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Nicotine alters lung branching morphogenesis through the α_7 nicotinic acetylcholine receptor

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Abstract

There is abundant epidemiological data linking prenatal environmental tobacco smoke with childhood asthma and wheezing, but the underlying molecular and physiological mechanisms that occur in utero to explain this link remain unelucidated. Several studies suggest that nicotine, which traverses the placenta, is a causative agent. Therefore, we studied the effects of nicotine on lung branching morphogenesis using embryonic murine lung explants. We found that the expression of α_7 nicotinic acetylcholine receptors, which mediate many of the biological effects of nicotine, is highest in pseudoglandular stage lungs compared with lungs at later stages. We then studied the effects of nicotine in the explant model and found that nicotine stimulated lung branching in a dose-dependent fashion. α -Bungarotoxin, an antagonist of α_7 nicotinic acetylcholine receptors, blocked the stimulatory effect of nicotine, whereas GTS-21, a specific agonist, stimulated branching, thereby mimicking the effects of nicotine. Explants deficient in α_7 nicotinic acetylcholine receptors did not respond to nicotine. Nicotine also stimulated the growth of the explant. Altogether, these studies suggest that nicotine stimulates lung branching morphogenesis through α_7 nicotinic acetylcholine receptors and may contribute to dysanaptic lung growth, which in turn may predispose the host to airway disease in the postnatal period.

Keywords

lung growth; nicotinic receptors

A relationship between environmental tobacco smoke and the subsequent development of wheezing and asthma has been identified in a number of epidemiological studies (8,12,27). In particular, maternal smoking has been independently associated with an increased risk for the development of asthma, diminished lower airway function, and the development of wheezing in infants, which can persist into the first few years of life (5,10). The underlying mechanisms by which these associations occur are not well understood, but it has been postulated that infants who are exposed to tobacco smoke have smaller airways that may predispose to airway obstruction (3). Since environmental tobacco smoke has already been established as a risk factor for abnormal fetal events such as stillbirth, premature birth, and low birth weight, it has been speculated that alterations in lung development in utero explain these changes seen in the lung (29).

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Although the composition of tobacco smoke is complex, nicotine is recognized as a major component that traverses the placental barrier. Spindel and colleagues (24,26) showed that chronic nicotine exposure in nonhuman primates results in alterations in collagen deposition around large airways and blood vessels in the fetus, increases in collagen mRNA expression in airway fibroblasts and smooth muscle cells, larger air spaces with less surface area, and increased type II cells. When lung function was examined, they found that these nicotine-induced alterations were associated with airflow limitation (25). These and related studies suggest that nicotine may affect lung development, thereby promoting airway dysfunction in the postnatal period. However, the mechanisms responsible for the effects of nicotine in fetal lungs and how they may affect lung development in utero remain unelucidated.

We postulated that nicotine affects lung branching morphogenesis, the process responsible for the formation of the primitive tubules that later become the airway tree, through effects on $\alpha 7$ nicotinic acetylcholine receptors (nAChRs). These ligand-gated ion channels represent the most common homomeric form of nAChRs in the body, and although their expression is highest in the central nervous system and muscle, they have been detected in fetal and adult lungs and in other organs (4). $\alpha 7$ nAChRs mediate the stimulatory effects of nicotine on matrix expression in lung fibroblasts (22). $\alpha 7$ nAChRs are also under transcriptional regulation by thyroid transcription factor (TTF-1), a transcription factor essential for the development of the lung (17). Multiple other functions of $\alpha 7$ nAChRs in lung cells have been reported, including bronchial epithelial cells, where $\alpha 7$ nAChRs appear to be involved in regulating cell-cell interactions, adhesion, and motility (31). Blockade of $\alpha 7$ nAChRs inhibits angiogenesis and endothelial network formation through VEGF-dependent pathways (11).

Thus, using embryonic murine lung explants, we set out to explore the role of nicotine in lung branching morphogenesis. We found that nicotine stimulates lung branching through $\alpha 7$ nAChRs, which are highly expressed during the pseudoglandular stage of lung development.

