

# Adverse Neurodevelopmental Effects of Dexamethasone Modeled in PC12 Cells: Identifying the Critical Stages and Concentration Thresholds for the Targeting of Cell Acquisition, Differentiation and Viability

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The use of dexamethasone (DEX) to prevent respiratory distress in preterm infants is suspected to produce neurobehavioral deficits. We used PC12 cells to model the effects of DEX on different stages of neuronal development, utilizing exposures from 24 h up to 11 days and concentrations from 0.01 to 10  $\mu$ M, simulating subtherapeutic, therapeutic, and high-dose regimens. In undifferentiated cells, even at the lowest concentration, DEX inhibited DNA synthesis and produced a progressive deficit in the number of cells as evaluated by DNA content, whereas cell growth (evaluated by the total protein to DNA ratio) and cell viability (Trypan blue exclusion) were promoted. When cell differentiation was initiated with nerve growth factor, the simultaneous inclusion of DEX still produced a progressive deficit in cell numbers and promoted cell growth and viability while retarding the development of neuritic projections as monitored by the membrane/total protein ratio. Again, even 0.01  $\mu$ M DEX was effective. We next assessed effects at mid-differentiation by introducing nerve growth factor for 4 days followed by coexposure to DEX. Although effects on cell number, growth, and neurite extension were still detectable, the outcomes were generally less notable. DEX also shifted the fate of PC12 cells away from the cholinergic phenotype and toward the adrenergic phenotype, with the maximum effect achieved at the outset of differentiation. Our results indicate that DEX directly disrupts neuronal cell replication, differentiation, and phenotype at concentrations below those required for the therapy of preterm infants, providing a mechanistic link between glucocorticoid use and neurodevelopmental sequelae. *Neuropsychopharmacology* (2006) **31**, 1647–1658. doi:10.1038/sj.npp.1300967; published online 30 November 2005

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## INTRODUCTION

Glucocorticoids are typically given prophylactically to pregnant women undergoing preterm labor between the 24th and 34th weeks of gestation, or directly to preterm infants, in order to reduce the incidence of respiratory distress (Gilstrap *et al*, 1995). Currently, 10% of all infants delivered in the US receive glucocorticoids, although only a small proportion are likely to have developed respiratory distress, so that hundreds of thousands of infants are treated unnecessarily (Matthews *et al*, 2002). Growing evidence indicates long-term liabilities from such treatment, including altered brain architecture (Murphy *et al*, 2001),

and subsequent cognitive deficiencies (Doyle *et al*, 2000; Seckl, 2001; Yeh *et al*, 2004). Accordingly, recent reviews point out the drawbacks of the blanket use of glucocorticoids (Blackmon *et al*, 2002; Coe and Lubach, 2005; Newnham, 2001; Raff, 2004; Seckl, 2004), but nevertheless, the major comorbidities associated with preterm delivery make it difficult to assign a clear cause-and-effect relationship.

Animal studies of glucocorticoid effects on brain development, which avoid these comorbidities, have clarified the issue considerably. By themselves, glucocorticoids administered during development can lead to persistent stunting of somatic growth, outright cerebral atrophy, and endocrine disruption, along with a host of behavioral anomalies (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). Nevertheless, most of the animal literature concerns doses of glucocorticoids well above those in clinical use and/or encompass prolonged exposure periods or periods outside the neurodeve-

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lopmental stages appropriate to mimic the use in humans. We recently developed a series of treatment models in the developing rat that take into account the stages of brain development appropriate to the targeted preterm human population, incorporating doses both within the therapeutic range and well below that level (Kreider *et al*, 2005a, b, 2006). Dexamethasone (DEX) treatments in late gestation or the early neonatal period that did not compromise long-term somatic growth, nevertheless disrupted neural cell acquisition, indices of neuritic outgrowth, synaptic activity, and cell signaling involved in trophic regulation of forebrain development (Kreider *et al*, 2005a), leading to long-term changes in cognition and motor activity (Kreider *et al*, 2005b), resembling those seen in models of prenatal stress (Bowman *et al*, 2004; Dean *et al*, 2001; Felszeghy *et al*, 2000; Muneoka *et al*, 1997). This provides strong evidence for a primary effect of glucocorticoids on brain development even at doses below those in clinical use.

