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Regulation of $\alpha 4\beta 2\alpha 5$ nicotinic acetylcholinergic receptors in rat cerebral cortex in early and late adolescence: sex differences in response to chronic nicotine

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Abstract

Chronic nicotine administration in animals, and smoking in humans, causes up-regulation of $\alpha 4\beta 2^*$ neuronal nicotinic receptors (nAChRs), which has been hypothesized to contribute to the addictive actions of nicotine. We used a rat model to test whether such up-regulatory effects differ in adolescents versus adults, and in males versus females. Following chronic treatment with nicotine or saline via subcutaneous osmotic minipumps, we measured $\alpha 4\beta 2$ and $\alpha 4\beta 2\alpha 5$ nAChRs in cerebral cortex using [³H]epibatidine to label assembled nAChRs, and selective antibodies to measure the individual subunits via immunoprecipitation. For the first time, we provide a detailed characterization of the response of both $\alpha 4\beta 2$ and $\alpha 4\beta 2\alpha 5$ nAChRs in female adolescent rat cerebral cortex. We found differences in nicotine-induced up-regulation between males and females in early adolescence that are absent in both late adolescence and adulthood. Males showed significant up-regulation at PN28 which was absent in age-matched females. These results demonstrate sex differences in the susceptibility of $\alpha 4\beta 2^*$ nAChRs to the effects of chronic nicotine exposure in the cerebral cortex based on age.

Keywords

Chronic nicotine; adolescence; nicotinic receptor subtypes; immunoprecipitation; $\alpha 5$ subunit

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1) Introduction

Adolescence is the most common period for initiation of recreational drug use (Spear et al., 2004). This use often begins with tobacco products, and evidence suggests that adolescents are differentially susceptible to the addictive and aversive effects of tobacco smoke (Levin et al., 2003; McQuown et al., 2007). Adolescent use of tobacco is associated with subsequent higher daily consumption and a lower probability of smoking cessation (Chen et al., 1998). It has also been demonstrated in animal studies that nicotine administration in early adolescence increased subsequent cocaine self-administration in rats (McQuown et al., 2007). Thus nicotine use during adolescence may have effects on brain function that are different and perhaps more prolonged than those that occur during adult tobacco use.

Increasing evidence indicates differences in smoking habits between males and females (Grunberg et al., 1991; Perkins 1999). When compared to males, females take fewer and shorter puffs from cigarettes, are less sensitive to some of the effects of nicotine, respond less to nicotine replacement therapy, and are more sensitive to smoking cues (Delfino et al., 2001). Sex differences in human response to nicotine could arise from multiple sources, including factors that directly affect tissue response to the drug (pharmacodynamics), factors that affect blood plasma levels of the drug (pharmacokinetics), factors that affect men and women's socially defined roles and factors that affect sex differences in conditioned responses to the drug (Jensvold et al., 1996). In this study, we attempt to address the pharmacodynamics differences between males and females during early and late adolescence.

To explore the effects of chronic nicotine in male and female adolescents, we used a well-studied model employing osmotic mini-pumps to administer nicotine in rats. Periadolescence in rats is defined as the time between the earliest detection of diurnal gonadotropin cycling (approximately postnatal day 28 [PN28]) and reproductive maturity (approximately PN38–42) (Spear et al., 1983; Ojeda and Urbanski, 1994). Adolescence in the rat can be further sub-divided into early adolescence [starting at PN28] and late adolescence [around PN42]. Research regarding nicotine addiction during adolescence has shown that male adolescent rats are more likely to self administer nicotine than adult rats (Leslie et al., 2004; Vastola et al., 2002; Beluzzi et al., 2005). These findings have been reproduced in female rats as well (Levin et al., 2003). Previous studies have also shown that adolescent rats experience aversive effects from nicotine differently compared to adults. Specifically, adolescents display selective loss of striatal (and hippocampal) AMPA receptors, an effect not obtained with comparable treatment in the adult (Adriani et al., 2004), damage to striatal serotonergic terminals (Xu et al., 2001) and lasting changes in striatal cell signaling (Abreu-Villaca et al., 2003). Indeed, the striatum and its dopaminergic projections are still undergoing active remodeling and restructuring during adolescence (Goldman-Rakic 1987), factors which may render this region especially vulnerable to toxicant injury. Neurochemical, neuroanatomical and behavioral changes that occur during this period in rats are similar to those seen in human adolescents (Slotkin et al., 2004).