METHODS

Animals

The C57BL/6J strain was selected as the control wild-type strain for the $\alpha 7$ nAChR-deficient strain *Chrna7^{tm1Bay}*, also in the C57BL/6J strain, obtained from Jackson Laboratories (Bar Harbor, ME). All animal protocols were approved by the Emory University Institute Animal Care and Use Committee.

Reagents

Nicotine and the $\alpha 7$ nAChR antagonist α -bungarotoxin were obtained from Sigma Chemicals (St. Louis, MO). The $\alpha 7$ nAChR agonist GTS-21 was generously donated by Dr. Richard Papke from the University of Florida (Gainesville, FL) through Taiho Pharmaceuticals in Japan. The anti- $\alpha 7$ nAChR rabbit antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The Vectastain ABC kit obtained from Vector Laboratories (Burlingame, CA) was used for immunohistochemistry. Blocking solution for immunohistochemistry was acquired from DakoCytomation California (Carpinteria, CA). For lung explant cultures, the DMEM was purchased from Mediatech (Herndon, VA), and the fetal bovine serum was purchased from Atlanta Biologicals (Norcross, GA). All other reagents were obtained from Sigma Chemicals (St. Louis, MO) or Fisher Scientific (Hampton, NH) unless otherwise specified.

Organ culture of murine lung explants and quantification of branching morphogenesis

The murine lung explant model has been previously described (1). Briefly, timed pregnant CD57BL/6J mice were killed to obtain embryos at embryonic *day 11* (E11). *Day 0* was

determined by the presence of a cervical plug. Lung rudiments were removed from the embryos in sterile PBS, placed on Costar Transwell plates, and cultured at the air-medium interface using DMEM-10% FBS. The cultures were incubated under 5% CO₂ at 37°C for 5 days, and the medium was changed daily. Nicotine (1 μM), the α₇ nAChR antagonist α-bungarotoxin (5 nM), and the α₇ nAChR agonist GTS-21 (3 μM) were added to medium at the time the cultures were established and with each subsequent medium change. Branching was determined by counting the total number of branching clefts each day at the same time for 5 days. Branching clefts were visualized in whole mount by transillumination on a dissecting microscope.

Statistical analysis of explant culture experiments

At least three to five lungs were included for each experimental condition, and all experiments were performed at least three times. Experimental results were consistent between multiple repeat experiments. Data from representative experiments were chosen for presentation in RESULTS; hence, the data are presented as means ± SD. Statistical significance of the difference in means was determined using the *t*-test method (criterion for significance, *P* < 0.05).

DNA content determination

DNA content was estimated using DNA fluorometry as previously described (1). After explants were cultured for 5 days, they were placed in 10× TNE buffer (100 mM Tris, 10 mM EDTA, 2 M NaCl, pH 7.4) and homogenized. Using Hoechst dye 33258, which binds double-stranded DNA and fluoresces at 465 nm, we estimated total DNA content per lung explant by following standard curve calculations generated using calf thymus DNA as the standard. A representative experiment was chosen for presentation in RESULTS.

Immunohistochemistry

Timed pregnant CD57BL/6J mice were killed at E11, E13, E15, E17, E19, and postnatal *day 1*. Lung rudiments were obtained from the embryos, fixed in 4% paraformaldehyde, and embedded in paraffin blocks. Sections (5 μm) were deparaffinized and rehydrated in graded alcohols. Endogenous peroxidase activity was blocked using 3% H₂O₂. Sections were washed in 1× TBS-0.05% Tween and blocked using antibody diluent and blocking solution (DakoCytomation) followed by overnight incubation at 4°C with 1:200 dilution of the anti-α₇ nAChR rabbit antibody (Santa Cruz Biotechnology). Sections were incubated with appropriate biotinylated secondary antibody according to materials and protocol provided by the Vectastain ABC kit (Vector Laboratories). Diaminobenzidine was used for chromogenic visualization (Vector Laboratories).

