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Disruption of Rat Forebrain Development by Glucocorticoids: Critical Perinatal Periods for Effects on Neural Cell Acquisition and on Cell Signaling Cascades Mediating Noradrenergic and Cholinergic Neurotransmitter/ Neurotrophic Responses

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Glucocorticoids are the consensus treatment for the prevention of respiratory distress in preterm infants, but there is evidence for increased incidence of neurodevelopmental disorders as a result of their administration. We administered dexamethasone (Dex) to developing rats at doses below or within the range of those used clinically, evaluating the effects on forebrain development with exposure in three different stages: gestational days 17–19, postnatal days 1–3, or postnatal days 7–9. At 24 h after the last dose, we evaluated biomarkers of neural cell acquisition and growth, synaptic development, neurotransmitter receptor expression, and synaptic signaling mediated by adenylyl cyclase (AC). Dex impaired the acquisition of neural cells, with a peak effect when given in the immediate postnatal period. In association with this defect, Dex also elicited biphasic effects on cholinergic presynaptic development, promoting synaptic maturation at a dose (0.05 mg/kg) well below those used therapeutically, whereas the effect was diminished or lost when doses were increased to 0.2 or 0.8 mg/kg. Dex given postnatally also disrupted the expression of adrenergic receptors known to participate in neurotrophic modeling of the developing brain and evoked massive induction of AC activity. As a consequence, disparate receptor inputs all produced cyclic AMP overproduction, a likely contributor to disrupted patterns of cell replication, differentiation, and apoptosis. Superimposed on the heterologous AC induction, Dex impaired specific receptor-mediated cholinergic and adrenergic signals. These results indicate that, during a critical developmental period, Dex administration leads to widespread interference with forebrain development, likely contributing to eventual, adverse neurobehavioral outcomes.
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INTRODUCTION

For over a decade, synthetic glucocorticoids like dexamethasone (Dex) have been the consensus treatment for the prevention of neonatal respiratory distress in preterm birth (Gilstrap *et al*, 1994). Such therapy has saved thousands of lives and enabled survival of very preterm infants, and antenatal glucocorticoid use currently encompasses nearly 10% of all US pregnancies (Matthews *et al*, 2002). Accordingly, *hundreds* of thousands of infants receive glucocorticoid treatment whose mothers do not end up delivering prematurely or who might not have developed respiratory distress. Even within the target population, the use of multiple glucocorticoid courses has become a common practice (Dammann and Matthews, 2001), and may even become a recommended treatment (Crowther and Harding, 2003), despite the fact that the original recommendation was for a single course (Gilstrap *et al*, 1994). It is increasingly suspected that excessive glucocorticoid treatment leads to life-long abnormalities of metabolic, cardiovascular, and behavioral function (Barrington, 2001; Seckl, 2001; Shinwell *et al*, 2000; Trautman *et al*, 1995; Yeh *et al*, 2004). However, epidemiologic studies are confounded by the comorbidities and multiple pharmacologic interven-

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tions inherent to the population born prematurely or undergoing therapies for preterm labor. In this regard, animal models become especially important to establish a cause-and-effect relationship between early glucocorticoid treatment and subsequent anomalies.

The adverse effects of glucocorticoids on brain development and behavioral outcomes have been well characterized but to date, most studies have focused on treatments that lie well above the dose range typical of therapeutic interventions in preterm infants, often sufficient to produce persistent stunting of somatic growth and outright cerebral atrophy 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). Models incorporating stress as a mechanism for increasing circulating glucocorticoids have also proven useful in characterizing neurodevelopmental defects, especially those influencing hypothalamus–pituitary–adrenal axis function (Dean *et al*, 2001; Felszeghy *et al*, 2000; Muneoka *et al*, 1997), but of course, these involve contributions of factors beyond glucocorticoids alone. Nevertheless, several recent studies in rats suggest that glucocorticoids, in doses commensurate with their use in preterm infants, produces lasting alterations in performance, encompassing motor activity, social behaviors, learning, and memory (Benesová and Pavlík, 1989; Kamphuis *et al*, 2003, 2004; Kreider *et al*, 2005), resembling some of the changes elicited by prenatal stress (Bowman *et al*, 2004). The current study establishes a cause-and-effect relationship between perinatal Dex treatment in rats and adverse effects on brain development by exploring doses well below (0.05 mg/kg) or within the therapeutic range (0.2 or 0.8 mg/kg), encompassing three different treatment windows that correspond to human neurodevelopment in the second to early third trimester (Dobbing and Sands, 1979; Rodier, 1988), the period in which glucocorticoids are most likely to be administered (Gilstrap *et al*, 1994): gestational days (GD) 17–19, postnatal days (PN) 1–3 and PN7–9. We evaluated developmental consequences in the forebrain 24 h after the last treatment. The forebrain contains the hippocampus, neocortex, and corpus striatum, regions that are highly enriched in glucocorticoid receptors and that are putative targets for developmental disruption by Dex (Kawata *et al*, 1998; Kitraki *et al*, 1996; Meaney *et al*, 1993; Owen and Matthews, 2003). We focused on families of biomarkers that characterize different mechanisms likely to contribute to adverse outcomes. First, we assessed the impact on neural cell acquisition and growth by measurements of DNA and cell protein fractions. Each neural cell contains only a single nucleus (Winick and Noble, 1965), so that the DNA content (amount of DNA in each brain region) reflects the total number of cells, and the DNA concentration (DNA per unit tissue weight) reflects the cell packing density (Bell *et al*, 1987; Slotkin *et al*, 1984; Winick and Noble, 1965). We also assessed the complement of cell proteins related to differentiation as opposed to cell numbers. As neurons specialize, they enlarge and develop axonal and dendritic projections. The ratio of total protein/DNA thus rises with the expansion of the cell (Qiao *et al*, 2003, 2004; Slotkin *et al*, 2005). The development of neuritic projections necessitates a rise in the contribution of membrane proteins relative to other cell proteins, and accordingly, we also assessed the membrane protein concentration and the ratio of membrane proteins to total cell proteins.

The second set of biomarkers focused on the potential impact on two specific neurotransmitter systems, acetylcholine and norepinephrine. Both of these transmitters play a critical role as trophic factors in development of the forebrain (Dreyfus, 1998; Hohmann, 2003; Hohmann and Berger-Sweeney, 1998; Lauder and Schambra, 1999; Whitaker-Azmitia, 1991) and are likely targets for developmental effects of glucocorticoids (Hu *et al*, 1996; Muneoka *et al*, 1997; Reznikov *et al*, 2004; Shi *et al*, 1998; Slotkin *et al*, 1982; Zahalka *et al*, 1993b). We evaluated choline acetyltransferase (ChAT) activity and the binding of [^{3}H]hemicholinium-3 (HC3) to the high-affinity presynaptic choline transporter. ChAT is the enzyme responsible for acetylcholine biosynthesis and the choline transporter regulates the rate-limiting step, availability of intracellular choline (Klemm and Kuhar, 1979; Simon *et al*, 1976). The transporter is overexpressed in the developing brain and is regulated by glucocorticoids (Zahalka *et al*, 1993a, b). We also characterized three neurotransmitter receptor-binding sites, β-adrenoceptor (βAR), α_2-adrenoceptor (α_2AR), and m_2-muscarinic acetylcholine receptor (m_2AChR). Each receptor has been implicated in the control of neural cell acquisition and differentiation (Duncan *et al*, 1990; Garofolo *et al*, 2003; Hodges-Savola *et al*, 1996; Kreider *et al*, 2004; Lidow and Rakic, 1995; Slotkin *et al*, 1988; Zhou *et al*, 2004) and, like the HC3-binding site, the βAR and α_2AR are overexpressed in the fetal brain (Kreider *et al*, 2004; Lidow *et al*, 1991; Slotkin *et al*, 1994b; Zahalka *et al*, 1993a). Notably, glucocorticoids have a direct effect on βAR expression (Davies and Lefkowitz, 1984; Tseng *et al*, 2002). Finally, we assessed the impact on cell signaling mediated by adenylyl cyclase (AC), the pathway that regulates production of the second messenger, cyclic AMP, a universal signal regulating replication and differentiation of virtually all eucaryotic and procaryotic cells. In addition to assessing basal AC activity, we evaluated the enzymatic response mediated by the three neurotransmitter receptors, as well as the relative coupling of receptors in comparison to glucocorticoid effects on total AC activity. The latter is especially important in light of our earlier report showing that, during development, glucocorticoids can enhance total AC activity, thus increasing cyclic AMP production in response to disparate receptor inputs (Slotkin *et al*, 1994a).