Nevertheless, these *in vivo* models necessarily involve potential contributions from effects on the maternal-fetal unit, maternal-neonatal interactions, maternal, and neonatal neuroendocrine status, and a host of other indirect factors that converge on brain development and behavior. A direct proof of a mechanistic connection between glucocorticoids and abnormal neuronal development thus requires an *in vitro* system. Primary neurons do not maintain cell division in culture and do not provide a uniform population either in terms of cell types or differentiation state. In the current study, we utilized rat pheochromocytoma PC12 cells, a model for evaluating neuronal cell replication and differentiation (Teng and Greene, 1994). In the presence of nerve growth factor (NGF), the cells gradually exit the mitotic cycle and begin to differentiate, developing axonal projections, electrical excitability, and the characteristics of two distinct phenotypes, cholinergic, and catecholaminergic neurons (Fujita *et al*, 1989; Song *et al*, 1998; Teng and Greene, 1994). These specific phenotypes are highly targeted by DEX treatment *in vivo* (Ebert *et al*, 1997; Hu *et al*, 1996; Kreider *et al*, 2005a, b, 2006; Reznikov *et al*, 2004; Slotkin *et al*, 1991; Zahalka *et al*, 1993). Accordingly, the PC12 model is particularly appropriate to evaluate the mechanisms underlying the effects of DEX on neural cell replication and differentiation into defined phenotypes. Indeed, the medium required to maintain PC12 cell replication, differentiation, and growth entails the use of sera containing endogenous glucocorticoids (Qiao *et al*, 2005; Song *et al*, 1998; Teng and Greene, 1994), so that, as with the situation *in vivo*, effects of DEX are exerted over and above the requisite normal concentration required to maintain neural cell development. At the same time, as with all *in vitro* models, the cell cultures lack many features common to the developing brain, including the ability to evaluate neuronal-glial interactions or more architectural aspects of regional development.

To recapitulate the effects seen in intact animals (Kreider *et al*, 2005a, 2006), we evaluated the effects of different concentrations and durations of exposure to DEX in undifferentiated and differentiated states, evaluating indices of cell replication (radiolabeled thymidine incorporation into DNA), cell number, cell growth, viability, and

phenotype. Each neural cell contains only a single nucleus (Winick and Noble, 1965), so that the DNA content reflects the total number of cells (Song *et al*, 1998); since DEX arrests mitosis in the G<sub>0</sub>/G<sub>1</sub> phase (Greenberg *et al*, 2002), this relationship holds true even where DEX treatment affects cell acquisition. Indices of growth were provided by measurements of protein subfractions related to cell size and membrane surface area (Thai *et al*, 1996). In replicating cells, the total protein/DNA ratio rises with cell enlargement and the membrane/total protein ratio falls as a consequence of the decreased surface-to-volume ratio. On the other hand, with the onset of differentiation, the development of neuritic projections necessitates a rise in the relative contribution of membrane proteins. The effects on cell number, size, and cell surface area were compared to those on viability, evaluated by Trypan blue exclusion. To characterize the cholinergic and catecholaminergic phenotypes, we assessed the activities of choline acetyltransferase (ChAT) and tyrosine hydroxylase (TH), the respective biosynthetic enzymes for acetylcholine and dopamine (Teng and Greene, 1994).

Our focus was on two specific issues surrounding the use of glucocorticoids in preterm infants: characterizing the actions exerted at or below the concentrations relevant to clinical use, and identifying the spectrum of effects at the most relevant stages of neuronal development. Previous studies of glucocorticoids in this model have typically used excessive concentrations and have not focused on specific neurodevelopmental stages (Berse and Blusztajn, 1997; Ebert *et al*, 1997; Hagerty *et al*, 2001; Lucas and Thoenen, 1977; Schubert *et al*, 1980; Unsicker *et al*, 1978). Accordingly, the present study utilized DEX concentrations as low as 0.01  $\mu$ M and up to 10  $\mu$ M. In humans, the typical levels after administration of a relatively low dose of 0.1–0.15 mg/kg is about 0.6  $\mu$ M when given directly to a preterm infant, or 0.06  $\mu$ M in the fetus when DEX is given to the mother (Charles *et al*, 1993; Osathanondh *et al*, 1977); as the recommended dose in preterm delivery is about three times higher (Gilstrap *et al*, 1995), our chosen dose range clearly spans the subtherapeutic to supratherapeutic range. We evaluated three different critical neurodevelopmental stages: undifferentiated cells that are undergoing active mitosis, early stages of differentiation in which cells are still dividing but are undergoing the transition to neuritic development and phenotypic specialization, and mid-differentiation in which the same processes are further developed. Again, these are stages that are most likely to be targeted by DEX, based on biochemical and morphological criteria from *in vivo* studies (Bohn, 1984; Murphy *et al*, 2001).