Semiquantitative bioluminescent RT-PCR

The determination of mRNA levels was done using a semiquantitative bioluminescence-based RT-PCR assay as previously described (15). RNA was extracted from E11, E13, E15, E17, E19, and postnatal *day 1* lungs by using RNA-Bee reagent (Tel-Test, Friendswood, TX). E11 and E13 lungs were pooled from entire litters because of the size of individual lungs; each litter was considered a single sample. Biotinylated PCR primers and probes for mouse α₇ nAChR were synthesized by Sigma-Genosys Biotechnologies (The Woodlands, TX). Primers and probes for α₇ nAChR were synthesized based on GenBank published sequences. They were as follows: 5'-GTAACCATGCGCCGTAGG-3', 3'-CCGAGGCTTGCTGCTGAC-5', and probe 3'-GGTGCTGGCGAAGTACTG-5'.

Reverse transcription of mRNA was done using the Promega Access RT-PCR kit (Promega, Madison, WI) with 1 μg of RNA sample and 1 μl (200 units) of SuperScript III RT (Invitrogen,

Carlsbad, CA) (see manufacturer's protocol). The first-strand cDNA synthesis reaction was carried out at 50°C for 1 h using 0.5 µl (0.5 units) of *Taq* DNA polymerase (Invitrogen). The thermal cycling parameters consisted of 95°C (3 min); 25 cycles of 94°C (1 min), 54°C (0.5 min), and 72°C (1 min); and a final extension at 72°C (5 min).

After amplification, the biotinylated PCR products were denatured, neutralized, and added to streptavidin-coated plates (Roche, Indianapolis, IN). To each well, we added 100 µl of hybridization buffer (62.5 mM Na₂HPO₄, 94 mM citric acid, 15 mM sodium azide, 0.0625% BSA, 94 mM NaCl, 0.0125% Tween 20, and 10 mM MgCl₂) containing 2 ng of the digoxigenin-labeled probes. Hybridization was done for 2 h at 42°C. After hybridization, the plates were washed, and 100 µl of assay buffer (25 M Tris base, 10 mM EDTA, 0.15 M KCl, 15 mM sodium azide, and 2 mg/ml BSA) containing 1 µl of anti-digoxigenin aequorin conjugate (AquaLite Chemicon, Temecula, CA) was added to each well. The plates were incubated for 30 min at room temperature and washed. Luminescence was measured on a Luminoskan Ascent microtiter plate luminometer (ThermoLabsystems, Franklin, MA) after triggering with 0.1 M calcium. Results were plotted as relative fluorescent units. Standardization reactions were done for the hypoxanthine phosphoribosyltransferase (HPRT) gene with probes synthesized based on GenBank published sequences. They were as follows: 5'-TGGTGGAGATGATCTCTC-3', 3'-CATCAACAGGACTCCTCG-5', and probe 3'-GAGAGGTCCTTTTCACCA-5'. RT-PCR experiments were repeated in triplicate, and the data are means ± SE.

Quantitative real-time PCR

Quantitative real-time PCR was done to determine mRNA expression in an additional manner as previously described (16). Total cellular RNA was extracted from lung explant cultures and fetal lungs obtained at selected gestational time points (E11, E13, E15, E17, E19), and reverse transcription reactions were performed as described above. Real-time PCR reactions were set up by adding the following reagents to Smart Cycler reaction tubes (Sunnyvale, CA): 5 mM MgCl₂, 0.2 µM forward primer, 0.2 µM reverse primer, 10× Master Mix (SYBR Green JumpStart Taq Ready-Mix for quantitative PCR, Sigma), and template cDNA (500 ng total). Primers for α₇ nAChR, fibronectin, and HPRT were synthesized based on GenBank published sequences. α₇ nAChR forward and reverse primers were 5'-GTAACCATGCGCCGTAGG-3' and 3'-CCGAGGCTTGTGCTGAC-5', respectively. Fibronectin forward and reverse primers were 5'-CTGTGACAACCTGCCGTAG-3' and 5'-ACCAAGGTCAATCCACAC-3', respectively. HPRT forward and reverse primers were 5'-TGGTGGAGATGATCTCTC-3' and 3'-GAGAGGTCCTTTTCACCA-5', respectively. Samples were briefly centrifuged and processed using the following cycle program with the Cepheid Smart Cycler: holding at 95°C for 600 s, followed by 40 cycles at temperatures of 95°C for 15 s, 68°C for 30 s (with Optics on), and 72°C for 30 s. Results of the log-linear phase of the growth curve were analyzed using the mathematical equation of the second derivative, and relative quantification was performed using the 2^{-ΔΔCT} method (13a).