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 were housed individually and given free access to food and water. For studies of gestational Dex exposure, dams received daily subcutaneous injections of Dex phosphate (0.05, 0.2, or 0.8 mg/kg) on GD17–19, whereas controls received equivalent volumes (1 ml/kg) of isotonic saline vehicle. On GD20, 24 h after the last Dex

treatment, dams were decapitated and fetuses were counted, weighed, and decapitated; the fetal forebrain was then dissected by making a cut rostral to the thalamus. This dissection, which follows the natural planes of the fetal and neonatal rat brain, includes the corpus striatum, hippocampal formation, and neocortex within the area designated as 'forebrain.' Forebrain samples from four fetuses derived from the same dam were then frozen together in liquid nitrogen and stored at −45°C. For studies of the effects of postnatal Dex treatment (0, 0.05, 0.2, or 0.8 mg/kg given on PN1–3 or PN7–9), pups were randomized at birth and redistributed to the nursing dams with litter size maintained at 10 to ensure standardized nutrition, with randomization repeated within treatment groups for each daily injection. At 24 h after the last injection, animals were selected from each litter and forebrain samples prepared combining two animals per litter on PN4 and one per litter on PN10. Group sizes consisted of six animals per treatment for determinations on GD20 (sex not determined), and six males and six females for each treatment for those on PN4 and PN10. To ensure that the treatment effects were unbiased by maternal differences, each dam or litter contributed only a single sample.

Biomarkers of Neural Cell Development

Tissues were thawed in 19 volumes of ice-cold 10 mM sodium–potassium phosphate buffer (pH 7.4) and homogenized with a Polytron (Brinkmann Instruments, Westbury, NY). DNA was assessed with a modified (Trauth *et al*, 2000) fluorescent dye-binding method (Labarca and Piagen, 1980). Aliquots were diluted in 50 mM sodium phosphate, 2 M NaCl, 2 mM EDTA (pH 7.4) and sonicated briefly (Virsonic Cell Disrupter, Virtis, Gardiner, NY). Hoechst 33258 was added to a final concentration of 1 μg/ml. Samples were then read in a spectrofluorometer using an excitation wavelength of 356 nm and an emission wavelength of 458 nm, and were quantitated using standards of purified DNA. The total concentration of tissue proteins was assayed from the original homogenate spectrophotometrically with bicinchoninic acid (Smith *et al*, 1985); in addition, we assessed the concentration of membrane proteins from the membrane preparations used for radioligand binding, as described below. For calculation of the ratio of membrane/total protein, the membrane protein value was averaged across the different membrane preparations.

Cholinergic Presynaptic Biomarkers

Aliquots of the original tissue homogenate were assayed in duplicate for ChAT using established procedures (Lau *et al*, 1988; Qiao *et al*, 2003, 2004). Each tube contained final concentrations of 60 mM sodium phosphate (pH 7.9), 200 mM NaCl, 20 mM choline chloride, 17 mM MgCl2, 1 mM EDTA, 0.2% Triton X-100, 0.12 mM physostigmine, 0.6 mg/ml bovine serum albumin, and 50 μM [^{14}C]acetylcoenzyme A. Blanks contained homogenization buffer instead of the tissue homogenate. Samples were preincubated for 15 min on ice, transferred to a 37°C water bath for 30 min, and the reaction was terminated by placing the samples on ice. Labeled acetylcholine was then extracted, counted and the activity determined relative to tissue protein (Smith *et al*, 1985). Preliminary determinations established that enzyme activity was linear with time and tissue concentration under these conditions.

For measurements of HC3 binding, an aliquot of the same tissue homogenate was sedimented at 40 000 *g* for 15 min and the supernatant solution was discarded. 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) containing 150 mM NaCl. Radioligand binding was evaluated with 2 nM [^{3}H]HC3 (Vickroy *et al*, 1984), 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 an excess concentration of unlabeled HC3 (10 μM) and binding values were expressed relative to membrane protein. Nonspecific binding averaged 35% on GD20 but rose to 65% postnatally.

Neurotransmitter Receptor Binding

Receptor binding was assessed in aliquots of the same original homogenate in sodium–potassium phosphate buffer as already described for the HC3 binding determinations. After the first sedimentation, we followed two different procedures for subsequent resuspension, washing, resedimentation, and final suspension. For βAR binding, α_2AR binding, and AC activity, the buffer consisted of 125 mM sucrose, 6 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.5), whereas for m_2AChR binding, the same sodium–phosphate buffer was used as for HC3. To evaluate βAR binding, aliquots of membrane preparation were incubated with [^{125}I]iodopindolol (final concentration 67 pM), in 145 mM NaCl, 2 mM $MgCl_2$, 1 mM Na ascorbate, 20 mM Tris (pH 7.5), for 20 min at room temperature in a total volume of 250 μl. Displacement of nonspecific binding was evaluated with 100 μM *d,l*-isoproterenol, comprising 30% of total binding on GD20 and 10% at postnatal age points. For α_2AR binding, the ligand was 2.5 nM [^{3}H]rauwolscine, which was incubated for 20 min at room temperature, with membrane preparation and final concentrations of 10 mM $MgCl_2$ and 50 mM Tris (pH. 7.5). Nonspecific binding was evaluated with 10 μM phentolamine, and constituted 30% of the total. Binding to m_2AChRs was evaluated with 1 nM [^{3}H]AFDX384, incubated for 60 min at room temperature in 10 mM sodium phosphate (pH 7.4), and nonspecific binding was evaluated with 1 μM atropine; nonspecific binding was 25% of the total on GD20, falling to 10% by PN10.

AC Activity

AC assessments were conducted by standard techniques published previously (Auman *et al*, 2000, 2001a; Zeiders *et al*, 1997, 1999a). Briefly, aliquots of the same membrane preparation used for the βAR-binding assays were incubated for 10 min at 30°C with final concentrations of 100 mM Tris-HCl (pH 7.4), 10 mM theophylline, 1 mM ATP, 2 mM $MgCl_2$, 1 mg/ml bovine serum albumin, and a creatine

phosphokinase-ATP-regenerating system consisting of 10 mM sodium phosphocreatine and 8 IU/ml phosphocreatine kinase, with 10 μM GTP in a total volume of 250 μl. The enzymatic reaction was stopped by placing the samples in a 90–100°C water bath for 5 min, followed by sedimentation at 3000 *g* for 15 min, and the supernatant solution was assayed for cyclic AMP using radioimmunoassay kits. Preliminary experiments showed that the enzymatic reaction was linear well beyond the assay period and was linear with membrane protein concentration; concentrations of cofactors were optimal and, in particular, higher concentrations of GTP produced no further augmentation of activity.

AC activity was evaluated in several different ways. First, we measured basal AC activity without addition of any stimulants. Next, we compared the responses of two direct AC stimulants, forskolin (100 μM) and Mn^{2+} (10 mM); these discriminate the effects of G_s-AC association, which selectively enhances the forskolin response (Limbird and Macmillan, 1981; Seamon and Daly, 1986), as well as allowing for detection of shifts in the AC isoform (Zeiders *et al*, 1999b). Finally, we compared the responses to activation of different neurotransmitter receptor stimulants, using 100 μM isoproterenol (βAR agonist), 100 μM clonidine (α_2AR agonist), or 100 μM carbachol (m_2AChR agonist). Since the latter two act in part on the inhibitory G-protein, G_i, their assessments were conducted in samples that were activated by addition of forskolin (Auman *et al*, 2001a, b; Garofolo *et al*, 2002; Slotkin *et al*, 1991b). These concentrations of each stimulant produce maximal responses, as assessed in earlier studies (Auman *et al*, 2000, 2001a; Zeiders *et al*, 1997, 1999a).

Data Analysis

Data are presented as means and standard errors. Differences between groups were first assessed by a global analysis of variance (ANOVA) (data log-transformed because of heterogeneous variance), incorporating all factors: drug treatment, treatment regimen, and sex (with the exception of GD17–19 Dex treatment, as already noted). This initial test was conducted across related measurements (considered as repeated measures, since they were all derived from the same homogenate) corresponding to each index class: cell development biomarkers (DNA and protein fractions), cholinergic presynaptic markers (ChAT, HC3), receptor binding (βAR, α_2AR, m_2AChR), and the multiple AC measures. Depending on the treatment interactions obtained in the global tests, data were then subdivided for lower order ANOVAs, followed where appropriate, by Fisher's Protected Least Significant Difference to establish effects comparing individual groups. Significance for main treatment 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). For convenience, some data are presented as the percentage change from control values; however, statistical evaluations were always carried out on the original data. For reference, control values are given in Table 1.

