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Mimicking Maternal Smoking and Pharmacotherapy of Preterm Labor: Fetal Nicotine Exposure Enhances the Effect of Late Gestational Dexamethasone Treatment on Noradrenergic Circuits

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Abstract

Smoking during pregnancy increases the risk of preterm delivery, which in turn necessitates the common use of glucocorticoids to prevent respiratory distress syndrome. Accordingly, there is a substantial population exposed conjointly to fetal nicotine and glucocorticoids (typically dexamethasone). We administered nicotine to pregnant rats throughout gestation, using a regimen (3 mg/kg/day by osmotic minipump) that maintains plasma nicotine levels within the range seen in smokers; on gestational days 17, 18 and 19, we gave 0.2 mg/kg of dexamethasone. We assessed norepinephrine levels in three brain regions (frontal/parietal cortex, brainstem, cerebellum) throughout adolescence, young adulthood and later adulthood, and contrasted the persistent effects with comparable measures in peripheral tissues (heart, liver). In adolescence, males showed initial deficits in the frontal/parietal cortex with either dexamethasone alone or the combined treatment, with resolution to normal by young adulthood; the group exposed to both nicotine + dexamethasone showed subsequent elevations that emerged in full adulthood and persisted through five months of age, an effect not seen with either agent separately. In females, the combined exposure produced an initial deficit that resolved by young adulthood, without any late-emerging changes. We did not see comparable effects in the other brain regions or peripheral tissues. This indicates that nicotine exposure sensitizes the developing brain to the adverse effects of dexamethasone treatment, producing sex-selective changes in innervation and/or activity of specific noradrenergic circuits. The fact that the combined treatment produced greater effects points to potentially worsened neurobehavioral outcomes after pharmacotherapy of preterm labor in the offspring of smokers.

Keywords

Brain development; Dexamethasone; Glucocorticoids; Nicotine; Norepinephrine; Preterm delivery

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INTRODUCTION

Preterm delivery is a leading cause of perinatal morbidity and mortality, and maternal smoking during pregnancy represents the single, most preventable factor that increases the risk [60,62]. In order to avoid neonatal respiratory distress syndrome, glucocorticoids (typically dexamethasone or betamethasone) are given as a consensus treatment for preterm labor occurring between 24 and 34 weeks of gestation [13]. Accordingly, there is a large population that is exposed prenatally to both nicotine and dexamethasone, two agents that, by themselves, are known to be neurodevelopmental disruptors. Nicotine evokes a change in the trajectory of cellular and synaptic development in the brain, leading to the later emergence of neurobehavioral deficits [10,11,14,34,47,48,51,52]. Similarly, fetal glucocorticoid treatment has an adverse impact on the immature brain, ultimately leading to impaired cognitive performance and increased risk of affective disorders later in life [1–3,5,7–9,23,25,28,32,33,36,38,39,56,59,61].

Numerous studies have been conducted with animal models of either fetal nicotine or dexamethasone administration separately but little attention has been paid to conjoint exposure. In two recent papers, we used established rat models to show that fetal nicotine exposure sensitizes the developing brain to the adverse impact of late gestational dexamethasone on cholinergic, dopaminergic and serotonergic synaptic development and activity [53,54]. The effects were both additive and synergistic, and showed emergence of persistent changes between adolescence, young adulthood and full adulthood. In the current study, we complete our survey of the effects of nicotine and dexamethasone by examining the impact on noradrenergic systems. We performed longitudinal comparisons of the effects of nicotine alone, dexamethasone alone, or the combined exposure, in three brain regions containing major noradrenergic cell bodies and projections, and contrasted the effects with those on peripheral sympathetic noradrenergic neurons. The treatment paradigms were chosen to match nicotine exposures associated with maternal smoking or transdermal nicotine patches [22,29,58], and dexamethasone as used in preterm delivery [53,54]. Nicotine was given throughout gestation via implanted osmotic minipumps at a dose of 3 mg/kg/day; we specifically chose a lower dose than in our previous work simulating heavy smoking [47,48], so as produce submaximal changes in order to leave room for interactions with dexamethasone. Dexamethasone was then given on gestational days (GD) 17–19, a developmental stage in the rat that corresponds to the period in which glucocorticoid therapy is recommended in preterm labor, using a dose (0.2 mg/kg) in the low therapeutic range [13]; the three-day regimen corresponds to multiple glucocorticoid courses, as used in approximately 85% of all cases [9]. Again, the dose was chosen to produce submaximal effects to allow for interactions with nicotine [17,19,27,49,50].