## METHODS

### PC12 Cell Culture and Treatments

As described previously (Song *et al*, 1998) PC12 cells (American Type Culture Collection, 1721-CRL) were grown in RPMI-1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% inactivated horse serum (Sigma Chemical Co., St Louis, MO), 5% fetal bovine serum (Sigma), and 50  $\mu$ g/ml penicillin-streptomycin (Invitrogen); the added sera thus expose the cells to endogenous glucocorticoids.

Cells were incubated with 7.5% CO<sub>2</sub> at 37°C and the medium was changed every 2–3 days. Because of the clonal instability of the PC12 cell line (Fujita *et al*, 1989), the experiments were performed on cells that had undergone fewer than five passages and studies were repeated several times with different batches of cells. For evaluation of DEX effects of different durations in the undifferentiated state, cells were plated on 60 mm poly-L-lysine-coated plates at various initial densities so as to achieve approximately 70% confluence at the time of each determination, in order to avoid complete confluence and excessive clumping at the longest time points. At 24 h after plating, the medium was changed to include concentrations of DEX phosphate (Sigma) ranging from 0.01 to 10 μM.

For studies involving DEX exposure in differentiating cells, the initial plating density was held constant at  $0.5 \times 10^6$  cells per 60 mm plate or  $3.5 \times 10^6$  cells per 100 mm plate. At 24 h after plating, the medium was changed to include 50 ng/ml of NGF (Sigma) with or without the addition of different concentrations of DEX. DEX effects were examined under two different conditions, the first in which DEX was present from the outset of differentiation (DEX added simultaneously with NGF), and the second in which cells were allowed to begin differentiation before the addition of DEX (4d NGF pretreatment followed by DEX). Each culture was examined under a microscope to verify the outgrowth of neurites after the addition of NGF.

### DNA Synthesis

To initiate the measurement of DNA synthesis, the medium was changed to include 1 μCi/ml of [<sup>3</sup>H]thymidine (specific activity, 2 Ci/mmol; GE Healthcare, Piscataway, NJ) along with the continued inclusion of DEX. After 1 h, the medium was aspirated and cells were harvested in 3.5 ml of ice-cold water. Duplicate aliquots of each sample were treated with 10% trichloroacetic acid and sedimented at 1000g for 15 min to precipitate nucleic acids and proteins. The resulting pellet was washed once with additional trichloroacetic acid and then with 75% ethanol. The final pellet was hydrolyzed with 1 M KOH overnight at 37°C, neutralized with 6 M HCl, and the DNA was precipitated with ice-cold 5% trichloroacetic acid and resedimented. The supernatant solution, comprising solubilized RNA and protein, was discarded. The DNA-containing pellet was hydrolyzed in 5% trichloroacetic acid for 15 min at 90°C, resedimented, and an aliquot of the supernatant solution was counted for radiolabel. Another aliquot was assayed for DNA spectrophotometrically by absorbance at 260 nm. Previous work has demonstrated quantitative recovery of DNA by these techniques (Bell *et al*, 1986; Slotkin *et al*, 1984). Incorporation values were corrected to the amount of DNA present in each culture to provide an index of DNA synthesis per cell.

### Cell Number, Size, and Viability

The medium was aspirated and the culture was rinsed with 1 ml of a buffer consisting of 0.9% NaCl and 10 mM sodium phosphate (pH 7.4). Cells were harvested in ice-cold buffer, homogenized (Polytron, Brinkmann Instruments, West-

bury, NY) and aliquots were withdrawn for measurements of DNA and total protein (Trauth *et al*, 2000). To prepare the cell membrane fraction, the homogenates were sedimented at 40 000g for 10 min and the pellet was washed and resedimented. Aliquots of the final resuspension were then assayed for membrane protein. Cell viability was assessed in separate cultures. The medium was changed to include trypan blue (Sigma; 1 volume per 2.5 volumes of medium) and cells were examined for staining under 400× magnification, counting an average of 100 cells per field in four different fields per culture.