Materials

Animals were purchased from Charles River Laboratories, Raleigh, NC. [^{14}C]Acetyl-CoA (specific activity 44 mCi/mmol,

Table 1 Control Values

		PN4		PN10	
Measure	**GD20**	**Male**	**Female**	**Male**	**Female**
Body wt (g)	2.61±0.06	11.2±0.2	10.8±0.1	23.5±0.5	20.8±0.5*
Forebrain wt (mg)	74±2	256±3	244±5	608±13	591±10
DNA concentration (mg/g)	4.4±0.1	2.16±0.02	2.23±0.01*	1.50±0.01	1.51±0.02
DNA content (μg/region)	324±8	552±7	557±10	913±20	894±16
Total protein/DNA (μg/μg)	12.0±0.3	24.1±0.4	23.2±0.3	39.1±0.5	38.2±0.5
Membrane/total protein (μg/μg)	0.21±0.01	0.237±0.007	0.242±0.008	0.321±0.013	0.281±0.009*
ChAT activity (pmol/mg protein/min)	52±2	66±1	70±2	246±6	254±3
HC3 binding (fmol/mg protein)	11.5±0.7	8.7±1.3	8.6±1.8	9.2±0.7	11.0±0.8
βAR binding (fmol/mg protein)	12.2±0.8	5.8±0.3	5.2±0.5	7.0±0.8	9.2±0.5
α_2AR binding (fmol/mg protein)	213±29	74±10	53±5	48±4	50±3
m_2AChR binding (fmol/mg protein)	97±6	138±4	139±4	398±7	414±8
Adenylyl cyclase (pmol/mg protein/min)					
Basal	132±7	214±7	212±11	130±19	145±12
Isoproterenol	146±7	235±9	233±8	142±18	139±13
Mn^{2+}	1322±58	1736±143	2004±144	1382±178	1813±63*
Forskolin	590±26	872±21	940±26*	531±100	702±55
Forskolin and clonidine	588±40	707±46	893±35*	630±96	772±52
Forskolin and carbachol	575±40	784±35	849±39	499±67	680±50*

*Significant difference between males and females.

diluted with unlabeled compound to 6.7 mCi/mmol), [^{3}H]HC3 (161 Ci/mmol), [^{125}I]iodopindolol (2200 Ci/mmol), [^{3}H]rauwolscine (78 Ci/mmol), and [^{3}H]AFDX384 (133 Ci/mmol) were obtained from Perkin–Elmer Life Sciences (Boston, MA). Cyclic AMP radioimmunoassay kits were purchased from GE Healthcare (Piscataway, NJ). Sigma Chemical Co. (St Louis, MO) was the source for all other reagents.

RESULTS

In control rat forebrain, the DNA concentration fell progressively from GD20 through PN10 whereas the DNA content rose (Table 1), commensurate with the normal ontogenetic decrease in cell packing density and the acquisition of new cells accompanying the brain growth spurt (Dobbing and Sands, 1979; Qiao *et al*, 2003; Rodier, 1988). Cell enlargement and neuritic extension also were demonstrable in the rise in protein/DNA and membrane/total protein ratios and similar increments were seen in ChAT activity and m_2AChR binding. In contrast, βAR, α_2AR, and HC3-binding sites exhibited fetal overexpression, with higher values on GD20 as compared to PN4, in keeping with earlier results (Kreider *et al*, 2004; Lidow *et al*, 1991; Slotkin *et al*, 1994b; Zahalka *et al*, 1993a). Isoproterenol evoked a small (10%) but statistically significant increase in AC activity ($p<0.002$), as would be expected from the relatively minor proportion of total enzyme activity that is linked to βARs. Accordingly, the response to direct activation of all AC molecules was far more substantial: a 10-fold increase for Mn^{2+} ($p<0.0001$) and a 4–5-fold increase for forskolin ($p<0.0001$). There was no net response to the α_2AR agonist, clonidine, on GD20 but significant inhibition (15% decrease, $p<0.01$) was seen on PN4 and stimulation on PN10 (20% increase, $p<0.007$). Carbachol also evoked significant AC inhibition (10% decrease, $p<0.002$) at PN4 but had little or no effect at other ages. There were only sporadic sex differences in any of the parameters.

In order to avoid type I statistical errors that might occur from repeated testing of the data sets, we first performed global ANOVAs on data groupings for each cluster of developmental characteristics: weights, cell development biomarkers, cholinergic presynaptic biomarkers, neurotransmitter receptor binding, and AC activity (Table 2). Each set of variables showed significant treatment differences that interacted with regimen (ie the different Dex treatment periods) and/or measure, indicating the need to separate the various measurements from each other and to examine the effects of the different Dex regimens separately. Accordingly, as described below, the data were subdivided by the two interactive variables and lower-order effects of treatment and treatment × sex interactions were re-examined. Where there was no treatment × sex interaction, sex was retained as a factor in the lower-order statistical analysis but the results for males and females were combined for presentation.

Dex Treatment on GD17–19

Maternal body weights at the end of Dex treatment on GD20 were not significantly different from control values: 340 ± 16 g in controls, 332 ± 13 g in the Dex 0.05 mg/kg group, 337 ± 9 g in the Dex 0.2 mg/kg group, and 314 ± 9 g in the Dex 0.8 mg/kg group. However, considering the weight gain during the period of Dex administration, even the lowest dose of Dex impaired maternal weight gain over the course from GD17 to GD20 (Figure 1a). Since the prepregnancy weights of the dams averaged 220 g, only the group receiving the highest Dex dose actually showed a reduced weight gain of greater than 10% over the course of gestation. Despite these effects on the dam, there was no effect on fetal viability or on the number of fetuses (Figure 1a). Similarly, fetal body weights were unaffected on GD20 and even the highest Dex dose elicited only a small reduction in forebrain weight (Figure 1b); the same pattern was seen when whole brain weight was considered (data not shown). Across all the cell development biomarkers, Dex treatment at 0.05 or 0.2 mg/kg had no net effect but at 0.8 mg/kg, there were significant overall deficits ($p<0.02$). The overall reduction clearly represented the contributions of impaired DNA content (Figure 2a) and membrane/total protein (Figure 2b).

Table 2 Global ANOVA

	Body and forebrain weight	Cell development biomarkers[a]	Cholinergic presynaptic biomarkers[b]	Neurotransmitter receptor binding[c]	AC activity[d]
Treatment	$p<0.0001$	$p<0.0001$	$p<0.002$	NS	$p<0.0001$
Treatment × regimen	$p<0.0001$	$p<0.03$	$p<0.006$	NS	$p<0.04$
Treatment × sex	NS	NS	NS	$p<0.0002$	NS
Treatment × measure	$p<0.0001$	$p<0.0001$	$p<0.0002$	$p<0.005$	$p<0.0008$
Treatment × regimen × measure	$p<0.04$	$p<0.0009$	$p<0.008$	NS	$p<0.08$
Treatment × sex × measure	NS	NS	NS	$p<0.0001$	$p<0.02$
Treatment × regimen × sex × measure	$p<0.07$	NS	NS	NS	NS

NS, not significant.
[a]DNA concentration, DNA content, Total protein/DNA, membrane/total protein.
[b]ChAT activity, HC3 binding.
[c]βAR, α_2AR, m_2AChR binding.
[d]Basal, isoproterenol, Mn^{2+}, forskolin, forskolin and clonidine, forskolin and carbachol.