Chronic administration of nicotine increases the density of nAChRs in rat and mouse brains (Schwartz and Kellar, 1983; Marks et al., 1983). This up-regulation occurs in more than two-thirds of rodent brain regions (Marks et al., 1992). However, the extent of this up-

regulation varies depending on the method of administration and brain region, with binding increased in the cerebral cortex by more than 60% (Kellar et al., 1989; Pauly et al., 1991; Marks et al., 1992; Perry et al., 2002; Nguyen et al., 2003). Similarly, studies of autopsied brains from human smokers and age-matched non-smokers (Benwell et al., 1988; Breese et al., 1997; Perry et al., 1999) found markedly higher densities of nAChR binding sites in the cerebral cortex of smokers compared to non-smokers. The cerebral cortex is of particular interest when studying nicotine addiction because of its probable role in cognition, memory, arousal, attention and anxiety. The cerebral cortex is known to be affected by nicotine (Levin, 1992). Indeed, acute nicotine improves performance on tasks regarding attention, cognition, learning and memory. When compared to adults, adolescents show increased novelty seeking behavior as well as elevated levels of impulsivity and restlessness, behaviors mediated by cortical activity (Laviola et al., 2003). Cortical development is not complete by adolescence and therefore may be disturbed by exposure to nicotine (Goriounova and Mansvelder 2012). In particular the medial prefrontal cortex (mPFC) which shows delayed development compared to other regions of the cortex is responsible for decision-making (Gogtay et al., 2004) and may play a role in the initiation of drug use in adolescents (Li et al., 1999).

Neuronal nAChRs are pentameric ligand-gated cation channels permeable to Na^+ , K^+ , and Ca^{2+} . The channels are comprised of combinations of α and β subunits assembled from one of nine α ($\alpha 2$ - $\alpha 10$) and three β ($\beta 2$ - $\beta 4$) subunits. The majority of these receptors are heteromeric receptors comprised of two α and two β subunits, and a fifth subunit that is either an α or β subunit (Albuquerque et al., 2009; Millar et al., 2009). The major nAChR subtype in the CNS is the $\alpha 4\beta 2^*$ nAChR, where the * indicates a variable fifth subunit (either $\alpha 4$, $\beta 2$ or $\alpha 5$). These receptors account for >50% of all nAChR subtypes in the brain (Perry et al., 2002; Marks et al., 2010). Unlike other α subunits, the $\alpha 5$ subunit requires at least one other α subunit as well as a $\beta 2$ and/or $\beta 4$ subunit to form a functional nAChR, (Ramirez-Latorre et al., 1996) and does not participate in agonist binding (Fucile et al., 1997; Gerzanich et al., 1998). Previous studies suggest the importance of these nAChRs for nicotine self-administration: animals that don't express $\alpha 4$ or $\beta 2$ nAChR subunits fail to self-administer nicotine and the re-expression of the knocked out subunit in the ventral tegmental area can restore self-administration (Picciotto et al., 1998; Pons et al., 2008). The inclusion of the $\alpha 5$ subunit nAChRs alters channel conductance rates as well as desensitization rates and pharmacological properties (Alkondon et al., 1998; Tapia et al., 2007). As the majority of these nAChRs are presynaptic and modulate the release of neurotransmitters (Vizi and Lendvai 1999), projections from the prefrontal cortex are known to target dopaminergic and GABAergic neurons in the ventral tegmental area (Carr and Sesack, 2000), and cortical $\alpha 5$ subunit nAChRs drive development and morphology of cortical neurons (Bailey et al., 2012), we chose to look at nAChR regulation in the cerebral cortex. Using this design we are able to determine changes in the density of these nAChRs in a key region of the brain involved in the functional output of nicotine behaviors as mentioned above.

In the cerebral cortex of adult male rats, ~90% of the heteromeric nAChRs are the $\alpha 4\beta 2^*$ subtype, and of these ~15% are the $(\alpha 4\beta 2)_2\alpha 5$ subtype (Mao et al., 2008). These nAChRs are highly sensitive to activation by nicotine (Brown et al., 2007) and are also highly Ca^{2+}

permeable (Tapia et al., 2007). Interestingly, in the adult rat brain, $\alpha 4\beta 2^*$ nAChRs that include the $\alpha 5$ subunit are resistant to the nicotine-induced up-regulation (Mao et al., 2008). Human genome-wide association studies have identified sequence variants in the region encoding the $\alpha 5$ subunit that increase the risk of nicotine dependence and lung cancer (Amos et al., 2008; Berrettini et al., 2008).

Previous work from our lab using autoradiography has indicated that adolescent and adult male rats differ significantly in the density of $\alpha 4\beta 2^*$ nAChRs following chronic nicotine exposure (Doura et al., 2008), which suggests that these receptors may vary in their response to nicotine based on age or stage in development. In the present study we used subunit-selective antibodies in immunoprecipitation procedures to further elucidate differences in cortical $\alpha 4\beta 2^*$ nAChR expression following chronic nicotine at different ages, comparing adults to early and late adolescence. In addition, we compared males and females at all three developmental stages. Our results indicate that both the age and the sex of the animal contribute to the net response of $\alpha 4\beta 2^*$ nAChRs to chronic nicotine.