MATERIALS AND METHODS

Animal treatments

All experiments were carried out humanely and with regard for alleviation of suffering, with protocols approved by the Institutional Animal Care and Use Committee and in accordance with all federal and state guidelines. Timed-pregnant Sprague-Dawley rats were shipped on GD2 by climate-controlled truck (total transit time < 1 h), housed individually and allowed free access to food and water. There were four treatment groups, each comprising 11–13 dams: controls (vehicle infusion + saline injections), nicotine treatment alone (nicotine infusion + saline injections), dexamethasone treatment alone (vehicle infusion + dexamethasone injections), and those receiving the combined treatment (nicotine infusion + dexamethasone injections). On GD4, before implantation of the embryo in the uterine wall, each animal was quickly anesthetized with ether, a small area on the back was shaved, and an incision made to permit s.c. insertion of a Model 2002 Alzet minipump. The pumps

contained a total volume of 247 μ l with a delivery rate of 0.48 μ l/h, thus providing a treatment duration of just over 21 days, terminating on postnatal day (PN) 4. In earlier work, we confirmed the termination of nicotine delivery coinciding with the calculated values [58]. Pumps were filled with nicotine bitartrate dissolved in bacteriostatic water so as to deliver 3 mg/kg/day of nicotine free base, determined by the initial body weights of the dams; because weights increased with gestation, the dose rate fell accordingly to 2.2 mg/kg/day, but the dose rates remained well within the range that produces nicotine plasma levels similar to those in moderate smokers [12,16,21,22,30,43–45,47]. The incision was closed with wound clips and the animals were permitted to recover in their home cages. Control animals were implanted with minipumps containing only the water and an equivalent concentration of sodium bitartrate. On GD17, 18 and 19, dams received subcutaneous injections of either saline vehicle or 0.2 mg/kg dexamethasone sodium phosphate, at the lower range recommended for therapeutic use in preterm labor [13]. Parturition occurred during GD22, which was also taken as PN0. After birth, pups were randomized within treatment groups and litter sizes were culled to 10 (5 males and 5 females) to ensure standard nutrition. Pups were weighed and litters re-randomized every few days (corresponding to the intervals for mandatory changes of bedding) so as to distribute differential effects of maternal caretaking equally among all litters, making sure that all the pups in a given litter were from the same treatment group to avoid the possibility that the dams might distinguish among pups with different treatments; cross-fostering, by itself, has no impact on neurochemical or behavioral effects of these treatments [37]. Animals were weaned on PN21.

On PN30, 60, 100 and 150, animals were decapitated and tissues were flash-frozen in liquid nitrogen. From the brain, we chose the frontal/parietal cortex, brainstem and cerebellum; other regions were reserved for future studies. In addition, on PN150, we also dissected the heart and one liver lobe. Tissues were stored at -45°C until assayed. For each treatment group, 12–24 animals were examined at each age point, equally divided into males and females, with each final litter assignment contributing no more than one male and one female to any of the treatment groups.

Assays and data analysis

Tissues were thawed on ice and deproteinized by homogenization in 0.1 N perchloric acid containing 3,4-dihydroxybenzylamine as an internal standard. Homogenates were sedimented at $26,000 \times g$ for 10 minutes, the supernatant solutions were decanted, and norepinephrine was then trace-enriched by alumina adsorption, separated by reverse-phase high performance liquid chromatography and quantitated by electrochemical detection [40]; values were corrected for recovery of the internal standard.

Data were compiled as means and standard errors. Statistical evaluations were carried out using an initial multivariate ANOVA incorporating all variables in a single test (treatment, age, brain region, sex) and lower-order tests were then carried out as permitted by interactions of treatment with the other variables. Individual groups that differed from control were identified with Fisher's Protected Least Significant Difference Test. For all tests, significance was assumed at the level of $p < 0.05$. The design of the studies required two different ways of analysis regarding treatment variables. To characterize the effects of nicotine alone, dexamethasone alone, or the combined treatment versus controls, the four treatment groups were first considered as a one-dimensional factor in the statistical design. To determine whether the effects of nicotine and development were interactive, the treatment factors were changed to a two-dimensional design. In this formulation, synergistic or less-than-additive effects appear as significant interactions between the two treatment dimensions, whereas simple, additive effects do not show significant interactions.

Materials

Animals were purchased from Charles River Laboratories (Raleigh, NC) and osmotic minipumps from Durect Corp. (Cupertino, CA). Bacteriostatic water was obtained from Abbott Laboratories (N. Chicago, IL) and all other chemicals came from Sigma Chemical Co. (St. Louis, MO).