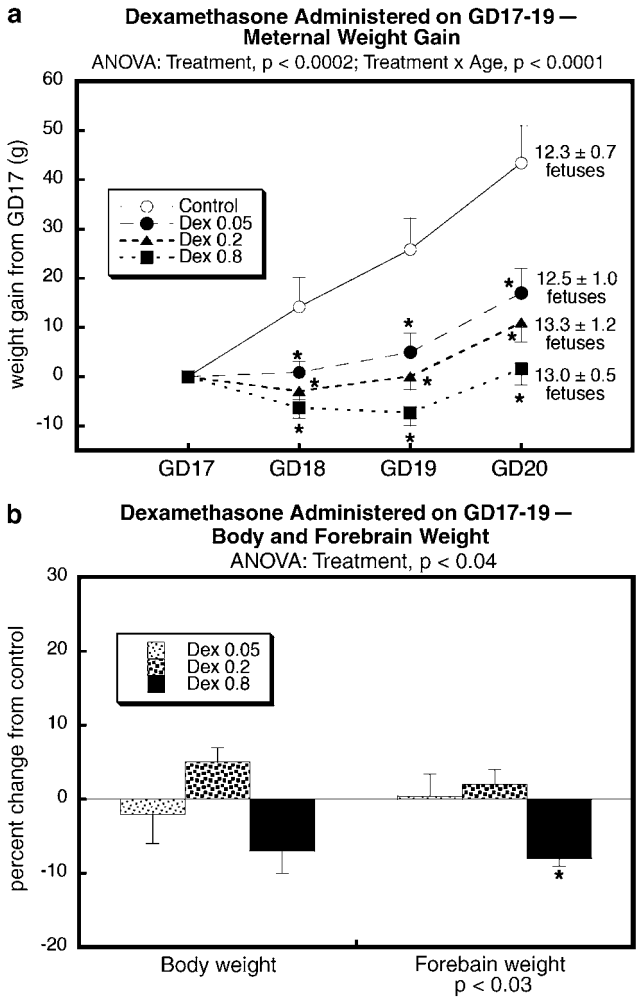

Figure 1 Effects of GD17–19 Dex treatment on maternal weight gain and litter characteristics (a) and on body and forebrain weights in the fetuses on GD20 (b). For maternal weight gain, ANOVA across all doses and time points appears at the top of the panel; for fetal weights, ANOVA across all doses and both measures appears at the top and ANOVA for each measure appears at the bottom. Asterisks denote individual values that differ significantly from the corresponding control. There were no significant differences in maternal weights prior to the first Dex injection on GD17 (313±6 g across all groups).

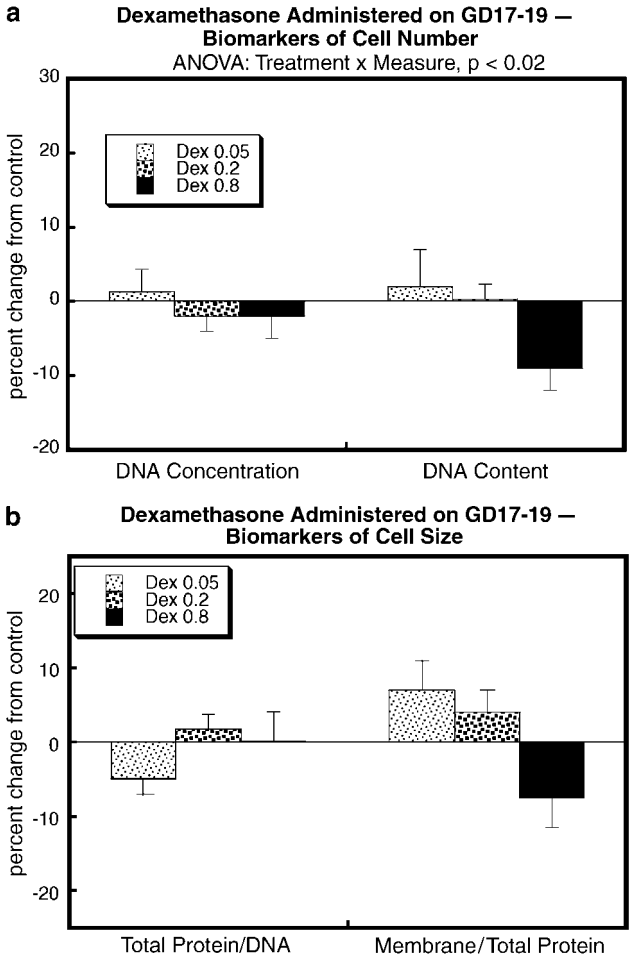

Figure 2 Effects of GD17–19 Dex treatment on biomarkers of neural cell development, evaluated on GD20: (a) DNA concentration and content and (b) protein/DNA ratio and membrane/total protein ratio. ANOVA across all doses and measures appears at the top of each panel. In addition, across all four measures, 0.8 mg/kg of Dex elicited a significant reduction ($p<0.02$) but values for each individual measure did not achieve significance.

Although there was little effect on biomarkers of presynaptic cholinergic development (Figure 3a), prenatal Dex administration elicited alterations in neurotransmitter receptor binding that differed among the various receptors ($p<0.07$ for the interaction of treatment × receptor type), with α_2ARs showing the largest effect, achieving statistically significant deficits at 0.8 mg/kg Dex (Figure 3b). Although the intermediate dose of Dex (0.2 mg/kg) produced a consistent increment in all measures of AC activity (Figure 3c), the effect did not achieve statistical significance.

Dex Treatment on PN1-3

In contrast to the relatively small effects of prenatal Dex administration on body and forebrain weights, treatment in the early postnatal period evoked marked growth retardation that was statistically significant at every dose (Figure 4). Brain sparing was evident by the smaller reduction in forebrain weight as compared to body weight ($p<0.02$), and again, the same pattern was seen for whole brain weight (data not shown). The effects of PN1–3 Dex treatment on indices of cell number were also robust (Figure 5a). DNA concentration showed a 15% increase at the highest dose of Dex, connoting an increase in cell packing density. When superimposed on the impairment of forebrain growth, the change in DNA content, signifying the total number of cells in the forebrain, showed significant deficits at all Dex doses. Across both indices of cell size (total protein/DNA ratio, membrane protein/total protein ratio), there was a significant reduction caused by the highest Dex dose ($p<0.0001$) but significance was not achieved with either ratio alone.

Dex administration on PN1–3 had significant effects on all aspects of synaptic development and cell signaling. For cholinergic presynaptic biomarkers, there was a small elevation of ChAT activity across all doses, at the margin of statistical significance (Figure 6a); in fact, across all three treatment regimens (GD17–19, PN1–3, PN7–9), there was a significant promotional effect on ChAT ($p<0.002$) that

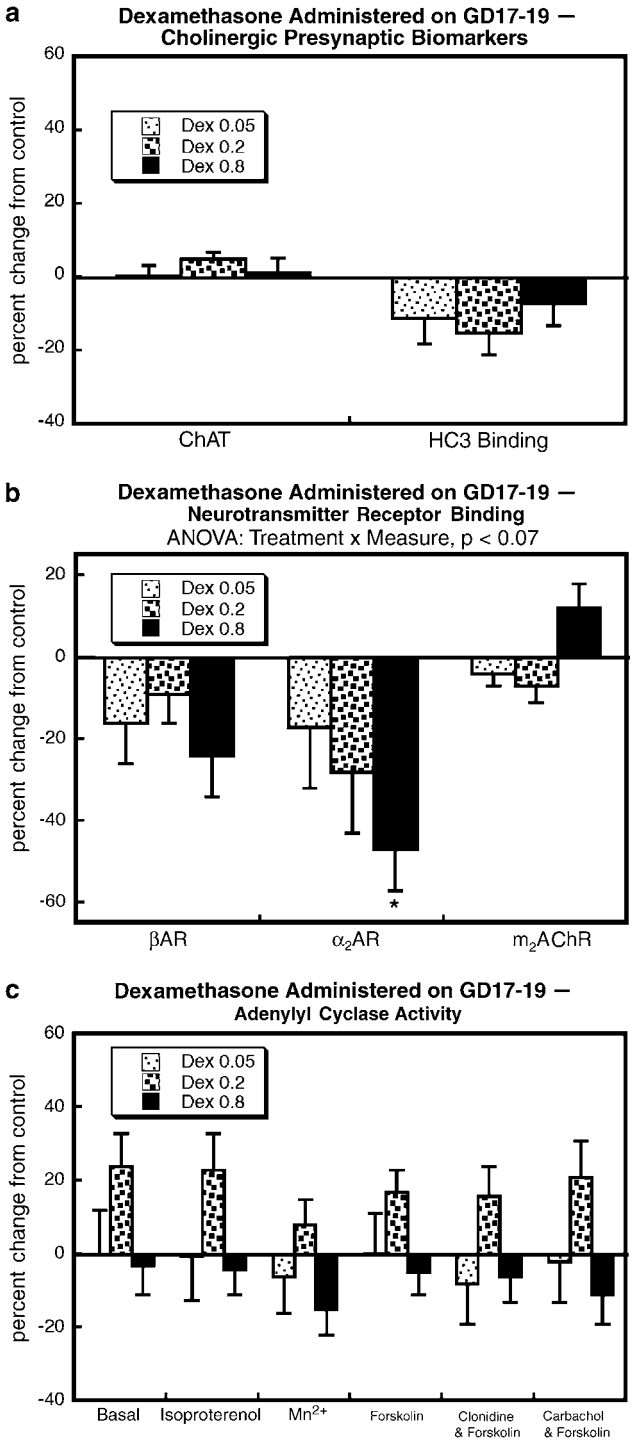

Figure 3 Effects of GD17–19 Dex treatment on biomarkers of synaptic development and responsiveness, evaluated on GD20: (a) presynaptic cholinergic indices, ChAT activity, and HC3 binding; (b) neurotransmitter receptor binding for βARs, α_2ARs, and m_2AChRs; and (c) indices of AC signaling. Receptor binding showed an interaction of Dex treatment × receptor type ($p<0.07$), necessitating separate lower-order analysis of the different receptors to identify individual group differences from control (asterisk).