2 Materials and Methods

2.1 Radioligands and antibodies

[³H]Epibatidine ([³H]EB; 56.3 Ci/mmol and 62.2 Ci/mmol) was obtained from Perkin Elmer Life Sciences (Boston, MA, USA). Rabbit antisera directed at peptide sequences of the cytoplasmic domains of the nAChR $\alpha 5$ subunit were kind gifts from Robert P. Yasuda (Georgetown University, Washington, DC, USA). A monoclonal antibody (mAb 270) to the chick $\beta 2$ subunit was made from hybridoma stocks (American Type Culture Collection, Manassas, VA, USA). This mAb was originally developed and characterized by Whiting and Lindstrom (1987). A monoclonal antibody selective for $\alpha 4$ subunits (mAb 299) (Whiting and Lindstrom 1988; Nakayama et al., 1995) was purchased from Santa Cruz Biotechnologies, Inc. (Santa Cruz, CA, USA). The antibodies used here are subunit-selective and their specificity for immunoprecipitation of radiolabeled nAChRs containing the corresponding subunits has been reported previously (Hernandez et al. 2004; Marritt et al. 2005; Perry et al. 2007). Additional studies to confirm the specificity of the $\alpha 5$ antibody have been previously published (Mao et al. 2008). Protein G Sepharose beads were purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Normal rabbit serum (NRS) was purchased from Santa Cruz Biotechnologies, Inc. (Santa Cruz, CA, USA). For simplicity, we use the term antibody to refer to unpurified antiserum, as well as to affinity purified antiserum and mAb.

2.2 Experimental Procedures

2.2.1 Animal treatment—Sprague–Dawley rats were obtained from Hilltop Lab Animals (Scottsdale, PA, USA) and delivered at specified ages based on the intended age of sacrifice. Osmotic minipumps (Alzet model 2002; Durect Corporation, Cupertino, CA, USA) were filled with sterile saline or with nicotine hydrogen tartrate (Sigma-Aldrich, St Louis, MO, USA) in saline. PN42 and PN84 animals were treated for 14 days. Implanting subcutaneous pumps can be technically challenging if it occurs prior to weaning. Therefore, in order to prevent issues with compromised nutrition or early weaning, PN28 animals were implanted

at weaning (PN21) and treated for 7 days rather than 14. Preliminary studies from our lab (Supplemental Figure 1) indicated that the same level of nAChR up-regulation could be achieved in adult males with 7 or 14 days of treatment, which indicated that 7 days of treatment would be sufficient to induce up-regulation at PN28 if it were to occur. We administered nicotine for 14 days, whenever possible, as this is the standard of chronic nicotine exposure in the field when using this exposure method. The minipumps used deliver solution at a rate of 12 μ L/day for a duration of 14 days (7 days for PN28). The nicotine tartrate concentration was calculated to provide a dose of 17 mg/kg/day (equivalent to 6 mg/kg/day nicotine free base = 37 μ mol/kg/day). As the animals age and pregnancy progresses, the animals will gain weight, and the dose received at the time of sacrifice is less than the dose at implantation. However, the animals are still receiving relatively high doses of nicotine at sacrifice. Based on our measurements, the average weight gained by our female dams was 105g $\pm$ 9g at the time of sacrifice. Therefore, these animals were still receiving a nicotine concentration between 5.39 mg/kg/day and 5.74 mg/kg/day nicotine free base. The starting dose has been shown to yield blood levels of 0.56 μ mol/L for nicotine and 3.5 μ mol/L for cotinine in adult rats (Nguyen et al. 2004), which are comparable to levels achieved in humans who are moderate to heavy smokers. Male and female rats were anesthetized with isoflurane and the minipumps were inserted under sterile conditions into a subcutaneous pocket via a small incision made between the shoulders. While under anesthesia, animals were administered buprenorphine (0.1 mg/kg, s.c.) for post-operative pain. After recovery from anesthesia (10–30 min), animals were returned to individual cages. Fourteen days after minipump implantation (7 days for PN28 animals), animals were anesthetized with isoflurane and killed by decapitation. The brains were then rapidly removed and dissected, and cerebral cortex frozen on dry ice and stored at -80°C until use. Animal use and procedures were approved by the George Washington University Medical Center Institutional Animal Care and Use Committees, and performed in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). Every effort was made to reduce animal suffering and to minimize the number of animals used.

2.2.2 Measurement of nAChR binding sites—Tissues were homogenized in 20 ml Tris buffer (50 mM Tris-HCl buffer, pH 7.4 at 4°C) using a Polytron Tissue Homogenizer (Brinkmann Instruments, Inc., Westbury, NY), at a moderate speed for 15–20 seconds in a large centrifuge tube. The samples were centrifuged twice at 30,000 G for 10 min in buffer and the membrane pellets were resuspended in fresh buffer. nAChR binding sites in the membrane homogenates from the tissues were then measured with 2 nM [^3H]EB. Incubations were carried out in 200 μ L Tris buffer for 24 h at 4°C . Bound nAChRs were separated from free ligand by vacuum filtration over Brandel GF/B glass-fiber filters (Gaithersburg, MD, USA) that were pre-wet with 0.5% polyethyleneimine; radioactivity on the filters was then measured in a liquid scintillation counter. Non-specific binding was determined in the presence of 300 μ M nicotine, and specific binding was defined as the difference between total binding and non-specific binding. Animal numbers were 7–12 for PN84, 6–11 for PN42, and 6–8 for PN28.

