control of attention behavior. While changes were observed in young mice near the completion of the *in vivo* nicotine treatment, differences remained prominent in fully mature mice that had not been exposed to nicotine for more than two months. The direction of the alterations in neuronal morphology and nAChR currents occurred in a *chrna5* genotype-dependent manner, highlighting an important role for $\alpha 5$ subunit-containing nAChRs in normal mPFC development and their potential as a target for the teratogenic effects of nicotine.

4.1. Consequences of developmental *in vivo* nicotine on nAChR currents

Further research is required to identify mechanisms underlying the observed changes in nAChR currents following developmental *in vivo* nicotine treatment, as nicotine exhibits a complex pharmacology at nAChRs including agonist activation and desensitization, and can also lead to changes in expression, trafficking and post-translational modification of receptor subunits (Changeux, 2010; Lester et al., 2009; Marks et al., 2011; Quick and Lester, 2002). Of note, the inclusion of the $\alpha 5$ subunit within the $\alpha 4\beta 2^*$ nAChR boosts its activation by nicotine and also protects to some degree against desensitization by this drug (Bailey et al., 2010). In the adult frontal cortex, chronic *in vivo* nicotine treatment leads to an upregulation of $\alpha 4\beta 2^*$ nAChRs containing $\alpha 4$ and $\beta 2$ subunits (Besson et al., 2007; Marks et al., 2011; Sparks and Pauly, 1999) but not those containing the $\alpha 5$ subunit (Mao et al., 2008). Similar results are seen in the developing brain, where chronic *in vivo* nicotine treatment increases expression of $\alpha 4$ and $\beta 2$ subunits without altering $\alpha 5$ subunit content (Counotte et al., 2012; Lv et al., 2008). Very little is known about the effects of the inclusion of an $\alpha 5$ subunit on the ability of *in vivo* nicotine to alter nAChR post-translational modification or its ability to pharmacologically chaperone nAChRs through the endoplasmic reticulum (Srinivasan et al., 2011). However, several of these mechanisms of nAChR modulation may contribute to the ability of developmental *in vivo* nicotine to create a lasting increase in nAChR currents in corticothalamic neurons of $\alpha 5^{-/-}$ mice expressing $\alpha 4$ and $\beta 2$ subunits.

4.2. Potential links between nAChR signaling and apical dendrite morphology

We find that developmental *in vivo* nicotine also exerts significant changes on the morphology of layer VI neurons in a manner strikingly dependent on *chrna5* genotype. In wildtype mice, developmental *in vivo* nicotine maintains an immature pattern of apical dendrite morphology into adulthood, and also results in decreased nAChR currents. Morphologically, it could be argued that mPFC layer VI neurons from wildtype mice exposed to developmental *in vivo* nicotine come to resemble neurons from vehicle control $\alpha 5^{-/-}$ mice in adulthood. Electrophysiologically, our recordings in the presence of acute nicotine suggest that the average nAChR currents in layer VI neurons of the developmentally-treated young wildtype mice are similar to vehicle control young $\alpha 5^{-/-}$ mice *in vivo*. Of relevance, the developmental period between the age of the young and adult mice is normally accompanied by significant cerebral cortical circuit refinement (Berardi et al., 2000; Hensch, 2004). The elevated nAChR signaling in layer VI neurons of normal,

young wildtype mice may be a necessary trigger for apical dendrite retraction. In particular, nAChR signaling can mediate retraction (Pugh and Berg, 1994) of neuronal processes in culture and, more specifically, is associated with a shortening of mPFC layer VI neuron apical dendrites during this time (Bailey et al., 2012). The electrophysiological results from this current study show that this normally-elevated nAChR signaling is decreased following developmental *in vivo* nicotine, suggesting a potential mechanism for our morphological findings in wildtype mice. It is also important to note that developmental *in vivo* nicotine exposure alters the morphology of apical but not basal dendrite trees, suggesting that nicotine interferes with signaling pathways that influence the maturation of apical dendrites only. Such pathways may underlie differential alterations in apical versus basal dendrite trees observed during normal postnatal development (Romand et al., 2011) and accelerated aging (Shimada et al., 2006) of rodent cerebral cortical pyramidal neurons.