achieved statistical significance at all doses but without a significant treatment × regimen interaction. Accordingly, it is correct to conclude that there is an increase in ChAT but the presence or absence of statistical significance for any

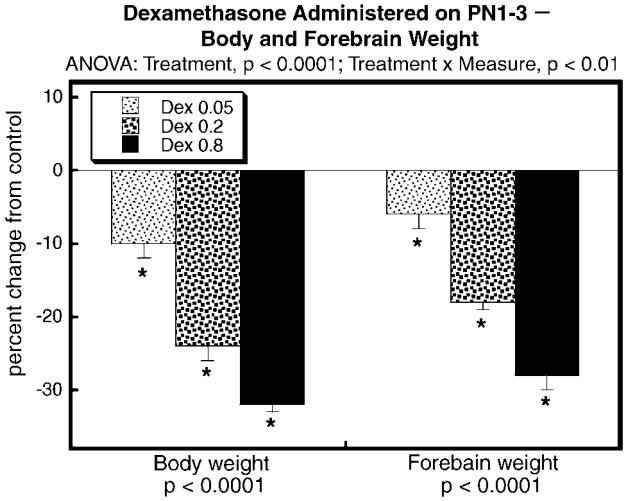

Figure 4 Effects of PN1–3 Dex treatment on body and forebrain weights on PN4. ANOVA across all doses appears at the top of the panel and ANOVA for each measure appears at the bottom; asterisks denote individual values that differ significantly from the corresponding control. Data for males and females were combined for presentation because of the absence of a treatment × sex interaction.

single regimen would need to be interpreted with caution. Unlike the small effects on ChAT, HC3 binding showed a massive increase at the lowest of Dex (Figure 6a). Accompanying the increased impairment of growth and cell acquisition at higher Dex doses, the promotional effect on HC3 binding disappeared. As with prenatal Dex treatment, administration on PN1–3 reduced α_2AR binding but the effect was sex-selective, expressed only in males (Figure 6b).

The effects on AC signaling were far more profound (Figure 6c). Dex treatment produced a dose-dependent increase across all AC measures, achieving 30–40% increases for the direct AC stimulants, Mn^{2+} and forskolin. Basal AC activity and the AC response to isoproterenol also showed increments of smaller magnitude. Owing to the significant interaction of treatment × measure ($p<0.03$), we also assessed the relative effects of the different neurotransmitter receptor stimulants in relationship to the response to direct AC stimulants. Dex elicited a significant loss of the isoproterenol response relative to the maximum AC activity achieved with Mn^{2+} ($p<0.009$) or forskolin ($p<0.01$). The ratio of isoproterenol/Mn^{2+} AC activity decreased from a control value of 0.128 ± 0.006 to 0.116 ± 0.005 in the 0.2 mg/kg Dex group, and to 0.111 ± 0.004 in the 0.8 mg/kg Dex group. Similarly, the isoproterenol/forskolin activity ratio dropped from 0.257 ± 0.008 to 0.237 ± 0.007 and 0.217 ± 0.008, respectively. Dex treatment also altered the AC response to m_2AChR stimulation. In control forebrain, addition of carbachol reduced forskolin-stimulated activity by about 10% (ratio of activity with/without carbachol, 0.89 ± 0.03). This inhibitory response was lost in the Dex groups ($p<0.02$), which showed no net effect of carbachol at 0.05 mg/kg (ratio of 0.96 ± 0.03) or 0.2 mg/kg (1.00 ± 0.02) with restoration of the response at the highest Dex dose (ratio of 0.88 ± 0.04).

In light of the profound effects of PN1–3 Dex administration, we performed an additional study in which animals received one additional day of treatment (PN1–4, with evaluation on PN5). The more prolonged regimen elicited

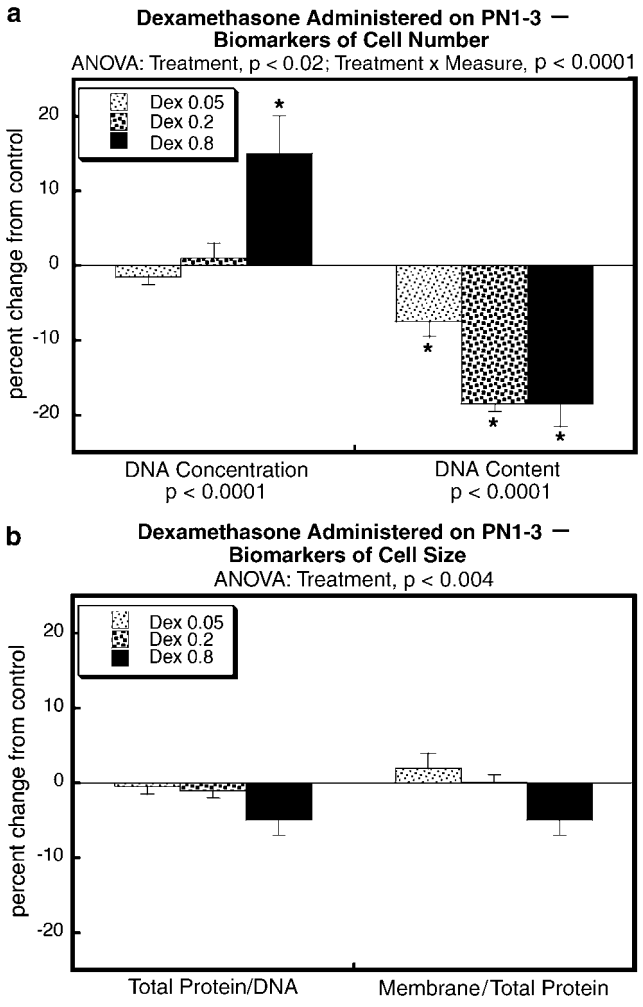

Figure 5 Effects of PN1–3 Dex treatment on biomarkers of neural cell development, evaluated on PN4: (a) DNA concentration and content and (b) protein/DNA ratio and membrane/total protein ratio. ANOVA across all doses and both measures appears at the top of each panel and ANOVA for each measure appears at the bottom; asterisks denote individual values that differ significantly from the corresponding control. The main effect in (b) reflects a significant deficit evoked by Dex 0.8 mg/kg ($p < 0.0001$) but neither ratio alone achieved statistical significance. Data for males and females were combined for presentation because of the absence of a treatment × sex interaction.

much larger deficits in body weight than were seen with PN1–3 treatment: a 20% deficit in the 0.05 mg/kg Dex group, 35% in the 0.2 mg/kg Dex group, and 45% in the 0.8 mg/kg Dex group (Table 3). The effects on forebrain growth were also correspondingly greater than seen with the three-dose regimen but brain sparing was evident from the smaller effect on the forebrain as compared to body weight. Brain weight deficits for the three treatments were 10, 25, and 35%, respectively. More importantly, the extended Dex treatment compromised subsequent viability, with significant mortality in the preweaning period, an effect not seen with the PN1-3 regimen (data not shown).