RESULTS

Distribution of α₇ nAChRs in developing murine lungs

We began by exploring the distribution and expression of α₇ nAChRs in developing lungs using semiquantitative RT-PCR, quantitative real-time PCR, and immunohistochemistry. RT-PCR and quantitative real-time PCR studies revealed α₇ nAChR mRNA and protein at all stages of lung development, but α₇ nAChR mRNA expression was highest in pseudoglandular stage lungs (E13–E15) and decreased by E17 (Fig. 1A). α₇ nAChR protein was identified on both epithelial and mesenchymal cells, particularly around the primitive airways (Fig. 1, B–

D), but not all cells stained positive. Staining for the $\alpha 7$ nAChR protein was consistent with higher expression of $\alpha 7$ nAChR at E11 through E15 (Fig. 1, B–D).

Nicotine stimulates lung branching morphogenesis through $\alpha 7$ nAChRs

To test the effects of nicotine on lung branching morphogenesis, we used the lung explant model. Lungs obtained at E11 were cultured with varying concentrations of nicotine. In this system, nicotine was found to stimulate branching, and this effect was most noticeable at days 4 and 5 of culture (Fig. 2, A and B). Despite the increased number of branches, the pattern of branching seemed equivalent between groups, and the morphology of the terminal buds was not different (Fig. 2A). The increase in branching occurred in a dose-dependent fashion, with more branches in 5 μ M nicotine than in 1 μ M nicotine (data not shown). Of note, the nicotine-treated lungs appeared larger than their untreated counterparts, suggesting an effect on explant growth (see below).

The stimulatory effect of nicotine on lung branching was entirely inhibited by α -bungarotoxin, a selective antagonist of $\alpha 7$ nAChRs (Fig. 2B). Culture with 5 nM α -bungarotoxin alone in the medium did not impair branching. To further test the role of $\alpha 7$ nAChRs, we treated lung explants with GTS-21, an $\alpha 7$ nAChR agonist also known as DMBX. Like nicotine, GTS-21 stimulated lung branching (Fig. 2C).

To confirm our hypothesis that $\alpha 7$ nAChRs mediate the effects of nicotine on branching, we obtained lung rudiments for explant culture from $\alpha 7$ nAChR-deficient mouse embryos (Jackson Laboratories) and exposed them to nicotine. After 5 days in culture, the explants from $\alpha 7$ nAChR-deficient mouse embryos did not demonstrate a stimulation in branching with nicotine treatment similar to that seen in wild-type CD57BL/6J mice. The untreated lungs from $\alpha 7$ nAChR-deficient embryos appeared to have slightly fewer branches than the wild-type at the end of 5 days, although the difference was not statistically significant. The branching pattern appeared similar (Fig. 3).

Nicotine stimulates lung explant growth

To determine the effects of nicotine on lung growth, we assessed total DNA content as a marker of overall explant growth. In the presence of 1 μ M nicotine, the lung explants grew significantly more compared with controls (Fig. 4). Although this effect was diminished by α -bungarotoxin, the antagonist was not as effective in diminishing growth as it was in blocking branching.

Nicotine exposure stimulates fibronectin expression in the pseudoglandular stage

Branching morphogenesis is a complex phenomenon that requires epithelial and mesenchymal interactions for an epithelial bud to form. Fibronectin is a known morphogen that is found in the branching clefts and is considered essential for proper branching (18, 22a). Interestingly, nicotine has been shown to stimulate fibronectin expression in fetal and adult lung fibroblasts (22). Thus we tested the effects of nicotine on fibronectin in our lung explant model. In the presence of 1 μ M nicotine, lung explants express significantly more fibronectin mRNA. Fibronectin expression is also increased in the lungs obtained from fetuses exposed to prenatal nicotine. The effect of prenatal nicotine exposure was found to be most dramatic during the pseudoglandular stage (E13 and E15), when branching morphogenesis occurs (Fig. 5).