In the $\alpha 5^{-/-}$ mice, the relationship between nAChR currents and apical dendrite morphology appears somewhat more complicated. Our initial results suggested the possibility that developmental *in vivo* nicotine restores normal morphological patterns through its seeming normalization of nAChR currents in these neurons during development. However, with exposure of the brain slice to acute nicotine, it appears that mPFC layer VI neurons $\alpha 5^{-/-}$ mice are even more sensitive to suppression of their nAChR currents. On average, it would seem that the nAChR currents in young $\alpha 5^{-/-}$ mice exposed to nicotine *in vivo* would remain similar to those in $\alpha 5^{-/-}$ mice treated with vehicle. Consistent with this interpretation, apical dendritic morphology of young $\alpha 5^{-/-}$ mice exposed to developmental *in vivo* nicotine did not differ significantly from young $\alpha 5^{-/-}$ vehicle control mice. However, after developmental nicotine exposure is complete, adult $\alpha 5^{-/-}$ mice show normalized apical dendritic morphology. This normalization suggests that having high nAChR currents at least part of the day during development may be sufficient to trigger dendritic retraction. Alternatively, it is possible that normalization of apical dendrite morphology in the adult $\alpha 5^{-/-}$ mice may reflect an entirely different set of neurobiological processes. For example, selective apical dendrite retraction of other populations of prefrontal cortical neurons has been demonstrated with the experience of stress (Brown et al., 2005; Cook and Wellman, 2004; Goldwater et al., 2009) or accelerated aging (Shimada et al., 2006). It is possible that wildtype and $\alpha 5^{-/-}$ offspring in this current study may have experienced distinct environments during development because they were generated from separate homozygous lines, however, these lines were less than five generations removed from shared heterozygous parents and there was no noticeable effect of genotype on maternal physiology or care for offspring.

4.3. Implications for prefrontal attention circuits

By altering the apical dendrite complexity of mPFC layer VI neurons, developmental *in vivo* nicotine has the potential to disrupt the circuitry that supports attention. Cortical neurons typically receive laminar-specific presynaptic local cortical and long-range inputs (Llano and Sherman, 2009). Furthermore, cortical layer VI neurons appear capable of detecting and responding to excitatory input received by their distal apical dendrites (Ledgergerber and Larkum, 2010; Zarrinpar and Callaway, 2006). Following chronic developmental *in vivo* nicotine in wildtype mice, the unusual preponderance of neurons with apical dendrites that

stretch across the width of the mPFC to the pial surface suggests changes in the overall profile of synaptic inputs to these neurons, likely significantly altering the function of prefrontal cortical circuits to which these neurons contribute.

Pyramidal neurons within layer VI of the mPFC are implicated in modulating attention behavior through their prominent projections both to more superficial mPFC layers and to the mediodorsal thalamus (Briggs and Usrey, 2008; Gabbott et al., 2005; Zikopoulos and Barbas, 2006). It should also be noted that some neurons in this population project to additional subcortical regions including the dorsal striatum and lateral hypothalamus (Gabbott et al., 2005). While nAChR signaling in mPFC layer VI neurons is important for normal attention performance in adult mice (Guillem et al., 2011), the implications of nAChR signaling during development for attentional performance are not well understood. Our results suggest that perturbation of developmental nAChR signaling in mPFC layer VI neurons will have repercussions both for the excitation and underlying morphological structure of a neuronal population that contributes to normal attention behavior. These findings suggest a potential neurobiological mechanism of the attention deficits observed in humans and in animal models following developmental exposure to nicotine *in vivo* (Counotte et al., 2011; Heath et al., 2010; Pauly and Slotkin, 2008; Schneider et al., 2011; Thakur et al., 2013).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

nAChR	nicotinic acetylcholine receptor
$\alpha 5^{-/-}$	genetic deletion for the nicotinic acetylcholine receptor $\alpha 5$ subunit (<i>chrna5</i> null)
<i>chrna5</i>	gene name for the nicotinic acetylcholine receptor $\alpha 5$ subunit
mPFC	medial prefrontal cortex