RESULTS

By itself, prenatal nicotine exposure did not evoke significant changes of norepinephrine levels in the frontal/parietal cortex of either males (Figure 1A) or females (Figure 1B). In contrast, late gestational dexamethasone treatment led to reduced levels in early adolescence (PN30) in males, with subsequent recovery to normal by young adulthood (PN60), and no further changes as the animals made the transition to full adulthood (PN100, PN150). In males, the combined exposure to nicotine + dexamethasone produced the same initial deficits as seen with dexamethasone alone (Figure 1A); however, in this case, elevations of norepinephrine emerged by PN100, persisting through PN150. The effect was synergistic, that is, statistically distinguishable from simple additive effects of the two individual treatments (nicotine $\times$ dexamethasone interaction, $p < 0.05$). In females, the combined treatment elicited a significant reduction in norepinephrine in early adolescence, an effect not seen with either treatment alone; but in this case, the net reduction was not distinguishable from additive (albeit nonsignificant) effects of the two individual treatments (no interaction of nicotine $\times$ dexamethasone). Unlike the situation in males, females did not show the later emergence of elevated norepinephrine.

In contrast to the frontal/parietal cortex, none of the treatments elicited statistically significant changes of norepinephrine levels in the brainstem (Figure 2). The lack of effect of on PN30 was statistically distinguishable from the significant reductions seen in the frontal/parietal cortex (treatment $\times$ region interaction: $p < 0.02$ for males, $p < 0.05$ for females). On PN150, we noted the same trend toward elevated norepinephrine levels resulting from the combined treatment in males and likewise compared the nonsignificant elevation in the brainstem to the significant increase in the forebrain; in this case, we were unable to distinguish between the effects in the two regions (main effect of treatment, $p < 0.04$; no significant treatment $\times$ region interact

In contrast to the persistent effect on norepinephrine in the frontal/parietal cortex of males, we did not find any significant differences in the cerebellum, nor were there any effects on norepinephrine in the heart or liver (Table 1).

We previously reported the effects of these treatments on maternal and litter characteristics, somatic growth and brain region weights on animals from the same cohort used in these determinations [53]. There were no treatment-related differences in the proportion of dams giving birth, litter size or sex ratio. Although birth weights were reduced significantly in all of the treatment groups, values recovered to within a few percentage points of normal values shortly after weaning in the nicotine group and by adolescence in the dexamethasone and combined treatment groups. At the time of sampling, brain region weights showed no significant deficits with any of the treatments.

DISCUSSION

In earlier studies, we showed that combined prenatal exposure to nicotine and dexamethasone elicited additive, synergistic and late-emerging effects on cholinergic, dopaminergic and serotonergic systems, involving specific regions and circuits [53,54]. The current results show that this extends similarly to noradrenergic systems. Accordingly,

prenatal nicotine exposure evokes widespread sensitization of the brain to the adverse effects of late gestational dexamethasone treatment, involving multiple regions, neurotransmitters and synaptic pathways. Importantly, in the current study, we also compared the effects on the brain to noradrenergic projections in the periphery, where we did not find any late-emerging changes. This emphasizes that the interaction of nicotine and dexamethasone involves specific effects on brain development and not simply global injury to developing noradrenergic neurons everywhere.

The dose of nicotine used here (3 mg/kg/day) was lower than in most of our previous work [31,44], specifically to produce submaximal effects so as to enable detection of interactions with dexamethasone, but the characteristics of the implanted pumps were also slightly different, producing nicotine exposure lasting through PN4, versus GD21 in the earlier experiments. The effects of nicotine alone were fairly minimal, reflecting both the lower dose and the more extended period of exposure. As described previously [53], the dual neurotoxic/neurotrophic actions of nicotine result in nonmonotonic dose effects on neuronal development [4,10,47,48]; in the current study, exposure continued into the neonatal period, a stage in which cholinergic-related trophic actions are particularly active [15,20], likely offsetting some of the direct, neurotoxic effects of gestational nicotine exposure. Nevertheless, nicotine clearly had an effect on the sensitivity of the developing brain to late gestational dexamethasone treatment. By itself, dexamethasone had only transient effects on frontal/parietal cortex norepinephrine levels, reducing the values in adolescence, with a return to normal by young adulthood. In contrast, the animals who had coexposure to nicotine showed a subsequent elevation that emerged between young adulthood and full adulthood, persisting through five months of age. The nicotine-dexamethasone interaction represented a synergistic effect, that is, the elevated norepinephrine in the group with the combined treatment was greater than could be accounted for by simple summation of the individual effects of nicotine and dexamethasone. Thus, nicotine sensitizes the developing brain to the adverse effect of dexamethasone, producing an outcome that could not otherwise have been anticipated.