DISCUSSION

Prenatal exposure to environmental tobacco smoke has been consistently associated with wheezing and the subsequent diagnosis of asthma (8,12,27). In addition, prenatal tobacco smoke exposure has been associated with decreased airflow in infants and children (5,7). These and related findings suggest that prenatal tobacco smoke potentially alters airway or lung

development in the fetus and subsequently manifests as decreased airflow or wheezing. Because of the complex composition of tobacco smoke and the question of which components actually penetrate into the fetal environment, it has been difficult to identify the specific etiologic agent for this association in epidemiological studies, but nicotine is considered a potential culprit.

Nicotine is a well-studied component of tobacco smoke that is known to cross the placental barrier to the fetal circulation and has been implicated as an etiologic agent in adverse fetal events related to environmental tobacco smoke (13). Although a link between exposure to nicotine alone during the prenatal period and airway dysfunction has not been established in humans, several studies have suggested a role for nicotine in other animals. For example, Sekhon et al. (25) showed that fetal monkeys exposed to nicotine in the prenatal period have altered pulmonary function similar to that seen in infants and children, including decreased forced mid-expiratory flow and increased airway resistance. In addition to the physiological differences, there were also histological changes in the lungs exposed to prenatal nicotine. Specifically, nicotine-treated animals exhibited lungs with less alveolar surface area available for gas exchange as a consequence of larger air spaces. The composition of the matrix of the airway wall was also different, particularly the cartilaginous airways, which showed a significant increase in the amount of collagen deposition in the airway wall (24,26). These findings illustrate that nicotine potentially alters the normal pattern of airways and parenchymal development, which may subsequently impact pulmonary function.

In view of the above information, we hypothesized that nicotine affects lung branching morphogenesis, thereby disrupting airway formation in ways that do not prevent further lung development but that might predispose the host to airway dysfunction after birth. To test this, we exposed pseudoglandular stage murine lung explants to nicotine and evaluated for branching. We used 1 μ M nicotine in the majority of our experiments to better approximate physiological concentrations seen by fetuses of smoking mothers. It has been reported that fetuses are exposed to higher concentrations of nicotine than their mothers. In the fetal serum, the concentration of nicotine ranges from 3 nM to 0.1 μ M (14). Our studies show that nicotine stimulated branching, and this effect was most evident at culture *days 4 and 5*. These findings are similar to those described by Wuenschell et al. (30), who found that nicotine stimulated branching and induced the expression of surfactant proteins A and C, suggesting an effect on epithelial cell differentiation and perhaps lung maturation. Of note, the dichotomous and monochotomous branching pattern of nicotine-treated lung explants did not differ from that of untreated explants, suggesting that nicotine may accelerate branching by an early induction of the same cellular processes involved in branching morphogenesis seen in the normal fetal lung such as fibroblast growth factor-10, bone morphogenetic protein-4, and Sonic hedgehog signaling (2).

We also tested the role of $\alpha 7$ nAChRs in nicotine-induced lung branching. $\alpha 7$ nAChRs are present in the fetal lung in a developmentally dynamic manner, and its gene transcription has recently been found to be regulated by TTF-1, an essential transcription factor for the initiation of lung morphogenesis (17). Prenatal nicotine exposure also increases expression of $\alpha 7$ nAChRs around airways (24). Using semiquantitative RT-PCR, we found that mRNA expression of $\alpha 7$ was highest at E13–E15, followed by a decrease at E17. This was confirmed by real-time PCR. Our own immunohistochemical studies showed expression of $\alpha 7$ protein in epithelial and mesenchymal cells between E11 and E13.