Dex Treatment on PN7–9

With a shift in the regimen toward later postnatal treatment, Dex elicited a smaller degree of growth impairment than

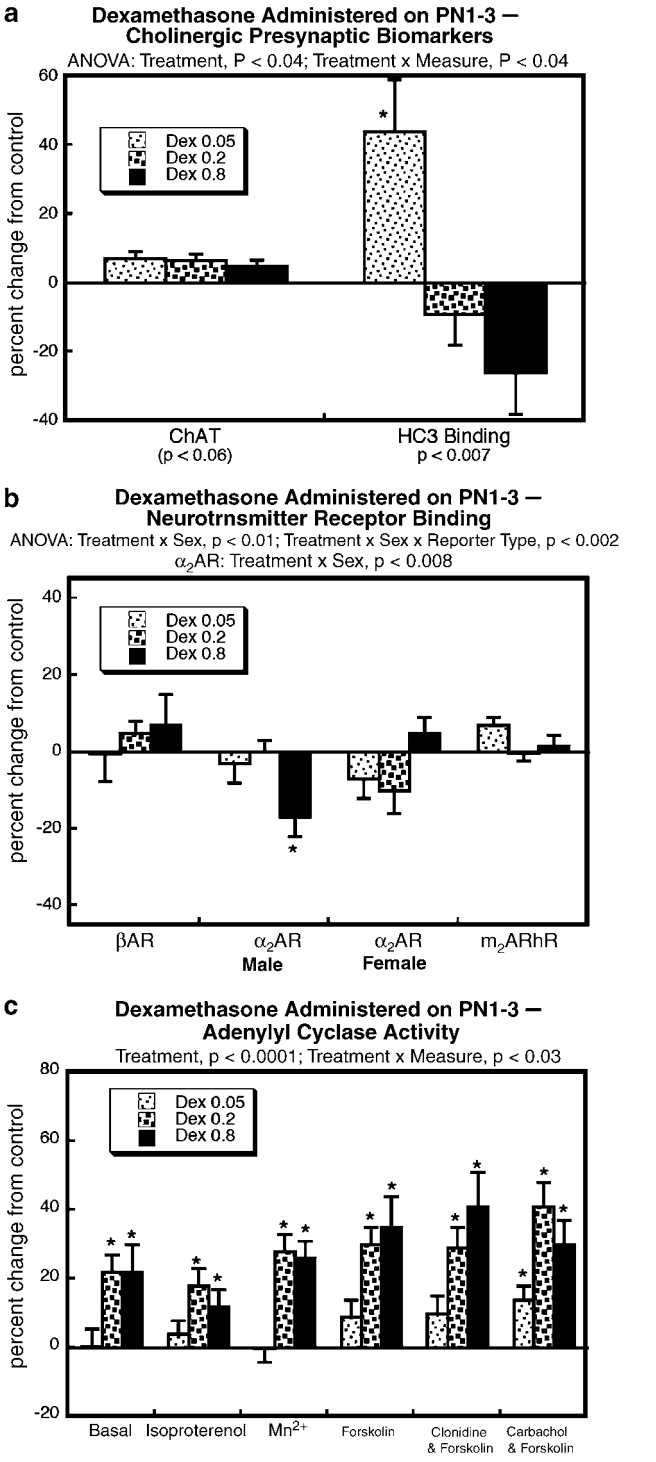

Figure 6 Effects of PN1–3 Dex treatment on biomarkers of synaptic development and responsiveness, evaluated on PN4: (a) presynaptic cholinergic indices, ChAT activity, and HC3 binding; (b) neurotransmitter receptor binding for βARs, α_2ARs, and m_2AChRs; and (c) indices of AC signaling. ANOVA across all doses and both measures appears at the top of each panel and ANOVA for each measure appears at the bottom; asterisks denote individual values that differ significantly from the corresponding control. For all determinations except α_2AR binding, data for males and females were combined for presentation because of the absence of a treatment × sex interaction.

Table 3 Dexamethasone Administration on PN1–4 (Four Injections)

	Control	Dex 0.05 mg/kg	Dex 0.2 mg/kg	Dex 0.8 mg/kg
Body weight (g)	12.6±0.2	10.2±0.2	8.0±0.2	6.7±0.1
Forebrain weight (mg)	294±4	262±5	223±5	185±4

Each treatment group consisted of 18 males and 18 females and evaluations were conducted 24 h after the last dose. ANOVA indicates significant deficits in body weight ($p<0.0001$) and forebrain weight ($p<0.0001$) and the effects were also significant for each individual Dex group compared to control.

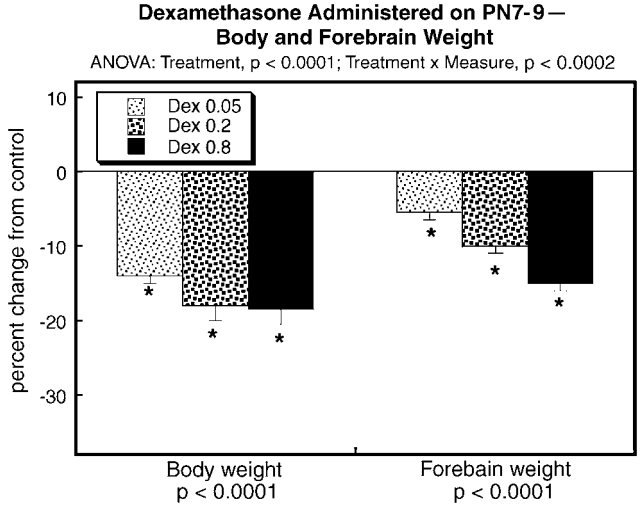

Figure 7 Effects of PN7–9 Dex treatment on body and forebrain weights on PN10. ANOVA across all doses and both measures appears at the top of the panel and ANOVA for each measure appears at the bottom; asterisks denote individual values that differ significantly from the corresponding control. Data for males and females were combined for presentation because of the absence of a treatment × sex interaction.

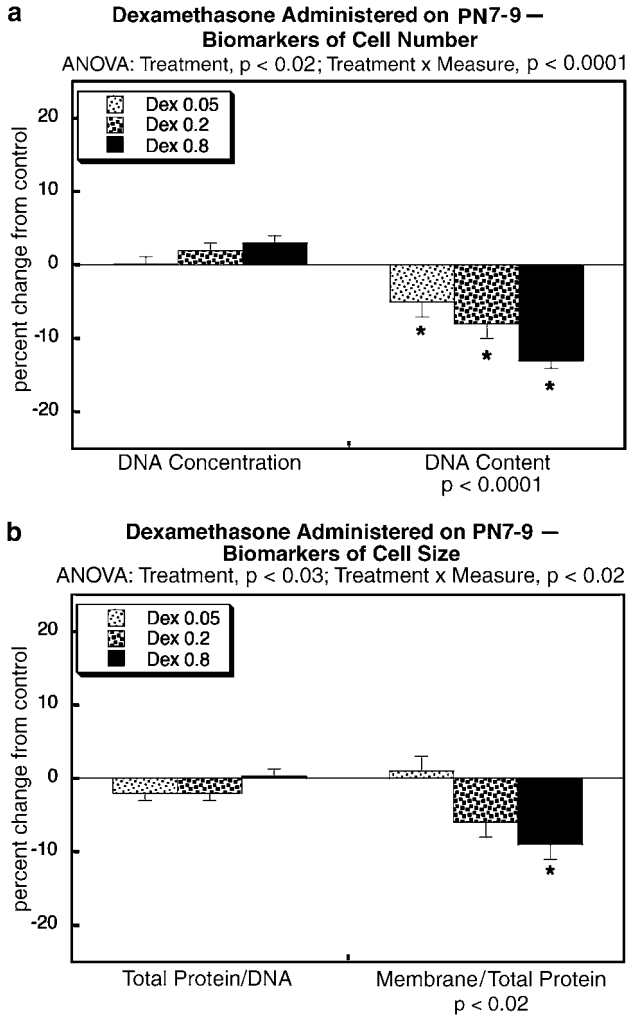

Figure 8 Effects of PN7–9 Dex treatment on biomarkers of neural cell development, evaluated on PN10: (a) DNA concentration and content and (b) protein/DNA ratio and membrane/total protein ratio. ANOVA across all doses and both measures appears at the top of each panel and ANOVA for each measure appears at the bottom; asterisks denote individual values that differ significantly from the corresponding control. Data for males and females were combined for presentation because of the absence of a treatment × sex interaction.

was seen on PN1–3. Although body and forebrain weights (and whole brain weights, not shown) were significantly reduced at all three doses of Dex, the maximum effect was only about half to two-thirds of that seen earlier (Figure 7). Again, brain sparing was evident from the significantly ($p<0.0001$) smaller effects on forebrain weight as compared to body weight. The adverse effects of Dex on biomarkers of neural cell development were also correspondingly reduced in the animals receiving treatment on PN7–9. There were no significant effects on the DNA concentration, and the deficits in DNA content ranged from 5 to 15% (Figure 8a). Nevertheless, the reduction in membrane/total protein ratio was just as large as had been seen with early postnatal Dex treatment (Figure 8b).

