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Gestational Dexamethasone Treatment Elicits Sex-Dependent Alterations in Locomotor Activity, Reward-Based Memory and Hippocampal Cholinergic Function in Adolescent and Adult Rats

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Glucocorticoids are the consensus treatment for preventing respiratory distress syndrome in preterm infants but there is emerging evidence of subsequent neurobehavioral abnormalities, independent of somatic growth effects. Pregnant rats were given 0.2 mg/kg of dexamethasone, a dose commensurate with clinical use, on gestational days 17–19 and behavioral evaluations were made on the offspring in adolescence and adulthood. The dexamethasone groups had the same body weights as the controls but nevertheless displayed long-term, sex-selective alterations in locomotor and cognitive behaviors. In the figure-8 activity apparatus, dexamethasone treatment ablated the normal sex differences in locomotor activity by reducing values in females to the lower level typical of males; habituation of activity similarly was impaired in females, reducing the profile to match that of control males, while male rats in the dexamethasone group showed a partially feminized pattern of habituation. In the 8-arm radial maze, control rats displayed typical sex differences, with male rats performing more accurately than females. Dexamethasone treatment eliminated this normal dichotomy, delaying learning in males while improving performance in females to the level normally seen in control males. Finally, we assessed hippocampal [3H]hemicholinium-3 binding as a biomarker for cholinergic synaptic activity, and again found loss of sex differences in the dexamethasone group: values in males were increased to the higher levels typical of females. These results indicate that gestational treatment with dexamethasone obtunds the normal sex differences in neurochemistry and behavior that are typically seen in adolescence in adulthood, thus producing sex-selective alterations in activity, learning, and memory.
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INTRODUCTION

Since the release of the NIH Consensus Report over a decade ago (Gilstrap *et al*, 1994), synthetic glucocorticoids have been routinely administered to prevent respiratory distress in preterm infants, currently utilized in 10% of all US pregnancies (Matthews *et al*, 2002). Although the original recommendation was for a single course of glucocorticoid treatment, a recent study showed that 85% of clinical use actually involves repeated courses (Dammann

and Matthews, 2001) and specific recommendations for prolonged or repeated use are beginning to emerge (Crowther and Harding, 2003), with some treatments over a period of several weeks (Crowther and Harding, 2003; Dammann and Matthews, 2001; Jobe, 2000). High doses of glucocorticoids, sufficient to elicit clear-cut growth retardation, are commonly given in refractory cases, despite an emerging suspicion that there might be long-term adverse consequences (Bakker *et al*, 2001; Finer *et al*, 2000; Jobe, 2000). Indeed, recent epidemiologic studies indicate an elevated risk of metabolic disorders, cardiovascular disease, and cognitive deficits after glucocorticoid use in preterm delivery (Barrington, 2001; Seckl, 2001; Shinwell *et al*, 2000; Trautman *et al*, 1995; Yeh *et al*, 2004).

Given the confounds in outcome assessments after preterm delivery, animal models are essential to establish both cause-and-effect relationships for the adverse effects of glucocorticoids and to evaluate whether neurobehavioral

deficits occur at clinically relevant doses and particularly with exposures that do not produce lasting somatic growth impairment. Unfortunately, the vast majority of studies on developmental effects of glucocorticoids concentrate on regimens and doses that lie well above those used in preterm labor, resulting in frank somatic effects and endocrine disruption (Bohn, 1984; Fuxe et al, 1994, 1996; Gilad et al, 1998; Gould et al, 1997; Maccari et al, 2003; Matthews, 2000; Matthews et al, 2002; McEwen, 1992; Meaney et al, 1996; Weinstock, 2001; Welberg and Seckl, 2001). Accordingly, studies have reported both increases and decreases in locomotor and other general activities, likely a reflection of different dosing paradigms with varying degrees of persistent somatic growth impairment (Benesová and Pavlík, 1989; Ferguson and Holson, 1999; Flagel et al, 2002; Neal et al, 2004). Nevertheless, neonatal rats exposed to dexamethasone also display impaired spatial learning (Kamphuis et al, 2003) as well as alterations in social behaviors (Kamphuis et al, 2004) and complex motor skills (Benesová and Pavlík, 1989) at doses simulating those used clinically.