### Enzyme Activities

Cells were harvested as already described and were lysed by homogenization in a ground-glass homogenizer fitted with a ground-glass pestle, using a buffer consisting of 154 mM NaCl and 10 mM sodium-potassium phosphate (pH 7.4). ChAT assays (Lau *et al*, 1988) were conducted with 40 μg of protein in 60 μl of a buffer consisting of 60 mM sodium phosphate (pH 7.9), 200 mM NaCl, 20 mM choline chloride, 17 mM MgCl<sub>2</sub>, 1 mM EDTA, 0.2% Triton X-100, 0.12 mM physostigmine (Sigma), and 0.6 mg/ml bovine serum albumin (Sigma), containing a final concentration of 50 μM [<sup>14</sup>C]acetyl-coenzyme A (specific activity 44 mCi/mmol, diluted with unlabeled compound to 6.7 mCi/mmol; PerkinElmer Life Sciences, Boston, MA). 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 terminated by placing the samples on ice. Labeled acetylcholine was then extracted, counted, and the activity determined relative to DNA or total protein.

TH activity was measured using [<sup>14</sup>C]tyrosine as a substrate and trapping the evolved <sup>14</sup>CO<sub>2</sub> after coupled decarboxylation with dopa decarboxylase (Lau *et al*, 1988). Homogenates were sedimented at 26 000g for 10 min to remove storage vesicles containing catecholamines, which interfere with TH activity, and assays were conducted with 100 μl aliquots of the supernatant solution in a total volume of 550 μl. Each assay (pH 6.1) contained final concentrations of 910 μM FeSO<sub>4</sub>, 55 μM unlabeled L-tyrosine (Sigma) 9.1 μM pyridoxal phosphate (Sigma), 36 μM β-mercaptoethanol, and 180 μM 2-amino-6,7-dimethyl-4-hydroxy-5,6,7,8-tetrahydropteridine HCl (Sigma), all in a buffer of 180 mM sodium acetate and 1.8 mM sodium phosphate (pH 6.1). Each assay contained 0.5 μCi of generally-labeled [<sup>14</sup>C]tyrosine (specific activity, 438 mCi/mmol; Sigma) as substrate and blanks contained buffer in place of the homogenate.

### Data Analysis

Data are presented as means and SE. For each type of study, treatment differences were first evaluated with a global analysis of variance (ANOVA) incorporating all variables: Dex concentration, time, and the presence or absence of NGF. Based on the interactions of Dex with the other factors, data were then subdivided for lower-order tests, followed by Fisher's Protected Least Significant Difference to establish individual values that differed from the corresponding controls. Because many of the comparisons

involved large changes, even over an order of magnitude, the multivariate tests required log-transformation of the data before ANOVAs could be run. To ensure that transformation was applied in an unbiased fashion, all data sets were evaluated with Bartlett's test prior to ANOVA and reevaluated after log-transformation to ensure that the transformation resulted in equal variances. For comparisons that passed Bartlett's test without transformation, we nevertheless conducted an additional ANOVA with log transformation to verify that statistical significance was maintained.

For all tests, significance was assumed at  $p < 0.05$ .

## RESULTS

### Cell Acquisition, Growth, and Viability in Undifferentiated Cells

In undifferentiated PC12 cells, DEX concentrations as low as  $0.01 \mu\text{M}$  elicited significant inhibition of mitotic activity, as shown by a reduction in [ $^3\text{H}$ ]thymidine incorporation into DNA (Figure 1). The effect plateaued at about 60% inhibition between 1 and  $10 \mu\text{M}$ . Prolonged exposure to DEX did not elicit desensitization of the effect. By 72 h, at which time the rate of DNA synthesis in control cells had slowed by nearly half, DEX nevertheless produced an inhibitory profile that was similar to that seen earlier (significant main effect of DEX dose but no significant interaction of dose  $\times$  time). Because of the reduced overall synthetic rate, the lowest DEX concentrations did not produce statistically significant effects at 72 h but the magnitude of the reduction was the same as, and statistically indistinguishable from, that seen at 24 h.

