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Research report

Mimicking maternal smoking and pharmacotherapy of preterm labor: Interactions of fetal nicotine and dexamethasone on serotonin and dopamine synaptic function in adolescence and adulthood☆

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A B S T R A C T

Fetal coexposure to nicotine and dexamethasone is common: maternal smoking increases the incidence of preterm delivery and glucocorticoids are the consensus treatment for prematurity. We gave pregnant rats 3 mg/kg/day of nicotine throughout gestation, a regimen that reproduces smokers' plasma levels, and then on gestational days 17, 18 and 19, we administered 0.2 mg/kg of dexamethasone. We evaluated developmental indices for serotonin (5HT) and dopamine synaptic function throughout adolescence, young adulthood and later adulthood, assessing the brain regions possessing major 5HT and dopamine projections and cell bodies. Males displayed persistent upregulation of $5HT_{1A}$ and $5HT_2$ receptors and the 5HT transporter, with a distinct hierarchy of effects: nicotine < dexamethasone < combined treatment. Females showed downregulation of the $5HT_{1A}$ receptor with the same rank order; both sexes displayed presynaptic hyperactivity of 5HT and dopamine pathways as evidenced by increased neurotransmitter turnover. Superimposed on these overall effects, there were significant differences in temporal and regional relationships among the different treatments, often involving effects that emerged later in life, after a period of apparent normality. This indicates that nicotine and dexamethasone do not simply produce an initial neuronal injury that persists throughout the lifespan but rather, they alter the developmental trajectory of synaptic function. The fact that the combined treatment produced greater effects for many parameters points to potentially worse neurobehavioral outcomes after pharmacotherapy of preterm labor in the offspring of smokers.

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1. Introduction

There is an incontrovertible connection between maternal smoking during pregnancy and the risk of preterm delivery [74,76], and at the same time, glucocorticoid administration is the consensus treatment for preterm infants in order to prevent neonatal respiratory distress syndrome [18]. Both these factors are likely contributors to the increased incidence of neurodevelopmental disorders. Fetal exposure to nicotine, over and above the morbidities associated with prematurity, evokes a change in the trajectory of cellular and synaptic development in the brain, leading to the later emergence of neurobehavioral deficits [14,15,19,41,53,54,58,59]. By the same token, it is increasingly clear that glucocorticoid treatment similarly has an adverse impact on the immature brain, ultimately leading to impaired cognitive performance and increased risk of affective disorders later in life [4,6,7,9,11–13,28,31,34,38,40,43,46,47,69,73,75].

Animal studies show that the outcomes from developmental exposure to nicotine or dexamethasone, the glucocorticoid most commonly used in preterm infants [18], converge on similar neural circuits, transmitter systems and behavioral deficits [22,23,54,56,58,61,77]. This suggested to us that exposure to both agents, simulating the use of glucocorticoids in preterm infants born to smokers, might heighten the liability as compared to either exposure by itself. In an earlier study, we demonstrated

Abbreviations: 5HIAA, 5-hydroxyindoleacetic acid; 5HT, 5-hydroxytryptamine, serotonin; $5HT_{1A}R$, $5HT_{1A}$ receptor; $5HT_2R$, $5HT_2$ receptor; 5HTT, 5HT transporter; ANOVA, analysis of variance; DA, dopamine; DOPAC, dihydroxyphenylacetic acid; GD, gestational day; HVA, homovanillic acid; PN, postnatal day.

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just such an interaction for cholinergic systems after exposure of fetal rats to nicotine and dexamethasone [60]. In the present work, we extended these studies to include serotonin (5HT) systems, the likely target underlying the affective and emotional disorders noted for each agent. As in our earlier report, we chose exposure paradigms specifically to mimic human exposures. Nicotine was given throughout gestation via osmotic minipump implants, at a dose (3 mg/kg/day) that produces nicotine plasma levels similar to those in moderate smokers [27,35,71]; we specifically chose a lower dose than in previous work simulating heavy smoking [53,54], 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 [18]; the three-day regimen corresponds to multiple glucocorticoid courses, as used in approximately 85% of all cases [13]. Again, the dose was chosen to produce submaximal effects to allow for interactions with nicotine [22,23,32,55,56].

We then performed longitudinal evaluations of the effects on 5HT systems spanning development from the immediate postnatal period, through adolescence, young adulthood and full adulthood. We evaluated multiple indices of 5HT synaptic function in all the brain regions comprising the major 5HT projections (frontal/parietal cortex, temporal/occipital cortex, hippocampus, striatum) as well as those containing 5HT cell bodies (midbrain, brainstem). We measured three 5HT synaptic proteins known to be highly affected by developmental exposure to both nicotine and dexamethasone [56,58,61,77], the $5HT_{1A}$ and $5HT_2$ receptors ($5HT_{1A}R$, $5HT_2R$), and the presynaptic 5HT transporter (5HTT). The two receptors play major roles in 5HT-related mental disorders, particularly depression [5,17,78,79], and the transporter, which regulates the synaptic concentration of 5HT, is the primary target for antidepressant drugs [29,37,39]. As an index of presynaptic neuronal activity, we assessed the concentration of 5HT as well as 5HT turnover. Finally, we compared effects on 5HT to those on dopamine (DA), a monoamine that has similar properties in terms of transmitter biosynthesis, storage and release, but that subserves different functions and whose cell bodies and projections have different regional distributions from those of 5HT.