In the current study, we explored the consequences of gestational dexamethasone treatment in rats, utilizing a dose (0.2 mg/kg) well within the clinically used range. Despite the absence of somatic growth impairment, we found sex-selective alterations in activity and cognitive performance in adolescence and adulthood. In addition, we conducted preliminary investigations of the synaptic mechanisms underlying the behavioral defects, focusing on the role of cholinergic innervation of the hippocampus in learning and memory, a likely target for developmental exposure to glucocorticoids (Takahashi and Goh, 1998; Zahalka et al, 1993b). We measured binding of [^{3}H]hemicholinium-3 (HC3) to the high-affinity presynaptic choline transporter, the protein that is rate-limiting in acetylcholine biosynthesis and whose expression changes in direct response to persistent increases or decreases in nerve impulse activity (Klemm and Kuhar, 1979; Simon et al, 1976); these assessments were modeled after the use of HC3 binding to characterize changes in cholinergic synaptic function elicited by developmental neurotoxicants (Aubert et al, 1996; Happe and Murrin, 1992; Navarro et al, 1989; Qiao et al, 2003; Slotkin et al, 1990; Zahalka et al, 1992, 1993a).

METHODS

Animal Treatments

All studies were performed in accordance with the Declaration of Helsinki and with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. Timed-pregnant Sprague–Dawley rats (Charles River Laboratories, Raleigh, NC) were housed individually and given free access to food and water. On gestational days 17, 18, and 19, dams were administered 0.2 mg/kg, subcutaneous (s.c.), of dexamethasone phosphate (Sigma Chemical Co., St Louis, MO), while controls received equivalent volumes of isotonic saline (1 ml/kg). At birth, pups were randomized and redistributed to the nursing dams within their respective treatment groups with litter sizes maintained at 10 pups. To distribute any maternal caretaking differences equally among all pups, randomization within treatment groups was carried out every 4 days until weaning on postnatal day (PN) 21, at which point the day–night cycle was reversed (lights on at 1800 h).

All behavioral testing was carried out during the dark phase, the most active period, but in a lighted environment so that the rats could access the visual cues necessary to perform the tasks. Tests were performed on 8–9 rats per sex per treatment group, utilizing no more than one male and one female from each litter. Behavioral assessments were begun with the figure-8 apparatus on PN31, 32, and 33, followed by the radial arm maze beginning on PN45 and continuing through PN68. This sequence was designed to permit multiple behavioral tests to be conducted without significant carry-over of effects from one test to the next (Icenogle et al, 2004; Levin et al, 2001, 2002; Slotkin et al, 1999). Tests were scored by a trained observer who was blinded to the animal treatments.

Figure-8 Activity

The apparatus, in the shape of a figure-8, consisted of a continuous, enclosed 10 × 10 cm alley, 70 cm long and 42 cm wide, with a 21 × 16 cm central arena, a 20 cm-high ceiling and two blind alleys extending 20 cm from either side. Data for locomotor activity were acquired with eight photo-beams, one located on each of the two blind alleys and three on each of the two loops of the Figure-8, crossing the alleys. Three 1-h sessions were evaluated on PN31, 32, and 33, with each session divided into twelve 5-min blocks. Overall activity was determined as the average number of beam breaks per block and habituation was evaluated as the linear trend for the decline in activity over the sequential blocks within each session (Crofton et al, 1991; Keppel, 1982; Levin et al, 2001, 2002).

Eight-Arm Radial Maze

Over the period of training and testing, animals had free access to water but food was restricted to approximately 15 g/day so as to provide an adequate stimulus to seek the food reward obtained in the maze. The maze, elevated 30 cm from the floor, consisted of a central platform 50 cm in diameter, with eight 10 × 60 cm arms projecting radially, each with a food cup 2 cm from the distal end. Tests were conducted in a quiet room with multiple extra-maze visual cues that always remained in the same locations relative to the maze. The rats were first familiarized with the food reinforcement by placing each rat in an opaque, plastic cylinder in the middle of the maze with a sugar-coated cereal (Froot Loops® halves), giving them up to 5 min to eat all the pieces. Subsequently, the rats were tested for 12 sessions conducted from PN45 to PN68. In each session, the arms were baited and the rat placed in the opaque cylinder on the central platform for 10 s. The session started when the cylinder was removed, and the rat was allowed free access to the arms. Recordings were made of arm choices, a single choice being recorded once the rat placed all of its paws past the threshold of the arm. Errors were defined as subsequent entries into a previously entered arm and presented as 'entries to repeat,' the number of arms entered