2.2.3 Immunoprecipitation assays—Brain tissues from the same animals were used for both standard homogenate binding (above) and immunoprecipitation assays. Homogenates of brain tissues were prepared as above. The membranes were solubilized by incubating the homogenates in 2% Triton X-100 in 50 mM Tris buffer, with gentle rotation for 2 h at 4°C. This procedure results in ~90% solubilization of heteromeric nAChRs in the brain area measured. After centrifuging the mixture at 30,000 g for 10 min, aliquots of the clear supernatant cerebral cortex (equivalent to 10–15 mg tissue depending on the age of the animal) were added to sample tubes containing 2 nM [³H]EB. As all known heteromeric nAChRs display an affinity for [³H]EB between ~30 and 250 pM, this concentration is expected to occupy 86–98% of the nAChRs (Mao et al., 2008). One of the subunit-specific antibodies at an optimal concentration, which had been determined previously by titration in our lab to precipitate the maximum number of nAChRs possible from solution (1:30 [7μl in 200μl total] of mab299, 1:20 [10μl in 200μl total] of mab270 and 1:15 of α5 polyclonal), or 4μl of NRS, or Tris alone was then added. The final volume of the assay was 200 μL. The samples were then rotated gently overnight at 4°C. The next day, 50 μL of a 50% slurry of Protein G Sepharose beads was added and the rotation of the samples at 4°C was continued for another hour. The samples were then centrifuged at 8,000 g for 1 min and the supernatants were removed and filtered over Brandel GF/B glass-fiber filters that had been pre-wet with 0.5% polyethyleneimine. Pre-treating with PEI ensured the retention of solubilized nAChRs onto filters (Mao et al., 2008), which were counted by liquid scintillation and used as an internal control to confirm efficient immunoprecipitation and capture of labeled nAChRs. The immunoprecipitated pellets were washed by resuspension in cold 1 mL 50 mM Tris–HCl buffer (pH 7.0), followed by centrifugation at 8,000 g for 1 min. The pellets were then dissolved in 200 μL of 0.1 N NaOH and the radioactivity was measured in a liquid scintillation counter. The counts precipitated in tubes containing NRS, which was used as control for non-specific precipitation of unpurified antiserum, or Protein G alone, which was used as a control for non-specific precipitation of mAbs, were subtracted. Immunoprecipitation assays were carried out to measure nAChR subtypes in the cerebral cortex from saline- and nicotine-treated rats. The calculated number of [³H]EB-labeled nAChRs immunoprecipitated by each antibody plus the remaining solubilized [³H]EB-labeled nAChRs in the supernatant were compared back to total radiolabeled nAChRs in the membrane binding assays. The former typically represented 75–80% of the latter measurements. This would be expected as some nAChRs would be lost due to repeated washing steps in the immunoprecipitation assay.

2.3 Statistics

The comparison between males and females under nicotine and saline treated conditions was made using a three-way ANOVA to define age × treatment × sex interactions based on the calculated density of nAChRs determined by immunoprecipitation (Table 1). We then performed two-way ANOVA in order to further define the age × treatment and sex × treatment interaction, followed by Tukey's test for post-hoc analysis. Statistical significance was defined as $p < 0.05$. These results are summarized in Table 2. Animal numbers were $n=7$ for nicotine and $n=12$ for saline treated PN84 males; $n=8$ for nicotine and $n=7$ for saline for PN84 females; $n=6$ for nicotine and $n=11$ for saline treated PN42 males; $n=6$ for both

nicotine and saline treated PN42 females; and n=7 for nicotine and n=6 for saline treated PN28 males; n=6 for saline and n=8 for nicotine treated PN28 females.

3 Results

3.1 Effect of Chronic Nicotine Treatment on nAChRs in Male Cortex

Immunoprecipitation experiments compared the effect of chronic nicotine on $\alpha 4\beta 2^*$ nAChRs in the cerebral cortex of male rats at PN84 (adult), PN42 (late adolescence) and PN28 (early adolescence) (Figure 1). These data are shown as the effect of nicotine based on the subunit composition. Statistical significance was determined using a Tukey's post-hoc following a 3-way ANOVA. We observed both $\alpha 4$ - and $\beta 2$ -containing nAChRs significantly up-regulate in response to chronic nicotine exposure in adult males, while $\alpha 5$ -containing nAChRs showed no response to chronic nicotine exposure in adult males (Figure 1c). These findings are in agreement with our previous report (Flores et al., 1992; Mao et al., 2008). Additional previous work from our lab suggested a reduced up-regulation in PN42 males in response to chronic nicotine (Doura et al., 2008). However, in our study we saw significant up-regulation in both $\alpha 4$ - and $\beta 2$ -containing nAChRs (Figure 1b), which was not different from the up-regulation seen in males at PN84. Similarly, in cortex from early adolescent male cortex (PN28), we found significant nicotine-induced increases in nAChRs immunoprecipitated by both the $\alpha 4$ and $\beta 2$ antibodies, and again no change in $\alpha 5$ -containing nAChRs (Figure 1a).