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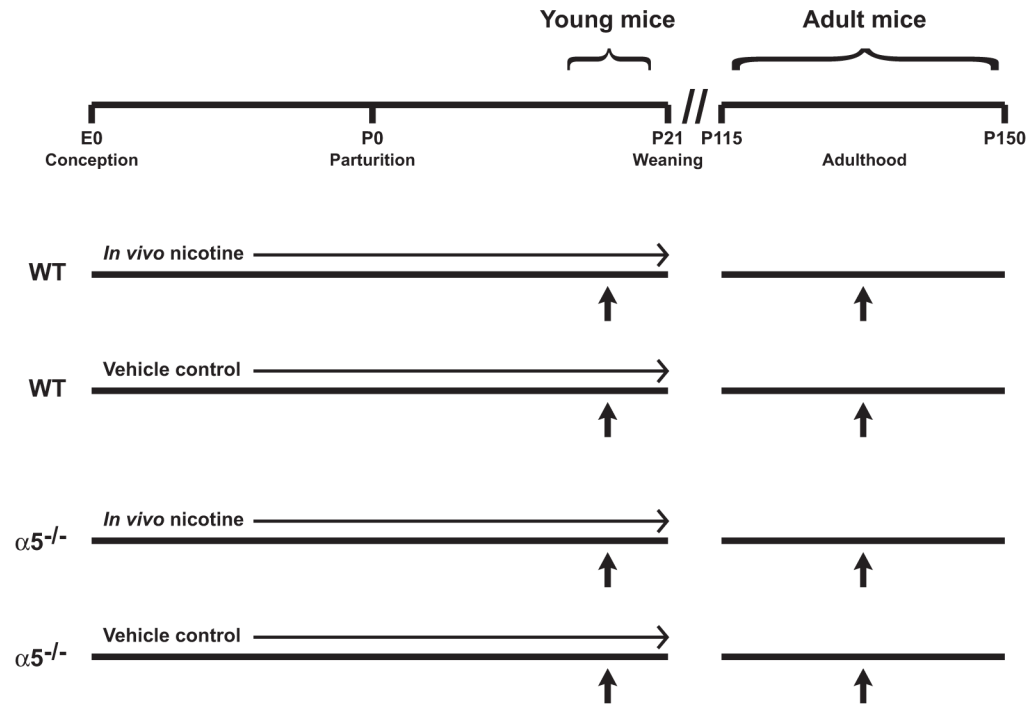
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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.neuropharm.2013.09.003>.

Developmental timeline and experimental groups:

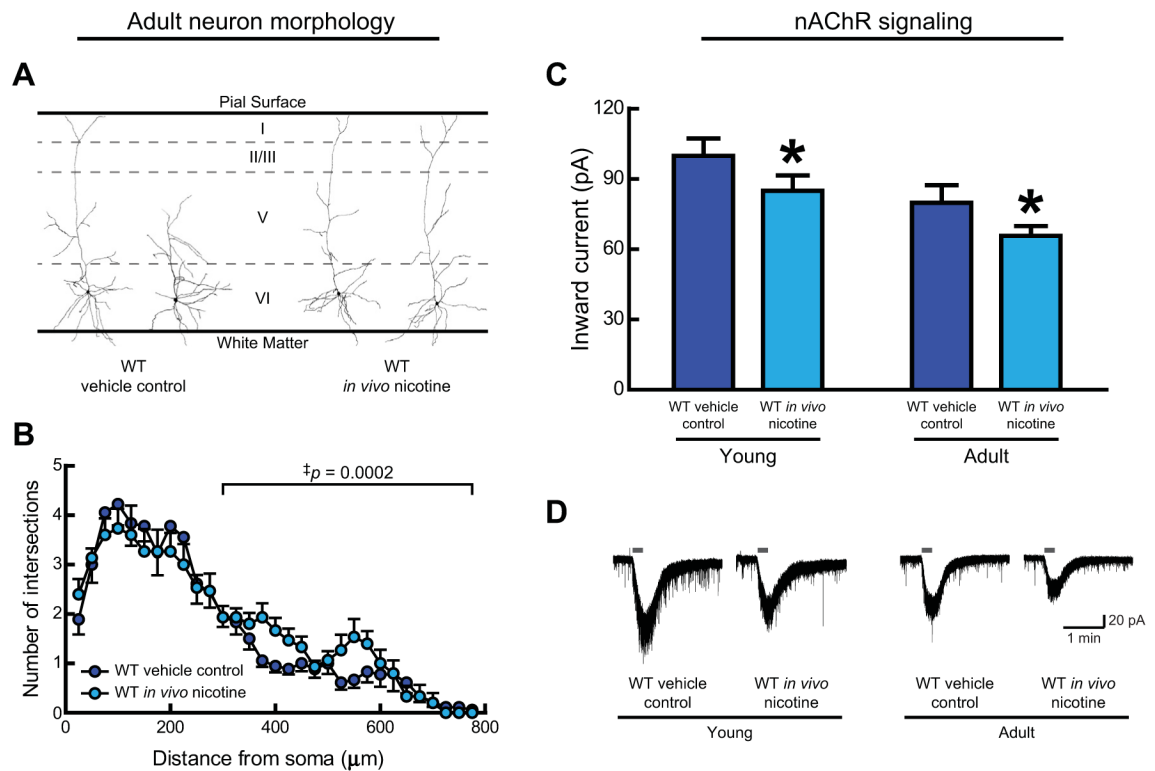


↑ Measurements taken for layer VI pyramidal neurons:

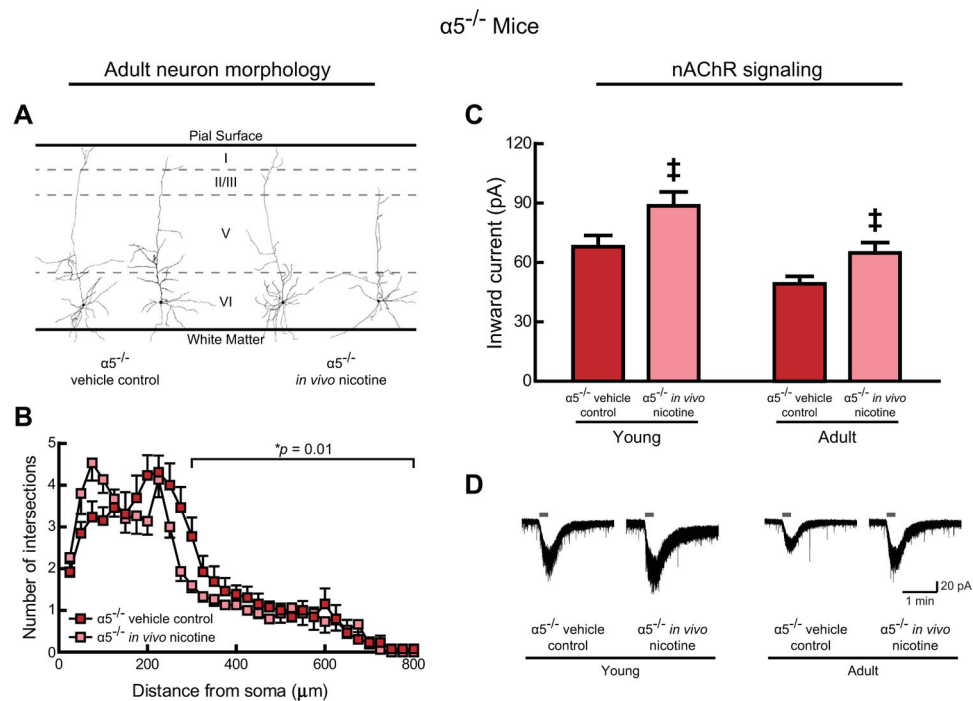
- dendrite morphology
- nAChR currents
- acute nAChR desensitization by nicotine

Fig. 1. Schematic illustration showing the timing of developmental chronic *in vivo* treatments in both genotypes, the ages at which young and adult male offspring were examined, and the measurements that were made from layer VI pyramidal neurons.