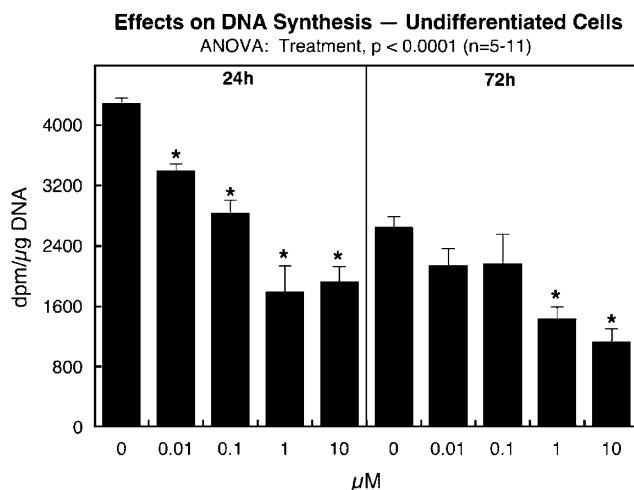
The consequences of reduced mitotic activity were readily evident in a progressive reduction in DNA content in

undifferentiated cells, indicative of reduced cell number (Figure 2a). After 168 h of DEX exposure, even the lowest concentration elicited reductions of over 30% and higher concentrations ranging from 0.1 to  $10 \mu\text{M}$  evoked deficits of more than 50%. In contrast, DEX did not impair cell growth and actually evoked significant increases in the total protein/DNA ratio, signifying larger cell size (Figure 2b). Again, these effects became progressively greater with time, were significant even at  $0.01 \mu\text{M}$  DEX and plateaued between 0.1 to  $10 \mu\text{M}$ . In keeping with perikaryal enlargement, the effects of DEX on the membrane/total protein ratio showed an inverse relation to the total protein/DNA ratio (Figure 2c); larger cells have a reduced surface-to-volume ratio, reflected in deficits in this index of membrane complexity. Finally, we used Trypan blue to evaluate the effects of the highest concentration of DEX ( $10 \mu\text{M}$ ) on viability in undifferentiated PC12 cells (Figure 2d). DEX did not decrease viability at any time and in fact, improved viability with prolonged exposure, as evidenced by a significant reduction in stained (nonviable) cells at 168 h.

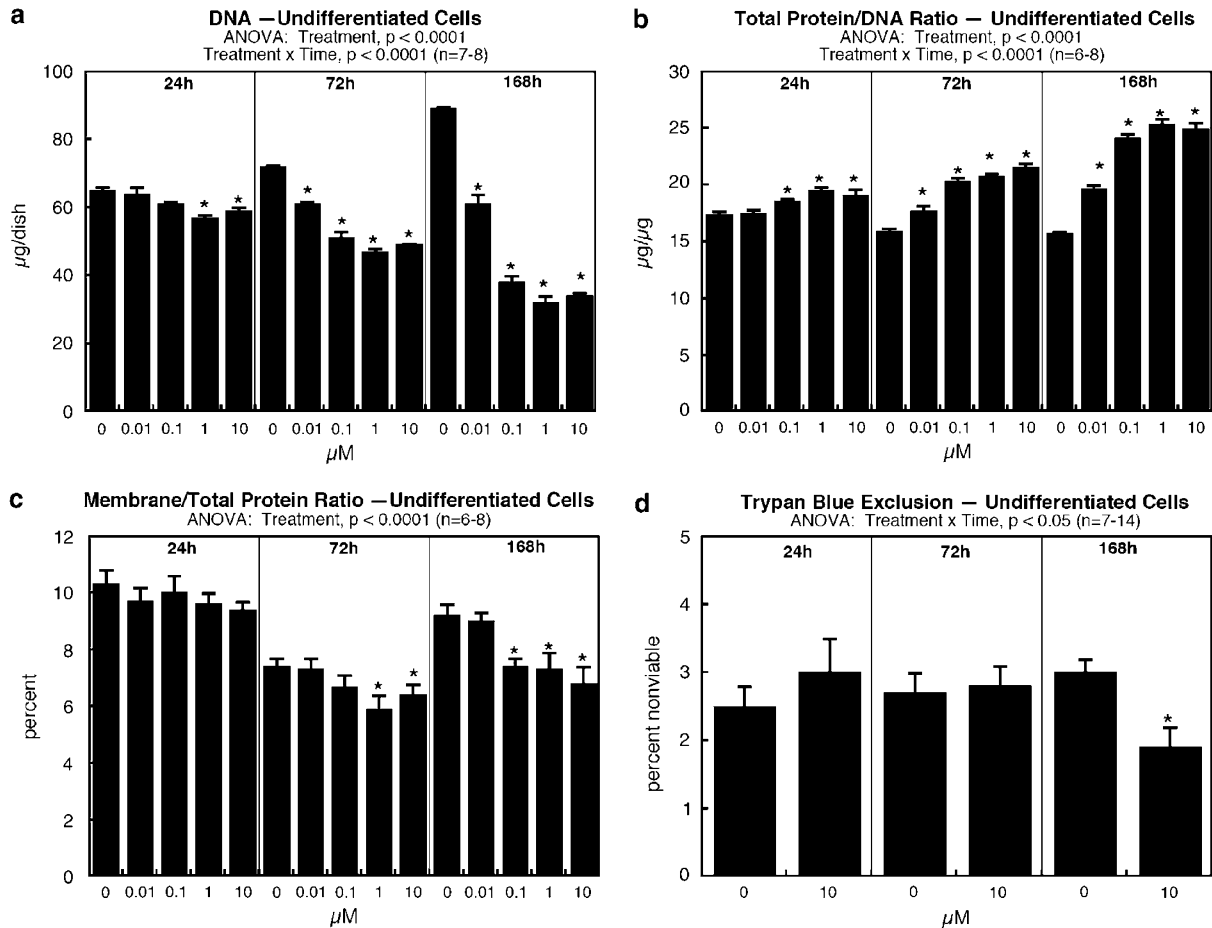
### Cell Acquisition, Growth, and Viability in Differentiating Cells