Confirming the immunoprecipitation data, conventional membrane binding studies with [3 H]EB to label all $\alpha 4\beta 2^*$ nAChRs expressed in the cerebral cortex of PN84 and PN42 and PN28 males indicated significant up-regulation in the number of assembled $\alpha 4\beta 2^*$ nAChRs at all three ages following chronic nicotine treatment (Figure 1f, 1e, and 1d respectively).

3.2 Effect of Chronic Nicotine Treatment on nAChRs in Female Cortex

In parallel experiments, immunoprecipitation assays were also performed on the cerebral cortex of female rats at PN84, PN42 and PN28 to define the effects of chronic nicotine on $\alpha 4\beta 2^*$ nAChR expression. As outlined above, statistical significance was determined using a two-way ANOVA to define age $\times$ treatment interactions. The calculated numbers of nAChRs under both nicotine and saline treated conditions were compared using a Tukey's test for post-hoc analysis. Up-regulation of [3 H]EB-labeled $\alpha 4$ - and $\beta 2$ -containing nAChRs was seen in PN84 female rats following chronic nicotine exposure (Figure 2a). Similar to males, no nicotine-induced change was seen in $\alpha 5$ -containing nAChRs in the adult females (Figure 2c). One of the notable differences in comparison to males was the highly significant nicotine-induced up-regulation of $\alpha 4$ - and $\beta 2$ -containing nAChRs at 42 days postnatal (Figure 2b). Also, unlike the effect seen in male cortex, no significant change was seen in any of the three subunit-containing nAChRs labeled with [3 H]EB at PN28 in female cortex (Figure 2a).

As was the case in our assessment of the male cerebral cortex, the immunoprecipitation results in the female cortex were mirrored by binding experiments using [3 H]EB to label all $\alpha 4\beta 2^*$ nAChRs. Specifically, the binding results revealed significant up-regulation of [3 H]EB-labeled nAChRs occurred following chronic nicotine exposure in adulthood and late

adolescence (Figure 2f and e respectively), which is in agreement with the up-regulation of $\alpha 4$ - and $\beta 2$ -containing nAChRs seen by immunoprecipitation (Figure 2c and b). In contrast, no significant change was seen at PN28 using either immunoprecipitation (Figure 2a) or receptor binding (Figure 2d) methodology.

3.3 Differences in Male and Female Adolescent Responses to Chronic Nicotine

There was an apparent pronounced difference in nicotine response between the sexes in early adolescence. This was evident by the large increase in $\alpha 4$ - and $\beta 2$ -containing nAChRs in the PN28 males (Figure 1a) that was not observed in the female cortex (Figures 2a). Additionally, although we see a nicotine-induced up-regulation of nAChRs in both males and females at PN42, females at PN42 displayed a significantly greater increase in both $\alpha 4$ - and $\beta 2$ -containing nAChRs in response to chronic nicotine than males at the same age as determined by two-way ANOVA. In concordance with previous literature, a significant up-regulation was seen in [^3H]EB-labeled nAChRs in the adult male cortex (PN84) (Figure 1c), and this was also seen in female cortex at the same age (Figure 2c).

In order to statistically confirm these observed differences, a three-way ANOVA was used to compare the immunoprecipitation results in males and females for each subunit at each age following nicotine or saline treatment (Table 1). These data suggested that there was a significant Sex $\times$ Age $\times$ Treatment interaction. In order to further clarify the interactions between these variables, we performed a two-way ANOVA. There was a significant treatment effect but no significant interaction (Sex $\times$ Treatment) at PN84, indicating no effect of sex on nicotine treatment in adult rats. At PN42, there was a significant sex $\times$ treatment interaction where nicotine treatment affected the levels of $\alpha 4$ - and $\beta 2$ -containing nAChRs in females to a greater extent than in males at the same age. At PN28, there was also a significant Sex $\times$ Treatment interaction in both the $\alpha 4$ - and $\beta 2$ -containing nAChRs, indicative of sex differences in response to chronic nicotine exposure. Similarly, Tukey's post-hoc analyses demonstrated there was a significant treatment effect on $\alpha 4$ - and $\beta 2$ -containing nAChRs of males that was absent in females at PN28. Taken together, these data suggest that females at PN42, and males at PN28, may be more sensitive to the effects of chronic nicotine exposure on nAChR density in the cerebral cortex than age-matched animals of the opposite sex.