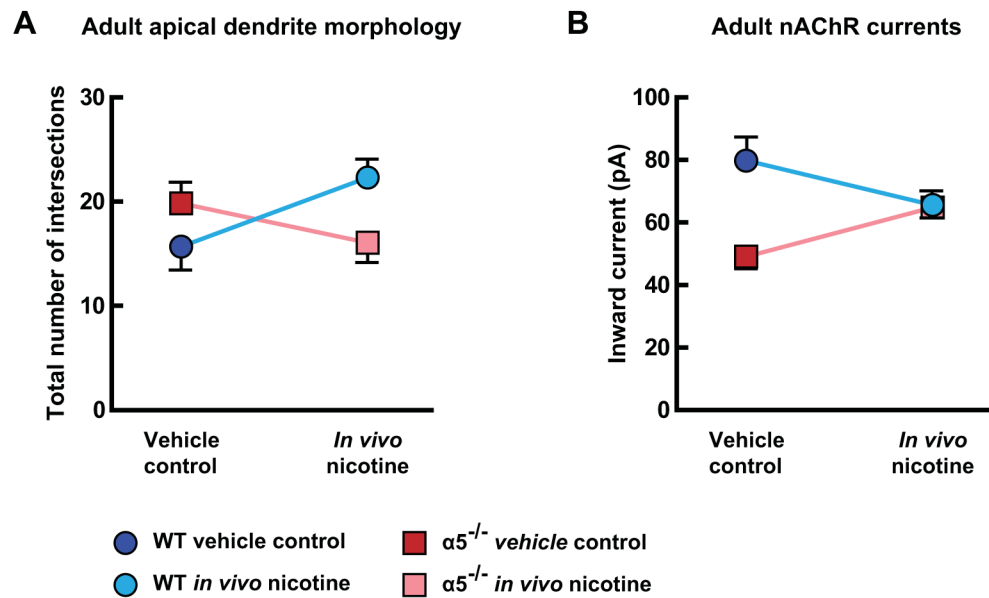
Wildtype Mice

**Fig. 2.**

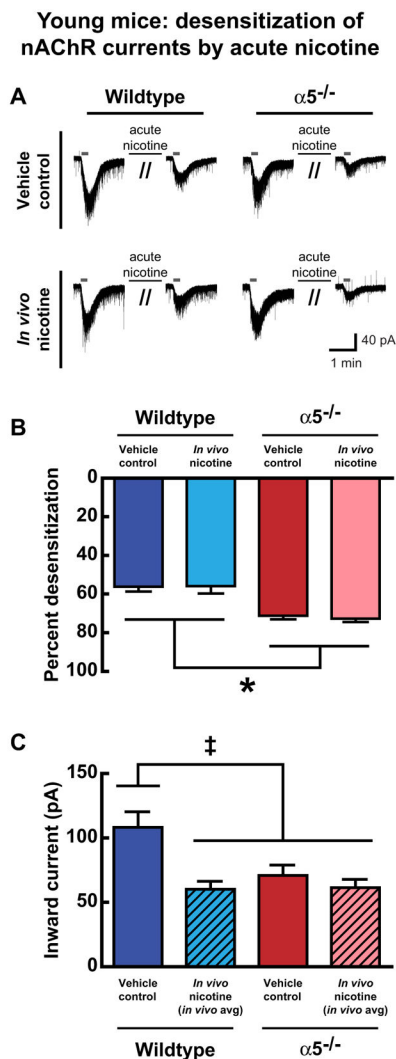
In wildtype mice, developmental *in vivo* nicotine alters adult morphology and nAChR signaling in medial prefrontal layer VI neurons. **A**, Representative tracings of adult neurons treated with either vehicle control or nicotine during development from conception to weaning on postnatal (P) day P21. **B**, Apical dendritic complexity is shown with three-dimensional Sholl analysis of the number of dendrite intersections at concentric spheres of varying distance from the soma for neurons from adult mice from both treatment groups. Developmental *in vivo* nicotine resulted in more complex distal apical dendrites in adulthood compared with vehicle control ($\ddagger p = 0.0002$). **C**, Whole-cell nAChR responses to 1 mM ACh (15 s, in the presence of 200 nM atropine) were significantly affected by age ($p = 0.006$) and were also significantly reduced by developmental *in vivo* nicotine ($*p = 0.04$). **D**, Example nAChR current recordings from young and adult mice of each experimental group.