prior to reentry into an arm in which the bait had already been consumed (Icenogle *et al*, 2004; Levin *et al*, 2001, 2002). Each session lasted until the rat entered all eight arms at least once, or for a maximum of 5 min; in order for a session to be counted, the rat had to have made a reentry, finished the maze perfectly or entered at least five arms. The response latency (time per entry) was calculated by dividing the total time of the session by the number of arms entered. Data for both entries to repeat and latency were compiled as results averaged from sequential pairs of sessions to obviate variability within individual sessions (Icenogle *et al*, 2004; Levin *et al*, 2001, 2002).

HC3 Binding

Littermates (six animals per sex for each treatment with no more than one male and one female used from each litter) of the animals used for behavioral assessments were decapitated on PN75 and the hippocampus was rapidly dissected, frozen with liquid nitrogen and maintained at -45°C until assayed. Tissues were thawed in 79 volumes of ice-cold 10 mM sodium–potassium phosphate buffer (pH 7.4), homogenized with a Polytron (Brinkmann Instruments, Westbury, NY), and sedimented at 40 000*g* for 15 min. The membrane pellet was resuspended (Polytron) in the original volume of buffer, resedimented, and the resultant pellet was resuspended using a smooth glass homogenizer fitted with a Teflon pestle, in 10 mM sodium–potassium phosphate buffer (pH 7.4) and 150 mM NaCl. Radioligand binding was evaluated (Vickroy *et al*, 1984) with 2 nM [³H]HC3 (specific activity 161 Ci/mmol; PerkinElmer Life Sciences, Boston, MA), with incubation for 20 min at room temperature, followed by rapid vacuum filtration onto glass fiber filters (presoaked for 30 min with 0.1% polyethyleneimine in buffer). The nonspecific component was defined as radioligand binding in the presence of 10 μM unlabeled HC3 (Sigma). Binding values were expressed relative to membrane protein (Smith *et al*, 1985). The selection of a single concentration of radioligand for the binding analysis enables the detection of changes in either binding affinity or capacity but does not permit distinction between effects on the two parameters. This strategy was necessitated by limitations in the amount of tissue available from each animal. However, the interpretation of results of the present study, which relate to HC3 binding as an index of neural activity, is valid for changes in either parameter (Aubert *et al*, 1996; Happe and Murrin, 1992; Navarro *et al*, 1989; Qiao *et al*, 2003; Slotkin *et al*, 1990; Zahalka *et al*, 1992, 1993a).

Data Analysis

Data are presented as means and standard errors. Treatment-related differences were evaluated with analysis of variance (ANOVA) incorporating all factors in an initial, global test: treatment, sex, and where appropriate to each behavior, session and block. Lower-order ANOVAs were then carried out where appropriate, as dictated by the interaction of treatment with the other variables. Significance for main effects was assumed at $p < 0.05$; however, for interactions at $p < 0.1$, we also examined whether lower-

order main effects were detectable after subdivision of the interactive variables (Snedecor and Cochran, 1967).

RESULTS

Dexamethasone had no significant effect on maternal weight gain during pregnancy, the proportion of dams giving birth, litter size, and viability, or the proportion of males and females in each litter (data not shown). On PN30, at the commencement of behavioral testing, there were no differences in body weights between the control and dexamethasone-treated groups. Control males weighed 104 ± 3 g, compared to 106 ± 3 g in the dexamethasone group; values in females were 98 ± 1 and 95 ± 3, respectively.