4 Discussion

Nicotine is one of the most socially acceptable drugs in our society, which makes it an early choice for adolescent experimentation with drugs of abuse. Epidemiological evidence suggests that earlier onset of drug experimentation is associated with an increased likelihood of the development of dependence (Schramm-Sapota et al., 2009). This study provides a detailed analysis of the differences in how male and female rats respond to chronic nicotine exposure in early and late adolescence. Our data suggest that both age and sex play a significant role in the response of $\alpha 4\beta 2^*$ nAChRs to chronic nicotine.

In adult male and female rat cerebral cortex, we saw a significant increase in $\alpha 4$ - and $\beta 2$ -containing nAChRs, but no significant change in $\alpha 5$ -containing nAChRs in either sex following nicotine administration. This confirms previous findings that indicated that $\alpha 5$ -

containing nAChRs are resistant to up-regulation in the presence of chronic nicotine administration (Mao et al., 2008). Work from other labs has suggested that there is a significant increase in $\alpha 5$ -containing nAChRs in the caudal cortex of adult male rats (Moretti et al., 2010). However, these data were specific to the caudal cortex, rather than the cortex as a whole. Our current data reconfirm the previous work from the Kellar lab that showing that in the adult male cortex these nAChRs are resistant to up-regulation (Mao et al., 2008). In addition, our results demonstrate that these nAChRs are also resistant to up-regulation in female adult rats. This is the first characterization of the response, at a subunit-specific level, of adult female nAChRs to chronic nicotine administration in the cerebral cortex. From this we are able to conclude that there was no significant difference between the adult male and female $\alpha 4\beta 2^*$ nAChR responses to nicotine.

Sex differences in human response to nicotine could arise from multiple sources, including factors that directly affect tissue response to the drug (pharmacodynamics), factors that affect blood plasma levels of the drug (pharmacokinetics), factors that affect men and women's socially defined roles and factors that affect sex differences in conditioned responses to the drug (Jensvold et al., 1996). Evidence regarding sex differences in pharmacokinetics suggests that, in rats, males display 3- to 5- fold higher hepatic nicotine metabolism than female rats (Kato 1974; Kato 1979; El Defrawy El Masry and Mannering 1974). These pharmacokinetic differences may, however, differ depending on the species studied (Boyland and de Knock 1966; Booth and Boyland 1967; Stalhandske et al., 1969; Jenner et al., 1973; Jenner et al., 1973b). In humans, it has been demonstrated that female nonsmokers excrete more nicotine and less cotinine in their urine than female smokers following IV administration of nicotine hydrogen tartrate (Beckett et al., 1971). In this same study, they emphasize that studying sexual dimorphisms in nicotine metabolism in human chronic smokers may be complicated by the metabolic changes induced by smoking. Therefore, although there are reported species-dependent sex differences in the pharmacokinetic profiles of males and females, it is unlikely that these differences explain all of the epidemiological differences between male and female adolescents in response to nicotine administration. Therefore, we chose to further investigate the possible pharmacodynamics differences between male and female adolescent rats.

Previous work from our lab and others has shown age- and sex-related differences in nAChR expression following nicotine exposure (Doura et al., 2008). Our present work examined nicotine effects on the density and subunit composition of $\alpha 4\beta 2^*$ nAChRs in the cerebral cortex in early and late adolescent male and female rats exposed to nicotine. Future studies in these ages and sexes are warranted to investigate possible differences in nAChR functionality in various brain regions following nicotine exposure.

Extensive work has shown that brain development is not complete by adolescence. In particular, growth in areas involved in cognition, like the medial prefrontal cortex (mPFC), continues throughout adolescence (Goriounova and Mansvelder, 2012). The development of cortical areas of the brain depends on age and experience and continues into adulthood (Sowell et al., 2003; Giedd, 2004). Because of these developmental differences and the complexity of the study design, we chose to look at the cortex as a whole to characterize $\alpha 4\beta 2$ and $\alpha 4\beta 2\alpha 5$ nAChRs in three ages and in both males and females. It will be interesting

to investigate whether sex- and age-related differences in nAChR density and subunit composition in the cerebral cortex also exist in other brain regions.

Developmental differences in nAChRs have also been reported previously (Azam et al., 2007). This study showed differences between male rats at PN20 and adult rats in nAChR mRNA. In addition, they found a gradual decline in [³H]nicotine binding after birth. Slotkin and colleagues also reported higher [³H]cytisine binding in the cerebral cortex of PN45 rats compared to PN60 or PN75 rats (Trauth et al., 1999). This group also found that overall binding was higher in females than in males, and that overall binding decreased with age. Additionally, work from Kota and colleagues (Kota et al., 2009) has shown that male mice exposed to nicotine in early adolescence (PND 28–34) showed enhanced reward to nicotine as measured by conditioned place preference (CPP) when compared to middle adolescent (PN 35–48), late adolescent (PN49–58) and adult mice (PN 70+).