There were two notable features of the nicotine-dexamethasone interaction. First, the effect was limited to males, and second, the long-term effect emerged after a period of apparent normality, during the transition from young adulthood (PN60) to full adulthood (PN100, PN150). Both these patterns were seen with studies of the impact of nicotine and dexamethasone, alone or in combination, on other neurotransmitter systems, brain regions and circuits [18,19,51–53]. The greater vulnerability of the male is not unexpected, reflecting protection of females against neurotoxicants by tonic estrogen receptor activation [26,46,55,57]. Further, the late-emergent effects are particularly notable because they emphasize the need for prolonged longitudinal studies after early-life exposure to toxicants; many studies terminate in young adulthood, before these later effects would emerge. As seen here, the consequences of nicotine and dexamethasone converge on the developmental *trajectory* of noradrenergic function, rather than eliciting an initial injury that simply continues or intensifies with age. In keeping with that interpretation, the effects were not shared equally by all noradrenergic projections, as would be expected from global injury to this particular type of neuron. Indeed, the fact that the alterations arise after adolescence points to the potential to design and administer therapies that could interdict the later emergence of neurobehavioral abnormalities.

The present results conclude a series of studies delineating the effects of combined exposure to nicotine and dexamethasone, encompassing four different neurotransmitter systems (acetylcholine, dopamine, serotonin, and here, norepinephrine), in a wide variety of brain regions and circuits. Although there are some notable differences in the impact on the diverse pathways, there is a consistent overall pattern: combined exposure produces a worsened

outcome, through either additive, or as seen here, synergistic effects and furthermore, neural circuits show global increases in indices of presynaptic activity, regardless of transmitter type. Given the increased incidence of preterm delivery in women who smoke during pregnancy [24,35,42,60,62], and the consensus use of glucocorticoids in preterm infants [13], our results are relevant to a substantial human population, especially in light of our use of treatment paradigms that closely mimic both the timing and plasma levels of corresponding human fetal exposures to nicotine and dexamethasone [13,34,47,48]. The findings reported here and in our earlier work [53,54] point to the likelihood of greater long-term liabilities in this group. Importantly, the offspring of women who smoke may have a *lower* risk of respiratory distress syndrome relative to gestational age [6] because nicotine accelerates type II lung cell differentiation [41]. Accordingly, the risk-benefit evaluations for glucocorticoid treatment might need to be reconsidered in this subgroup. Finally, our studies provide a proof-of-principle that early-life drug or chemical exposures that represent large proportions of the human population can enhance the subsequent vulnerability to later exposures to otherwise unrelated drugs or chemicals. Although much attention has been paid to underlying genetic differences that delineate subpopulations that are vulnerable to toxicants, our findings point to the need to explore equally the epigenetic changes that are wrought by early-life exposures and that likewise engender heightened vulnerability later in life.

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Abbreviations

ANOVA	analysis of variance
GD	gestational day
PN	postnatal day

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Highlights

- Prenatal nicotine exposure sensitizes the developing brain to adverse effects of dexamethasone.
- The effects involve noradrenergic circuits but also other neurotransmitters
- These may contribute to adverse outcomes for preterm babies of smokers.

Figure 1.

Norepinephrine concentrations in frontal/parietal cortex of control animals (Con) and animals exposed prenatally to nicotine (Nic), dexamethasone (Dex), or both (Nic+Dex): (A) Males, (B) females. Data represent means and standard errors obtained from 6–12 animals in each group at each age. Multivariate ANOVA indicates a significant interaction of treatment $\times$ age $\times$ sex ($p < 0.02$); after subdivision by sex, results of two-factor ANOVA (treatment, age) are shown at the top of each panel; asterisks denote individual values that differ significantly from the corresponding control.

Figure 2.

Norepinephrine concentrations in brainstem of control animals (Con) and animals exposed prenatally to nicotine (Nic), dexamethasone (Dex), or both (Nic+Dex): (A) Males, (B) females. Data represent means and standard errors obtained from 6–12 animals in each group at each age. Two-factor ANOVA (treatment, age), shown at the top of each panel, failed to find significant differences (NS, not significant), so lower-order tests for individual groups were not carried out. The absence of significant effects on PN30 was statistically distinguishable from the reductions seen in the frontal/parietal cortex (treatment $\times$ region, $p < 0.02$); however, on PN150, the nonsignificant increase in the male brainstem for the Nic +Dex group was not distinguishable from the significant increase seen in the frontal/parietal cortex ($p < 0.04$ for the main treatment effect but no significant treatment $\times$ region interaction).

TABLE 1

Norepinephrine concentrations (ng/g) in cerebellum, heart and liver

Tissue	Age	Control		Nicotine		Dexamethasone		Nicotine + Dexamethasone	
		male	female	male	female	male	female	male	female
Cerebellum	PN100	187±9	210±17	193±14	202±10	191±13	212±11	186±9	208±6
	PN150	206±9	235±14	215±12	225±11	214±9	212±12	217±12	223±9
Heart	PN150	1956±195	2785±299	1807±160	2457±312	1982±194	2457±249	1868±183	2633±266
	PN150	134±15	231±20	126±11	213±24	140±10	222±17	128±11	191±17

Data represent mean ± SE obtained from 6–12 animals in each group. ANOVA indicates no significant treatment effects or interaction of treatment with other variables.