Since the highest detectable expression of $\alpha 7$ nAChRs is during the pseudoglandular stage of lung development (E11–E15), when branching morphogenesis takes place, one can infer a potential role of $\alpha 7$ nAChRs during branching. That is exactly what we found. Of note, the antagonist of $\alpha 7$ nAChRs, the snake venom α -bungarotoxin, inhibited nicotine-induced lung

branching in our system. Furthermore, a specific agonist of $\alpha 7$ nAChRs, GTS-21, stimulated branching, thereby mimicking the effects of nicotine. Finally, lung explants deficient in $\alpha 7$ nAChRs were not found to respond to nicotine stimulation during branching morphogenesis. Interestingly, mice deficient in $\alpha 7$ nAChRs do appear to have normal lung development, indicating that $\alpha 7$ nAChRs are not essential for branching morphogenesis, but this needs to be confirmed with careful morphometric and functional studies. Independently, our data suggest that perturbing the normal expression and signaling pathways of $\alpha 7$ nAChRs, particularly by nicotine, can affect branching.

We then turned our attention to organ growth. Since lung explants cultured in nicotine appeared larger in two-dimensional size, we questioned whether nicotine affected growth. Indeed, we found that nicotine stimulated overall growth. This is not surprising in view of reports linking organ growth with branching potential (21). Of note, we were unable to entirely block that growth with a specific $\alpha 7$ nAChR antagonist, α bungarotoxin. Furthermore, growth in the $\alpha 7$ nAChR-deficient explants was not different between the untreated and nicotine-treated conditions. Thus $\alpha 7$ nAChRs play an important role in branching morphogenesis, but their role in regulating growth needs to be explored further.

Although branching morphogenesis is a complex program with multiple epithelial and mesenchymal interactions, we attempted to look at one morphogen in particular: fibronectin. Fibronectin is usually mesenchymal in origin but also has been described in the epithelium during branching morphogenesis (22a). Fibronectin expression in fetal lung fibroblasts already has been shown to increase with exposure to nicotine through $\alpha 7$ nAChR-mediated signals (22). We found that both nicotine treatment of lung explant cultures in vitro and prenatal nicotine exposure in vivo results in increased fibronectin expression in the pseudoglandular stage lung. Thus increasing fibronectin expression may be one way that prenatal nicotine exposure increases branching.

Our results showing nicotine stimulation of branching morphogenesis and stimulation of growth may provide an explanation to the decreased pulmonary function found in offspring with prenatal nicotine exposure. Many of the decreased flows observed in monkeys treated with nicotine likely reflect an airway, rather than a parenchymal, etiology, since there was increased deposition of collagen around the airways (26). It is possible that changes in the structure of conducting airways alone can result in overall decreased airflow with increased resistance. Since branching morphogenesis creates the template for conducting airway formation, any alteration in branching may also have an impact on the number and size of conducting airways that can result in abnormal expiratory flows. In our model, prenatal nicotine exposure may actually result in greater total airway length based on unpublished data using stereological analysis.

The dissociation between growth and branching raises the concept of dysanaptic growth. Dysanaptic growth refers to the disproportionate growth between conducting airway and alveolar parenchyma first described to explain variability in expiratory flow volume curves (9). In our lung explant model, branching mediated through the $\alpha 7$ nAChR appears to be dissociated from the overall growth, suggesting that dysanaptic growth is occurring in this pathway. Simply having dysanaptic growth may lead to alterations in lung function studies. Some epidemiological studies have shown that infants exposed to prenatal tobacco smoke have different physiological lung function irrespective of airway hyperresponsiveness (28). Dysanaptic growth also has been proposed as a potential mechanism that alters lung function in other models involving different growth conditions (6,23). The abnormal lung function associated with prenatal nicotine exposure may be a consequence of dysanaptic growth by changing branching morphogenesis without an equal change in parenchymal growth.

In summary, our results suggest that nicotine directly impacts branching morphogenesis during the pseudoglandular stage through effects on $\alpha 7$ nAChRs. Nicotine's effects on branching morphogenesis may be partially mediated by the stimulation of fibronectin expression, but this needs to be explored further. This interaction may explain why some infants and children exposed to prenatal environmental tobacco smoke demonstrate abnormal pulmonary function. Our studies continue to emphasize the importance of eliminating environmental tobacco smoke exposure for expectant mothers, infants, and children, as well as raising the question whether an alternative to nicotine replacement therapy for pregnant smokers is needed.

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Fig. 1.