As before, there was a small, but statistically significant increase in ChAT activity in the Dex-treated animals but in this case there was no promotional effect on HC3 binding (Figure 9a). However, actions directed toward neurotransmitter receptors were far more prominent (Figure 9b). All three receptor types showed significant increases in the Dex groups but with clearcut sex selectivity and a distinctly nonmonotonic response. Receptor binding was augmented by Dex far more in males than in females ($p<0.005$) and in addition, the effect peaked at 0.2 mg/kg and then declined at the highest Dex dose ($p<0.02$ comparing 0.2–0.8 mg/kg across all three receptors). Superimposed on this general pattern, the magnitude of the increase was much larger for the adrenergic receptors (40% maximum increase) than for m_2AChRs (10% increase). The effects of PN7–9 Dex treatment on AC signaling were similarly much larger than had been seen with PN1–3 treatment, achieving increases of as much as 60% at the highest dose (Figure 9c). As before, the biggest changes were seen for responses to direct AC stimulants (Mn^{2+}, forskolin with or without other additions) as compared to basal AC activity or the response to isoproterenol. In light of the interaction of Dex treatment × AC measure ($p<0.02$), we again compared responses to neurotransmitter stimulants relative to total AC activity assessed with Mn^{2+} or forskolin. In males, Dex at all doses reduced the response to isoproterenol relative to

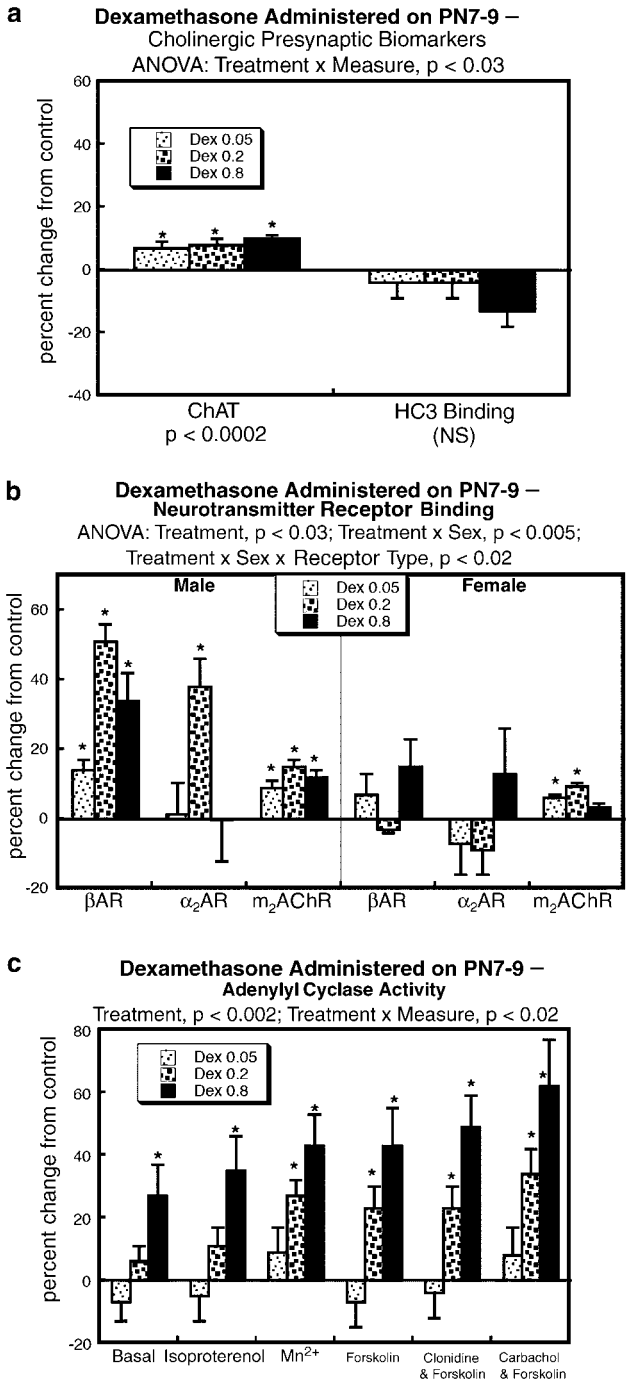

Figure 9 Effects of PN7–9 Dex treatment on biomarkers of synaptic development and responsiveness, evaluated on PN10: (a) presynaptic cholinergic indices, ChAT activity, and HC3 binding; (b) neurotransmitter receptor binding for βARs, α_2ARs, and m_2AChRs; and (c) indices of AC signaling. ANOVA across all doses and both measures appears at the top of each panel and ANOVA for each measure appears at the bottom; asterisks denote individual values that differ significantly from the corresponding control. For (a) and (c), data for males and females were combined for presentation because of the absence of a treatment × sex interaction.

Mn^{2+}-stimulated AC ($p<0.02$): 0.103 ± 0.006 in controls, 0.082 ± 0.007 in the 0.05 mg/kg Dex group, 0.076 ± 0.003 in the 0.2 mg/kg Dex group, 0.084 ± 0.005 in the 0.8 mg/kg Dex group. Effects on the isoproterenol/forskolin ratio were also statistically significant ($p<0.04$) and the corresponding values were 0.29 ± 0.03, 0.22 ± 0.02, 0.22 ± 0.01 and 0.22 ± 0.01, respectively.

DISCUSSION

Our results indicate that Dex, at doses commensurate with its use in preterm labor, and also at doses well below therapeutic levels, alters neural cell development through multiple mechanisms that can contribute a broad spectrum of potential, long-term effects. Superimposed on this general vulnerability, we found evidence for a critical window of heightened sensitivity to adverse effects of Dex on the developing brain, centered around the first 10 days postpartum in the rat, corresponding to the period in human fetal neurodevelopment in which glucocorticoids are likely to be used (Dobbing and Sands, 1979; Gilstrap *et al*, 1994; Rodier, 1988).

Of the three different regimens, Dex administration on GD17–19 elicited the smallest degree of somatic growth retardation, deficits of forebrain weight, or alterations of neurochemical markers, despite the fact that the treatment produced clearcut reductions of maternal weight gain. To some extent, the lowered sensitivity in gestation may represent pharmacokinetic differences engendered by maternal catabolism of Dex, thus reducing the effective dose delivered to the fetus. Indeed, although Dex readily crosses the rat placenta, fetal levels are considerably lower than those in the dam (Seckl, 2004; Varma, 1986). Nevertheless, superimposed on this basic difference, the comparative dose–effect relationships across the different treatment windows can be used to evaluate the relative sensitivity of the brain itself to the effects of Dex. Treatment with 0.8 mg/kg on GD17–19 produced the same degree of somatic and forebrain growth inhibition as was seen at lower doses on PN1–3 or PN7–9, yet was devoid of the robust changes seen in neural cell acquisition, cholinergic biomarkers, receptor binding, and cell signaling parameters, all of which were prominent after postnatal Dex administration. However, we did obtain evidence for some alterations with gestational treatment, including a small degree of neural cell loss and prominent suppression of α_2ARs, a receptor type that is overexpressed and linked to cell replication during neurodevelopment (Duncan *et al*, 1990; Kreider *et al*, 2004; Lidow *et al*, 1991; Slotkin *et al*, 1994b). Furthermore, because we examined the entire forebrain, this leaves open the likelihood of missing more focal effects of Dex that may be diluted by the inclusion of large amounts of unaffected subregions. Indeed, we have already identified persistent changes in hippocampal cholinergic indices and related behavioral performance after GD17–19 treatment with 0.2 mg/kg of Dex, a dose that did not elicit detectable changes at the 24 h time point in any of the parameters evaluated here (Kreider *et al*, 2005). Accordingly, although the present findings indicate a lesser sensitivity to Dex as compared to later developmental stages, these results should not be interpreted as total sparing from the adverse effects of Dex during this period.