Nicotine is a psychoactive compound that may disturb the normal course of brain maturation (Richards et al., 2003). Prior work from our lab using autoradiography indicated that adolescent and adult male rats differ significantly in the density of $\alpha 4\beta 2^*$ nAChRs following chronic nicotine exposure (Doura et al., 2008). This detailed study allowed regional and nAChR subtype specificity using subtype-specific ligands [¹²⁵I] α -conotoxin MII, [¹²⁵I]-5-iodo-3-(2(S)-azetidylmethoxy)pyridine, and [¹²⁵I] α -bungarotoxin. In that study, substantially reduced up-regulation of $\alpha 4\beta 2^*$ nAChRs in the cerebral cortex of adolescents was observed compared to adults. Specifically, adolescents exhibited significant up-regulation in only 4 out of 10 cortical regions, compared to the up-regulation seen in all 10 of the same regions in adult males. These data suggest that nAChRs may vary in their response to nicotine based on age, and that adolescent males may have reduced receptor sensitivity to the effects of nicotine compared to adult males. The data presented in this study do not fully recapitulate the findings reported by Doura and colleagues (Doura et al., 2008), albeit the variations in these data may be explained by the differences in methodology employed by the two studies. In addition, our study examined the cortex as a whole using an immunological approach to define subtype specificity with particular emphasis on the response of $\alpha 5$ -containing nAChRs to chronic nicotine at these ages. The present data showed significant up-regulation in the overall binding to $\alpha 4\beta 2^*$ nAChRs in males during mid-adolescence (PN42) following nicotine treatment, however no differences were seen in the proportion of $\alpha 5$ -containing nAChRs at this age. We did see a significant increase in $\alpha 4$ - and $\beta 2$ -containing nAChRs at that age in male rats; however using a two-way ANOVA analysis we were able to demonstrate a significant difference in the response to chronic nicotine of both $\alpha 4$ - and $\beta 2$ -containing nAChRs between male and female rats at PN42. Similarly, female $\alpha 4\beta 2^*$ nAChRs showed pronounced up-regulation at PN42. We also observed that saline treated females express a significantly lower density of $\alpha 4\beta 2^*$ nAChRs compared to saline treated males at every age (see Supplemental Table 1 for summary). These findings indicate that the sex difference in $\alpha 4\beta 2^*$ nAChR densities in saline treated animals is consistent throughout all three developmental stages and therefore unlikely to account for the sexual dimorphism of nicotine response observed at PN28 and PN42.

Very little other work has investigated the receptor response to chronic nicotine in female rats. Trauth and colleagues used [³H]cytisine to measure the response of membranous $\alpha 4\beta 2^*$ nAChRs in the midbrain, cerebral cortex and hippocampus to chronic nicotine in PN45 and, after cessation of treatment, in adult PN75 male and female rats (Trauth et al., 1999). They saw no significant difference between males and females in the overall binding of these receptors in adolescence with continuous treatment. Our data agree with what was observed in this paper with continuous treatment in both male and female rats in mid-adolescence. However, the study by Trauth et al., did not investigate the effect of nicotine on early adolescence in either males or females.

There has been virtually no work done on the effect of nicotine on nAChRs at PN28. This may be due to the technical difficulty of treating animals so soon after weaning. We were able to confirm, prior to starting these experiments that 7 days of treatment with nicotine was sufficient to induce nAChR up-regulation in adult male rats equivalent to that seen after the standard 14 day treatment (S1). Therefore, we were able to look at the effect of nicotine at the beginning of adolescence (PN28) in male and female rats. Unlike the results from PN84 and PN42 animals, we observed a clear sex difference to nicotine exposure in PN28 rats. Using a two-way ANOVA we found a significant interaction between sex and treatment in the $\alpha 4$ -containing nAChRs of PN28 animals. Although no interaction was seen in the $\beta 2$ -containing nAChRs of PN28 rats, there was still a significant treatment and sex effect for this subtype. As with adult and late adolescent rats, there was no significant change in the density of $\alpha 5$ -containing nAChRs following nicotine treatment in PN28 male or female rats. This suggests that, in early adolescence, males display a heightened sensitivity to the receptor effects of nicotine that is absent in females of the same age. These data are in agreement with the CPP observations made by Kota and colleagues (Kota et al., 2009), and may provide a pharmacodynamics explanation for the enhanced rewarding effect observed in males in early adolescence.

4.1 Conclusions

These data emphasize the necessity of looking at both male and female subjects when investigating the effects of nicotine during development. Further work in this area should focus on a more detailed mechanism of action for receptor regulation that involves possible hormonal influences. These data also suggest that nicotine self administration behavior may differ between male and female rats during adolescence, which may, in part, be due to the differential effect of nicotine on nAChRs at these ages. In humans, this may help clarify some of the mechanisms of addiction, and may help explain the behavioral differences observed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

EB	Epibatidine
mPFC	medial prefrontal cortex
NRS	normal rabbit serum
PN	postnatal day
s.c	sub-cutaneous