Distribution and expression of $\alpha 7$ nicotinic acetylcholine receptors ($\alpha 7$ nAChRs) in the developing murine lung. Murine lungs were harvested at different stages of gestation followed by processing for RT-PCR/quantitative real-time PCR (A) or immunohistochemistry (B–D) for $\alpha 7$ nAChRs. A: $\alpha 7$ nAChR mRNA expression. Semiquantitative RT-PCR and quantitative real-time PCR (inset) for $\alpha 7$ nAChR showed the highest mRNA expression during the pseudoglandular stage (E13 and E15, days 13 and 15 of gestation) of development with a low level of expression remaining for the remainder of gestation. B–E: $\alpha 7$ nAChR distribution. We found abundant staining for $\alpha 7$ nAChRs in the early pseudoglandular stage of lung development (B, E11 lung; C, E13 lung), where expression was detected in both epithelial and

mesenchymal cells. Expression of α_7 nAChRs diminished at the end of the pseudoglandular stage (*D*, E15 lung), remaining in the endothelium and epithelium, and continued to decrease as the fetal lung matured into the neonatal period (data not shown). Image in *E* represents IgG control of an E15 lung.

Fig. 2.

Nicotine stimulates branching morphogenesis through α_7 nAChRs. *A*: effect of nicotine on ex vivo lung development. Lung explants obtained at E11 were placed in culture for 5 days and evaluated daily for the presence of branching clefts. Photographs of untreated (controls) and nicotine (1 μ M)-treated lung rudiments were obtained at culture days 2 and 5. *B*: effect of nicotine on the branching of lung explants. Lung rudiments harvested at E11 were cultured for 5 days in regular medium (control) or in the presence of nicotine (1 μ M), α -bungarotoxin (α -BGT; 5 nM), or both; branching clefts were counted each day. Note that nicotine stimulated lung branching clefts, and this was most noticeable at days 4 and 5 (* P < 0.05). α -Bungarotoxin alone had no effect but inhibited the stimulatory effect of nicotine. *C*: effect of GTS-21 on the

branching of lung explants. Lung rudiments were cultured as described above with nicotine (1 μ M) or GTS-21 (3 μ M) for 5 days, and the branching clefts were counted each day. Note that both nicotine and GTS-21 stimulated branching ($*P < 0.05$). Data are from an experiment representative of a set of triplicate experiments with similar results.

Fig. 3.

Lung explants from $\alpha 7$ nAChR-deficient mice are unresponsive to nicotine-stimulated branching. **A** and **B**: lung rudiments were harvested from wild-type (WT) and $\alpha 7$ nAChR-deficient mice (Chrna7) at E11 and cultured in the presence of 1 μ M nicotine for 5 days. Branching clefts were measured to assess branching morphogenesis. Explants from $\alpha 7$ nAChR-deficient mice did not increase branching in response to nicotine, but the WT explants showed a significant increase in branching when treated with nicotine as demonstrated previously (* $P < 0.05$). Data are from an experiment representative of a set of triplicate experiments with similar results.

Fig. 4.

Nicotine-stimulated growth is mediated through non- α_7 nAChR-related pathways. Lung rudiments harvested at E11 were cultured in the presence of nicotine (1 μ M), α -bungarotoxin (5 nM), or both for 5 days. Afterwards, the lungs were collected and processed for DNA fluorometry. Note that nicotine-treated explants contained more DNA, whereas those treated with the combination of nicotine and α -bungarotoxin showed reduced DNA content ($*P < 0.05$). Rudiments treated with α -bungarotoxin alone also showed reduced DNA content. Data are from a representative experiment. α BuTX, α -bungarotoxin; Nic, nicotine.

Fig. 5.

Nicotine stimulates fibronectin expression during branching morphogenesis and in the pseudoglandular stage. Lung rudiments harvested at E11 were cultured with and without nicotine (1 μ M) for 5 days. At the end of 5 days, the lungs were collected for semiquantitative RT-PCR analysis. Lung explants cultured in the presence of nicotine had increased fibronectin expression ($*P < 0.05$) in this branching model. The *inset* shows that fibronectin expression is increased by quantitative real-time PCR in pseudoglandular stage lungs (E13 and E15) taken from fetuses exposed to prenatal nicotine.