As found in earlier studies (Levin *et al*, 2001, 2002; Marmendal *et al*, 2004; Moraes *et al*, 2004), control rats showed marked sex differences in activity (Figure 1a) and habituation (Figure 1b) in the figure-8 apparatus. Males
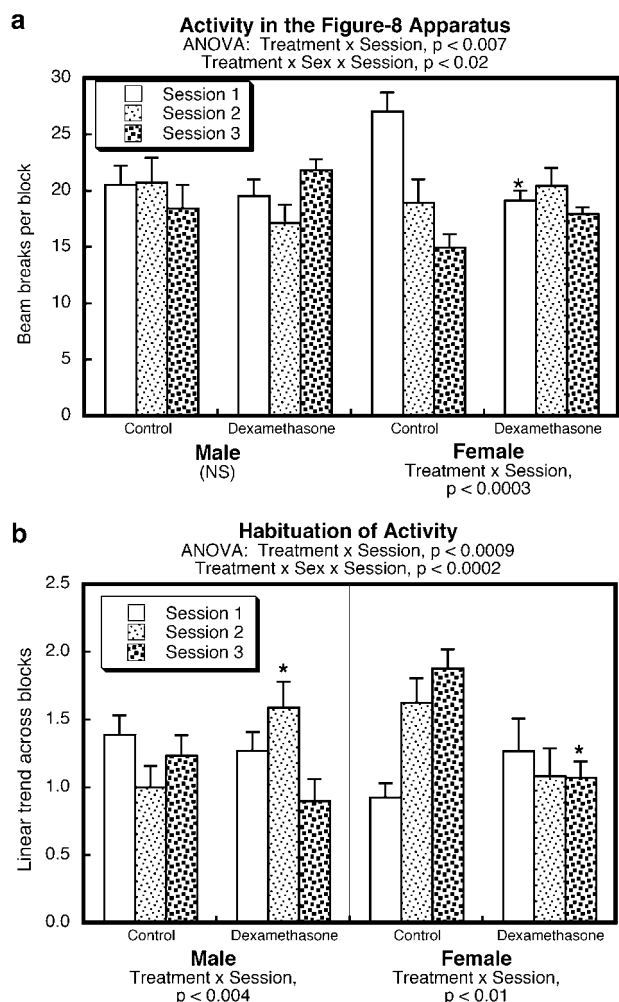
Figure 1 Effects of dexamethasone on activity (a) and habituation (b) in the figure-8 apparatus. ANOVA across treatments, sexes, and sessions appears at the top of each panel. As e result of the interaction of treatment with sex, data were separated for males and females, and lower-order ANOVAs were then conducted as shown at the bottom of each panel. Asterisks denote the individual values for which the dexamethasone groups differ from the corresponding controls. NS = not significant.

showed no change in activity over the three sessions but females showed much higher initial activity and a progressive decline over the subsequent sessions ($p < 0.004$ for the main effect of sex in the control group). A similar sex difference was seen for habituation: control males showed no significant change over sessions whereas females exhibited a marked increase in the rapidity of habituation ($p < 0.009$). Prenatal dexamethasone treatment completely ablated the sex difference in activity (no significant difference between males and females in the dexamethasone group) by masculinizing the pattern in females. Dexamethasone had similar effects on habituation, converting the pattern in the treated females to match that ordinarily seen in control males, that is, no differences in habituation over successive test sessions. In the males, dexamethasone produced partial feminization, with an increase in activity

during the second session ($p < 0.0002$ for the main effect of session in the dexamethasone group).

In the eight-arm radial maze, control males performed better than females, making fewer re-entries and thus exhibiting a higher value for entries to repeat (Figure 2a; $p < 0.0001$ for the main effect of sex). Again, this replicated established, normal sex differences in behavior (Levin et al, 2001, 2002). Both sexes showed significant improvement over the 12 training sessions ($p < 0.008$ for the main effect of session in males, $p < 0.05$ in females). Dexamethasone treatment had opposing effects on performance in the two sexes, slowing the learning in males, while masculinizing performance in females by evoking an overall increase in entries to repeat. Accordingly, unlike the controls, there was no significant sex difference in the animals treated with dexamethasone. The sex-selective effects on learning did not reflect underlying changes in locomotor activity in the maze, as shown by measurements of latency (Figure 2b). In controls, males showed higher overall activity than females as evidenced by lower latency ($p < 0.004$ for the main effect of sex) and this difference was still fully evident in the dexamethasone-treated group ($p < 0.0001$). In males, dexamethasone elicited a significant overall reduction in latency whereas females tended toward an increase.

Sex differences were also apparent for HC3 binding to membranes prepared from the hippocampus on PN75 (Figure 3). In controls, females displayed significantly higher values than males ($p < 0.0007$). Dexamethasone treatment increased HC3 binding in males to achieve levels indistinguishable from those in females without eliciting a corresponding change in females. Accordingly, after prenatal dexamethasone treatment, there was no longer any sex difference.