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Highlights

- Male rats at PN28, $\alpha 4\beta 2^*$ nAChRs in the cortex up-regulate after chronic nicotine
- No up-regulation of $\alpha 4\beta 2^*$ nAChRs in female cortex at PN28 after chronic nicotine
- Sex differences in nicotine-induced $\alpha 4\beta 2^*$ nAChR were observed at PN42
- No differences were observed in $\alpha 5$ -containing nAChRs at any age or in either sex
- Both age and sex play a role in $\alpha 4\beta 2^*$ nAChR regulation in the cortex of rats

Figure 1. Male Immunoprecipitation and Specific Binding of nAChRs (Double Column)
 Immunoprecipitation of $[^3\text{H}]\text{EB}$ -labeled nAChRs using subunit specific antibodies in the cerebral cortex of male rats at PN28 (a), PN42 (b), and PN84 (c) following chronic treatment with either nicotine or saline control. Specific binding of $[^3\text{H}]\text{EB}$ in the cerebral cortex of male rats at (d) PN28, (e) PN42, and (f) PN84 after chronic treatment with either nicotine or saline control. N=6–12, statistical significance determined with ANOVA, *p<0.05, **p<0.01, ***p<0.001.

Figure 2. Female Immunoprecipitation and Specific Binding of nAChRs (Single Column)
Immunoprecipitation of [3 H]EB-labeled nAChRs using subunit specific antibodies in the cerebral cortex of female rats at PN28 (a), PN42 (b), and PN84 (c) following chronic treatment with either nicotine or saline control. Specific binding of [3 H]EB in the cerebral cortex of female rats at (d) PN28, (e) PN42, and (f) PN84 after chronic treatment with either nicotine or saline control. N=6–11, statistical significance determined with ANOVA, *p<0.05, **p<0.01, ***p<0.001.

Table 1
Three-way ANOVA of Immunoprecipitation Data (1.5 Column)

Three-way ANOVA of all immunoprecipitation data. The interactions of the independent variables 'Sex', 'Age', 'Treatment' and 'Subunit' are examined and reported here. The F and *p* values for each analysis are reported, and statistical significance was determined as $p < 0.05$ and designated by *.

Three-Way ANOVA		
Source	F	Significance
Sex	18.726	$p < 0.000^*$
Age	12.648	$p < 0.000^*$
Treatment	88.235	$p < 0.000^*$
Subunit	418.399	$p < 0.000^*$
Sex $\times$ Age	6.630	$p < 0.000^*$
Sex $\times$ Treatment	0.772	$p < 0.000^*$
Sex $\times$ Subunit	3.375	$p < 0.036^*$
Age $\times$ Treatment	6.103	$p < 0.003^*$
Age $\times$ Subunit	2.901	$p < 0.023^*$
Treatment $\times$ Subunit	22.109	$p < 0.000^*$
Sex $\times$ Age $\times$ Treatment	11.662	$p < 0.000^*$
Sex $\times$ Age $\times$ Subunit	1.405	$p = 0.233$
Sex $\times$ Treatment $\times$ Subunit	1.174	$p = 0.311$
Age $\times$ Treatment $\times$ Subunit	1.068	$p = 0.373$

Dependent variable is amount of [^3H]epibatidine-bound nicotinic acetylcholine receptor

Table 2
Two-way ANOVA of Immunoprecipitation Data (1.5 Column)

Two-way ANOVA of results shown in Figures 1 and 2. The effect of ‘treatment’, ‘sex’ and ‘treatment × sex’ interactions for each test (F and *p* values) are reported. Results were further analyzed by Tukey’s post-hoc test to identify differences between group means. Statistical results from post-hoc analysis are shown as asterisks in Figures 1 and 2.

Two-Way ANOVA		
Test	Effect	Significance
PN84 α4 Male vs. Female	treatment sex treatment × sex	F=21.81, <i>p</i> <0.0001* F=3.370, <i>p</i> =0.0763 F=0.0007295, <i>p</i> = 9.786
PN84 β2 Male vs. Female	treatment sex treatment × sex	F=19.77, <i>p</i> =0.0001* F=1.879, <i>p</i> =0.1810 F=1.386, <i>p</i> =0.2486
PN42 α4 Male vs. Female	treatment sex treatment × sex	F=36.94, <i>p</i> <0.0001* F=0.0006630, <i>p</i> =0.9797 F=22.52, <i>p</i> <0.0001*
PN42 β2 Male vs. Female	treatment sex treatment × sex	F=74.33, <i>p</i> <0.0001* F=1.395, <i>p</i> =0.2507 F=27.56, <i>p</i> <0.0001*
PN28 α4 Male vs. Female	treatment sex treatment × sex	F=5.573, <i>p</i> =0.0271 F=14.91, <i>p</i> =0.0008* F=8.079, <i>p</i> =0.0092*
PN28 β2 Male vs. Female	treatment sex treatment × sex	F=7.635, <i>p</i> =0.0111* F=23.65, <i>p</i> <0.0001* F=3.055, <i>p</i> <0.0938

Dependent variable is amount of [³H]epibatidine-bound nicotinic acetylcholine receptor