**Fig. 3.**

In nicotinic acetylcholine receptor (nAChR) $\alpha 5$ subunit knockout mice ($\alpha 5^{-/-}$), developmental *in vivo* nicotine alters adult morphology and nAChR signaling in medial prefrontal layer VI neurons. **A**, Representative tracings of adult neurons treated with either vehicle control or nicotine during development from conception to weaning on postnatal (P) day P21. Note, layer VI neurons from vehicle control $\alpha 5^{-/-}$ mice retain long apical dendrites into adulthood (Bailey et al., 2012). **B**, Three-dimensional Sholl analysis shows the number of dendrite intersections at concentric spheres of varying distance from the soma for neurons from adult mice of both treatment groups. Developmental nicotine exposure resulted in less complex distal apical dendrites in adulthood compared with vehicle control (* $p = 0.01$). **C**, Whole-cell nAChR responses to 1 mM ACh (15 s, in the presence of 200 nM atropine) were significantly affected by age ($p = 0.0006$) and were also significantly increased by developmental *in vivo* nicotine ($\ddagger p = 0.003$). **D**, Example nAChR current recordings from each experimental group of $\alpha 5^{-/-}$ mice.

**Fig. 4.**

Interactions between nicotinic acetylcholine receptor (nAChR) $\alpha 5$ subunit genotype and developmental *in vivo* nicotine in medial prefrontal layer VI neurons of adult mice. *A*, The mean number of Sholl intersections for distal apical dendrites (300 μm from the soma to the terminal in 25 μm increments) was altered in adult mice by developmental *in vivo* nicotine in a manner that depends on the presence of the $\alpha 5$ nAChR subunit (effect of interaction, $p = 0.04$). *B*, Whole-cell nAChR responses to 1 mM ACh (15 s, in the presence of 200 nM atropine) were also altered in adult mice by developmental *in vivo* nicotine in a manner that depends on the $\alpha 5$ nAChR subunit (effect of interaction, $p = 0.01$). Data are presented as: wildtype vehicle control (dark blue), wildtype developmental *in vivo* nicotine (light blue), $\alpha 5^{-/-}$ vehicle control (dark red), and $\alpha 5^{-/-}$ developmental *in vivo* nicotine (light red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

**Fig. 5.**

In young mice, the influence of $\alpha 5$ subunit genotype and developmental *in vivo* nicotine on acute nicotine-induced nAChR desensitization in brain slice. *A*, Example current recordings of the response to ACh before and after acute nicotine application are shown for one neuron from each experimental group. *B*, Within individual neurons, nAChR desensitization was calculated by measuring nAChR current responses to 1 mM ACh (15 s, in the presence of atropine) before and after the brain slice was exposed to 300 nM nicotine (10 min). Percent desensitization by acute nicotine exposure was significantly affected by $\alpha 5$ subunit genotype ($*p < 0.0001$), but not by developmental nicotine exposure ($p = 0.8$). *C*, Theoretical *in vivo* average nAChR currents experienced in mPFC layer VI neurons of mice repeatedly exposed to nicotine during development were calculated by averaging nAChR currents measured before and after receptor desensitization by acute exposure to 300 nM nicotine, and compared with normal, non-desensitized nAChR currents in the vehicle control groups. This approach was based on the assumption that the strong diurnal variation in drinking water consumption in mice (Dole et al., 1983) would result in roughly equal periods of high and low nicotine exposure. The resulting nAChR current means were significantly different

among the experimental groups ($\dagger p = 0.0007$): the vehicle control wildtype group was significantly different from the other three groups (each comparison $p < 0.05$), and the other three groups were not significantly different from each other (each comparison $p > 0.05$).

Table 1

Neuron electrophysiological properties.