2. Materials and methods

2.1. 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 μl with a delivery rate of 0.48 μl/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 [71]. 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 [16,21,26,27,36,50–53]. 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 [18]. 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 [45]. Animals were weaned on PN21.

On PN30, 60, 100 and 150, animals were decapitated and the brain was dissected into frontal/parietal cortex, temporal/occipital cortex, hippocampus, striatum and midbrain; other regions and tissues were reserved for future studies. We used an additional set of animals on PN2, examining the entire forebrain (combined frontal/parietal cortex, temporal/occipital cortex, hippocampus and striatum) and the midbrain + brainstem. Tissues were frozen in liquid nitrogen and stored at −45 °C until assayed. For each treatment group, at least 12 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.

2.2. 5HT receptors and transporter

All of the ligand binding methodologies used in this study have appeared in previous papers [3,57,62,63], so only brief descriptions will be provided here. Tissues were thawed and homogenized (Polytron, Brinkmann Instruments, Westbury, NY) in ice-cold 50 mM Tris (pH 7.4), and the homogenates were sedimented at 40,000 × *g* for 15 min. The pellets were washed by resuspension (Polytron) in homogenization buffer followed by resedimentation, and were then dispersed with a homogenizer (smooth glass fitted with Teflon pestle) in the same buffer. An aliquot was assayed for measurement of membrane protein [66].

Two radioligands were used to determine 5HTR binding: 1 nM [^{3}H]8-hydroxy-2-(di-n-propylamino)tetralin for the $5HT_{1A}R$, and 0.4 nM [^{3}H]ketanserin for the $5HT_2R$. Binding to the presynaptic 5HTT site was evaluated with 85 pM [^{3}H]paroxetine. For the $5HT_{1A}R$ and 5HTT sites, specific binding was displaced by addition of 100 μM 5HT; for the $5HT_2R$, we used 10 μM methylsergide for displacement.

2.3. Neurotransmitter concentration and turnover

Tissues were thawed and homogenized in ice-cold 0.1 M perchloric acid and sedimented for 20 min at 40,000 × *g*. The supernatant solution was collected and aliquots were used for analysis of 5HT, 5-hydroxyindoleacetic acid (5HIAA), DA, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) by high-performance liquid chromatography with electrochemical detection [65,77]. Concurrently-run standards, containing each of the neurotransmitters and metabolites were used to calculate the regional concentration of each neurochemical. Transmitter turnover was calculated as the ratio of metabolites to transmitter, i.e. 5HIAA/5HT, and either DOPAC/DA or (DOPAC + HVA)/DA; since HVA contributed significantly to total metabolites in only one region (striatum), the ratio was calculated as DOPAC/DA in the other regions.

2.4. Data analysis

Data were compiled as means and standard errors. Because we evaluated multiple neurochemical measures that were all related to 5HT synapses, the initial comparisons were conducted by a global ANOVA (data log-transformed because of heterogeneous variance among regions and measures) incorporating all the variables and measurements so as to avoid an increased probability of type 1 errors that might otherwise result from multiple tests of the same data set. The variables in the global test were infusion treatment (control, nicotine), injection treatment (saline, dexamethasone), brain region, age and sex, with multiple dependent measures ($5HT_{1A}R$, $5HT_2R$ and 5HTT binding for the receptor and transporter determinations; neurotransmitter levels and turnover for the HPLC determinations); in both cases, the dependent measures were treated as repeated measures, since multiple determinations were derived from the same sample. Where we identified interactions of treatment with the other variables, data were then subdivided for lower-order ANOVAs to evaluate treatments that differed from the corresponding control. As permitted by the interaction terms, individual groups that differed from control were identified with Fisher's Protected Least Significant Difference Test. Significance was assumed at the level of $p < 0.05$. However, for interactions at $p < 0.1$, we also examined whether lower-order main effects were detectable after subdivision of the interactive variables [67]. The criterion for interaction terms was not used to assign significance to the effects but rather to identify interactive variables requiring subdivision for lower-order tests of main effects of the drug treatments, the variables of chief interest. Where treatment effects were not interactive with other variables, we report only the main treatment effects without performing lower-order analyses of individual values.

To enable ready visualization of treatment effects across different regions, ages and measures, the results are given as the percent change from control values, but statistical procedures were always conducted on the original data. The control values

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