DISCUSSION

The results of the current study indicate that dexamethasone, in a dose commensurate with its use in preterm labor,
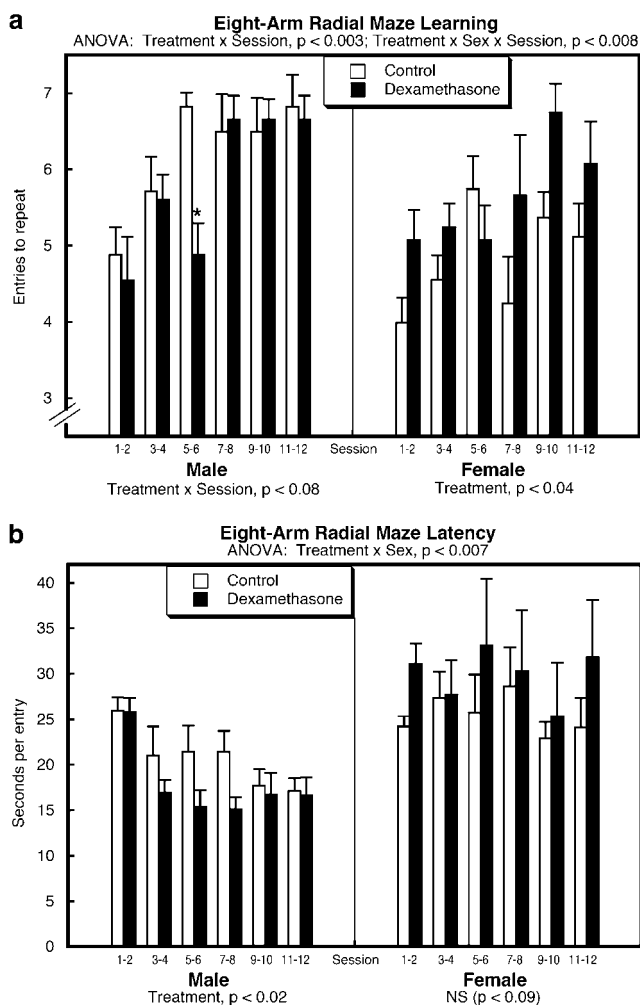

Figure 2 Effects of dexamethasone on entries to repeat (a) and latency (b) in the eight-arm radial maze. ANOVA across treatments, sexes, and sessions appears at the top of each panel. As a result of the interaction of treatment with sex, data were separated for males and females and lower-order ANOVAs were then conducted as shown at the bottom of each panel. Asterisk denotes the individual male value for entries to repeat, for which the dexamethasone group differs from the corresponding controls; for females, only the main treatment effect was evaluated because of the absence of a treatment × session interaction. Similarly, only main treatment effects appear for latency. NS = not significant.
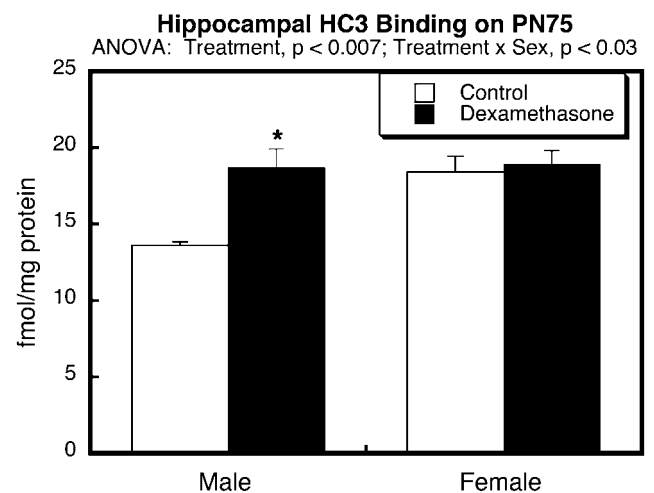

Figure 3 Effects of dexamethasone on HC3 binding in membrane preparations from the hippocampus. ANOVA across treatments and sexes appears at the top of the panel and, because of the interaction of treatment × sex, significance was analyzed separated for males and females, with the asterisk denoting the significant difference in males.

and in the absence of persistent somatic growth inhibition, elicits long-term behavioral alterations in conjunction with changes in hippocampal synaptic function. Sex selectivity is by far the most prominent feature of these effects, with distinctly different outcomes for males and females. Thus, in the Figure-8 apparatus, locomotor activity and habituation were reduced in females but not males and in fact, the latter showed increases in habituation. In the eight-arm radial maze, only the males displayed cognitive impairment as evidenced by a slowing of learning, whereas females actually showed improved performance; similarly, males and females showed changes in opposite directions for latency. Our results thus point to the importance of evaluating sex-dependence of the long-term effects of prenatal dexamethasone exposure, rather than conglomerate assessments of both sexes or, as in many studies, evaluation of effects only in male offspring.