	Wildtype mice			$\alpha 5^{-/-}$ mice		
	Vehicle control	Developmental <i>in vivo</i> nicotine	24	Vehicle control	Developmental <i>in vivo</i> nicotine	25
Young						
Number of neurons	26		24	25		21
Number of mice	5		5	5		5
Membrane potential (mV)	-76.9 ± 0.7		-76.5 ± 0.5	-77.3 ± 0.7		-74.9 ± 1.1
Input resistance (M Ω)	164.5 ± 12.7		188.1 ± 16.2	198.6 ± 22.8		177.5 ± 14.9
Spike amplitude (mV)	100.8 ± 1.6		100.1 ± 1.6	99.1 ± 1.6		96.6 ± 2.3
sEPSC frequency (Hz)	1.0 ± 0.1		1.2 ± 0.1	1.0 ± 0.1		1.0 ± 0.1
sEPSC amplitude (pA)	15.4 ± 0.8		14.4 ± 0.6	13.9 ± 0.8		15.4 ± 1.3
Adult						
Number of neurons	19		17	16		16
Number of mice	4		4	4		4
Membrane potential (mV)	-76.8 ± 0.8		-78.2 ± 0.5	-78.2 ± 0.5		-78.1 ± 1.0
Input resistance (M Ω)	132.7 ± 11.2		143.3 ± 5.3	129.6 ± 8.4		139.6 ± 10.4
Spike amplitude (mV)	97.2 ± 1.3		94.2 ± 0.9 ^a	97.0 ± 1.4		92.8 ± 1.5 ^a
sEPSC frequency (Hz)	0.9 ± 0.2		0.8 ± 0.1	0.9 ± 0.2		0.9 ± 0.1
sEPSC amplitude (pA)	17.2 ± 0.4		16.7 ± 0.7	16.8 ± 0.7		17.5 ± 0.7

^a $p = 0.008$ for persistent effect of developmental *in vivo* nicotine treatment to decrease spike amplitude in adulthood (two-way ANOVA).

Table 2

Summary of apical dendrite phenotype and nAChR current amplitudes in young and adult wildtype and $\alpha 5^{-/-}$ mice treated developmentally with *in vivo* nicotine or vehicle control.

	Young mice	Adult mice
Wildtype (Vehicle control)	<ul style="list-style-type: none"> Immature pattern of apical dendrite morphology High nAChR currents (108 ± 12 pA) 	<ul style="list-style-type: none"> Mature pattern of apical dendrite morphology Medium nAChR currents (80 ± 8 pA)
Wildtype (<i>In vivo</i> nicotine)	<ul style="list-style-type: none"> Exaggerated immature pattern of apical dendrite morphology Low nAChR currents as “<i>in vivo</i> average” (60 ± 6 pA). Extremely low nAChR current after acute nicotine (36 ± 4 pA). Medium nAChR current on nicotine washout (84 ± 9 pA). 	<ul style="list-style-type: none"> Immature pattern of apical dendrite morphology Low nAChR currents (66 ± 4 pA)
$\alpha 5^{-/-}$ (Vehicle control)	<ul style="list-style-type: none"> Immature pattern of apical dendrite morphology Low nAChR currents (71 ± 8 pA) 	<ul style="list-style-type: none"> Immature pattern of apical dendrite morphology Very low nAChR currents (50 ± 4 pA)
$\alpha 5^{-/-}$ (<i>In vivo</i> nicotine)	<ul style="list-style-type: none"> Immature pattern of apical dendrite morphology Low nAChR currents as “<i>in vivo</i> average” (61 ± 6 pA). Extremely low nAChR current after acute nicotine (26 ± 3 pA). High nAChR current on nicotine washout (97 ± 10 pA). 	<ul style="list-style-type: none"> Mature pattern of apical dendrite morphology Low nAChR currents (65 ± 5 pA)

Bold, italic text is used to indicate specific nAChR current amplitude hypothesized to influence adult apical dendrite morphology. High nAChR currents in young mice may trigger the apical dendrite retraction necessary for the mature pattern of adult apical dendrite morphology.