With a shift in the treatment window to PN1–3, Dex elicited a much greater degree of somatic and brain growth inhibition. By itself, even transient growth retardation enhances the subsequent incidence of a broad range of

cardiovascular and metabolic disorders (Barker, 2003; Khan *et al*, 2003; Lackland *et al*, 2003; Phillips, 2002). Similarly, Dex treatment of preterm infants increases the likelihood of hypertension and hyperglycemia (Le Cras *et al*, 2000; Nyirenda *et al*, 2001; Ortiz *et al*, 2003), which is in accord with our findings for growth retardation in the rat model. All the same, the most striking effects seen here were on biomarkers of neural cell acquisition, synaptogenesis, and synaptic signaling, displaying significant effects of Dex even at 0.05 or 0.2 mg/kg, doses below or at the lower end of those used clinically. Superimposed on this basic finding, there was a distinct shift in cellular target when Dex was given on PN1–3 as compared to PN7–9. With the early neonatal treatment, there was greater impairment of forebrain growth and cell acquisition, whereas receptors and cell signaling showed the greatest sensitivity with the later treatment. To a large extent, this dichotomy is a logical consequence of the specific events surrounding the two different periods: the early neonatal period involves a more rapid brain growth spurt with the attendant need for generation of new cells, whereas the later phase involves neural cell differentiation and synaptogenesis, with the attendant development of synaptic signaling (Dobbing and Sands, 1979; Rodier, 1988). Taken together, these differences reinforce the idea that Dex affects brain development through multiple mechanisms, each of which has a distinct peak of developmental sensitivity.

Accordingly, the long-term outcomes of Dex are likely to be quite different depending upon the critical window in which exposure occurs. Indeed, our results for the biomarkers of cell number and size also indicate fundamental differences in the outcomes of the different Dex regimens. Although there were only minor effects after treatment on GD17-19, the group given Dex on PN1–3 showed a decrease in DNA content, signifying deficits in the total of number of neural cells. At the highest dose, the cell packing density (DNA per gram tissue) was increased whereas indices of cell size indicated a relative decline. This pattern allows for inferences to be drawn about the specific cellular targets. Since glia are smaller than neurons, neuronal loss accompanied by partial glial replacement will produce precisely this archetype, representing reactive gliosis that is a characteristic of neuronal injury (O'Callaghan, 1993). With the shift to treatment on PN7–9, the decrease in total cell number was not accompanied by a drop in the total protein/DNA ratio but only by a decrease in the membrane/total protein ratio, suggestive of impaired development of neuritic projections, as might be expected from targeting of later events in neurodevelopment. Obviously, these conclusions from biochemical findings need to be confirmed by morphological investigations but our findings point the way to the specific types of alterations that are likely to be found.

The effects of Dex on indices of synaptic development, neurotransmitter receptor expression, and cell signaling are particularly important in light of the trophic roles of acetylcholine and norepinephrine in forebrain development (Dreyfus, 1998; Hohmann, 2003; Hohmann and Berger-Sweeney, 1998; Lauder and Schambra, 1999; Whitaker-Azmitia, 1991). Dex administration on PN1–3 enhanced ChAT activity and HC3 binding, suggesting an accelerated differentiation of cholinergic nerve terminals. Shifting the treatment window to PN7–9 still produced the increase in ChAT but the effect on HC3 binding was lost, suggesting a lessened effect of Dex on this particular developmental event. Indeed, the augmentation of cholinergic development appears to occupy a very restricted time-frame and dose requirement, as even with the PN1–3 treatment, raising the dose of Dex into the therapeutic range (0.2 or 0.8 mg/kg) reversed the promotional effect. This biphasic response has been noted before (Slotkin *et al*, 1991a; Zahalka *et al*, 1993b) and the interpretation is relatively straightforward: enhanced differentiation evoked by Dex is offset when the dose is raised sufficiently to impair general development consequent to growth inhibition. Indeed, in the current work, we observed similar biphasic responses for effects of PN7–9 Dex administration on adrenergic receptors, indicating that this is a more general phenomenon.

Interestingly, the largest effects on receptors involved those that are overexpressed during brain development (βAR, α_2AR), rather than the m_2AChRs, which are not. These findings suggest a specific link between Dex administration and effects mediated by neurotransmitter receptors with specific trophic roles in the developing brain. Indeed, the sharp dichotomy in the effects of α_2AR stimulation on AC activity point out the different roles of the overexpressed receptors during specific phases of development: clonidine was inhibitory on PN4 but stimulatory on PN10. Nevertheless, it is the enhancement of βAR expression by Dex that may be of particular relevance. Terbutaline, a βAR agonist, is frequently administered to arrest preterm labor (Lam *et al*, 1998), and this drug also penetrates to the fetus to stimulate βARs in the developing brain, impairing cell acquisition and producing lasting synaptic and structural anomalies (Meyer *et al*, 2005; Rhodes *et al*, 2004a, b; Slotkin *et al*, 2003). Dex and terbutaline are almost always given together in the therapy of preterm labor, and our findings suggest the strong likelihood of additive or synergistic effects on neurodevelopment, a possibility highlighted for future study (Gilstrap *et al*, 1994). Of further interest, the effects of Dex on adrenergic receptor expression and on βAR-mediated AC stimulation were among the only ones in which we found a sharp distinction between males and females. Dex influences testosterone synthesis and metabolism (Reznikov *et al*, 2004) and in turn, testosterone regulates the expression of α_2ARs (Dygalo *et al*, 2002). Although parallel studies have not been conducted for sex-dependent effects on βAR expression, there are steroid-binding sites in the βAR promoter (Cornett *et al*, 1998), so that such differences may also exist for this receptor. In either case, the sex-selective effects of Dex on adrenergic receptors and corresponding cell signaling during this critical period are likely to contribute to long-term effects. Both α_2ARs and βARs exert direct control over neural cell replication and apoptotic cell elimination as required for architectural assembly of the brain (Duncan *et al*, 1990; Garofolo *et al*, 2003; Kreider *et al*, 2004; Lidow and Rakic, 1995; Popovik and Haynes, 2000; Slotkin *et al*, 1988; Zhu *et al*, 1998). Glucocorticoid effects on adrenergic receptor expression and signaling may thus provide a contributory mechanism to the sex-selectivity seen for the ultimate behavioral outcomes (Bowman *et al*, 2004; Kreider *et al*, 2005).

In keeping with an earlier report (Slotkin *et al*, 1994a), gestational Dex treatment had only minor effects on AC signaling, but in contrast, we found that postnatal treatments evoked massive increases in all aspects of signal transduction through this pathway. Since the largest effects were seen for the response to direct AC stimulants (Mn^{2+}, forskolin), it is most likely that Dex induces the expression of AC, leading to substantial increases in the number of membrane-associated AC molecules; we did not find significant changes in the relative response to forskolin *vs* Mn^{2+}, suggesting that Dex does not alter the AC isoform being expressed, but rather simply increases the total number of AC molecules. Accordingly, Dex administration produces 'heterologous' sensitization of the pathway, increasing the ability of all inputs to evoke an increase in cyclic AMP formation, as demonstrated here for basal AC activity and disparate neurotransmitter receptor stimulants. Given the pivotal role played by cyclic AMP in the switch from cell replication to differentiation, and the fact that prolonged overproduction elicits apoptosis (Claycomb, 1976; Gu *et al*, 2000; Hultgårdh-Nilsson *et al*, 1994; Slotkin *et al*, 2003), the global induction of AC is thus likely to play a direct role in the adverse effects of Dex on cell acquisition, and also to sensitize developing neural cells to other stimuli that may then contribute to additional or later-emerging effects. We also found evidence for impaired G-protein signaling, as evidenced by changes in the response to carbachol (loss of m_2AChR-mediated inhibitory actions) and isoproterenol (reduced proportion of total AC activity responding to βAR stimulation), so that Dex may also compromise specific receptor-mediated responses, superimposed on the general increases in AC. Notably, too, Dex-induced changes in receptor-mediated AC signaling were generally unrelated to effects on receptor expression, in keeping with earlier observations that G-protein coupling *per se* is a more important determinant of the net response to receptor stimulation (Slotkin *et al*, 2003; Vatner *et al*, 1998).

In conclusion, Dex administration during a critical developmental window evokes a wide range of cellular defects in the developing forebrain, comprising actions directed toward cell acquisition, synaptic development, neurotransmitter receptor expression, and cell signaling. Importantly, these effects are all exerted with Dex doses at or below those used clinically in the management of preterm infants. Given the fact that hundreds of thousands of infants receive such treatment annually in the US, our findings suggest the need for a careful evaluation of the long-term neurodevelopmental liabilities engendered by glucocorticoid use.

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