Earlier studies identified effects on behavior and hypothalamo–pituitary–adrenal axis function in male offspring after developmental exposure to higher doses or longer durations of glucocorticoid treatment, regimens that elicit persistent growth retardation (Dean *et al*, 2001; Holson *et al*, 1995; Machhor *et al*, 2004). Similarly, prolonged prenatal stress and the associated release of endogenous maternal glucocorticoids are known to feminize behavioral responses in male rats (Holson *et al*, 1995; Ward, 1972). Our results, however, indicate that effects involve sexes and occur at a lower threshold than for persistent growth deficits. There are two important ramifications of these findings. First, the presence or absence of somatic effects is not an adequate predictor of whether a given regimen of antenatal glucocorticoids will spare brain development or behavior. More importantly, though, we found partial masculinization of behavioral performance in females, and feminization of performance in males, so that by and large, the normal sex differences disappeared in the dexamethasone-exposed animals. Interestingly, the same interference with expression of sexually dimorphic behaviors was recently reported for prenatal stress, which also elevates maternal glucocorticoid levels (Bowman *et al*, 2004). The elimination of sex differences thus points to an underlying mechanism, namely interference with sexual differentiation of the brain, which reaches a peak stage during the period of dexamethasone exposure used here (MacLusky and Naftolin, 1981; Mac-Lusky *et al*, 1985; McCarthy, 1994), rather than interference targeted singly to androgen or estrogen-related factors, although certainly the differential expression of estrogen receptors in sexually dimorphic areas such as the hippocampus is likely to play a significant role (Kawata *et al*, 1998). In keeping with this interpretation, prenatal stress or glucocorticoid administration, albeit with growth-retarding regimens, has been found to eliminate sex differences in aromatase activity and in brain morphology (Reznikov *et al*, 1999, 2004). Accordingly, although much less attention has been paid to outcomes in female offspring, the present results indicate that they are equally likely to show long-term anomalies, albeit with a different, and even opposite, spectrum of effects from those seen in males. Indeed, some of the effects of antenatal glucocorticoids may then resemble those of environmental estrogens, which are known to alter cognitive performance and motor activity

in females (Golub *et al*, 2004; Masuo *et al*, 2004). The pattern seen here, negation of sex differences, mimics that seen with chlorpyrifos, an organophosphorus insecticide completely unrelated to dexamethasone, but that similarly interferes with sexual differentiation of the brain (Levin *et al*, 2001, 2002; Slotkin, 2005).

Finally, we determined whether prenatal dexamethasone exposure similarly negates sex differences in neurotransmitter pathways known to be involved in spatial learning, focusing on the hippocampus, in which glucocorticoid receptor expression is prominent in late gestation (Levitt *et al*, 1996; Speirs *et al*, 2004), and in which cholinergic projections play a key role (Levin *et al*, 2001, 2002). Using HC3 binding as a biomarker for cholinergic synaptic activity, we found feminization in males. However, given an underlying mechanism of interference with sexual differentiation of the brain, there is no reason to expect that this is the only brain region or neurotransmitter affected by prenatal dexamethasone treatment, or that only males are targeted. Indeed, prenatal stress eliminates sex differences in monoamine levels (Reznikov and Nosenko, 1996), so it is likely that dexamethasone treatment will have similar actions for these pathways. A detailed neurochemical survey of different brain regions and neurotransmitter systems will be forthcoming in a separate study.

There is growing concern about the long-term sequelae after dexamethasone therapy in preterm infants. Our results support the idea that the beneficial effects on birth outcomes come at a long-term cost of disrupted neurodevelopment, even at doses at or below those in clinical use, and below the threshold for persistent growth deficits. Further, the present findings point to underlying mechanisms for these effects and, equally important, the need to evaluate sex differences in outcomes.

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