Unlike the studies in undifferentiated cells, in the experiments with addition of NGF, cells were plated at the same initial density regardless of the evaluated time point, so that longitudinal changes reflect the progression of differentiation. Previous studies indicate that cell replication continues, albeit at a reduced rate, for at least 10 days after the addition of NGF (Song *et al*, 1998), and in keeping with that finding, DNA content continued to rise through 264 h in culture (Figure 3a); indeed, the total number of cells approximately doubled between 168 h and 264 h. When DEX was administered from the outset of differentiation (ie simultaneously with the addition of NGF), it still elicited significant reductions in cell number, albeit to a lesser extent than had been seen in undifferentiated cells. The lowest concentration produced about 10% inhibition whereas deficits were about 30% at higher DEX concentrations.

In keeping with its ability to enhance growth and differentiation, NGF by itself produced more than a doubling in the total protein/DNA ratio as compared to undifferentiated cells but DEX administered from the start of differentiation nevertheless was still able to promote growth even further (Figure 3b). As before, significant effects were seen even at  $0.01 \mu\text{M}$  DEX with plateauing of the effect at the higher concentrations, showing a maximal increase of about 25%. In accord with neurite formation, NGF by itself increased the membrane/total protein ratio by 50% above the levels seen in undifferentiated cells (15% membrane protein *vs* 10% in undifferentiated cells), whereas simple perikaryal enlargement would have reduced the ratio. DEX administration evoked deficits in the membrane/total protein ratio, although concentrations above  $0.01 \mu\text{M}$  were required to produce a significant effect and the magnitude of the decrements was slightly smaller than for the other indices (Figure 3c). Trypan blue exclusion did not demonstrate any decrement in viability in the DEX-exposed cells (Figure 3d). On the contrary, there was a significant overall increase in



**Figure 1** Concentration–response and time–response relationships for the effects of DEX on [ $^3\text{H}$ ]thymidine incorporation into DNA in undifferentiated cells. Cells were plated at different initial densities to achieve approximately the same degree of confluence at the two time points (see Methods). ANOVA across all concentrations and both time points is shown at the top of the panel and the number of determinations at each time and concentration is shown in parentheses. \* Denotes individual values that differ from the corresponding control ( $0 \mu\text{M}$ ).



**Figure 2** Concentration–response and time–response relationships for the effects of DEX on cell number, cell size and viability in undifferentiated cells: (a) DNA concentration, (b) total protein/DNA ratio, (c) membrane/total protein ratio, and (d) Trypan blue exclusion. Cells were plated at different initial densities to achieve approximately the same degree of confluence at the various time points (see Methods); accordingly, apparent time-related changes in the absolute values for DNA are not meaningful. ANOVA across all concentrations and time points is shown at the top of each panel and the number of determinations at each time and concentration is shown in parentheses. \* Denotes individual values that differ from the corresponding control (0  $\mu\text{M}$ ).

viability (reduced staining); values for the individual time points did not achieve significance separately because of the small sample size but the effect across the two time points was significant. Just as reported previously (Qiao *et al*, 2005) NGF treatment alone produced a progressive rise in nonviable cells, which likely involves the increase in oxidative stress attending the early stages of differentiation and neurite formation.

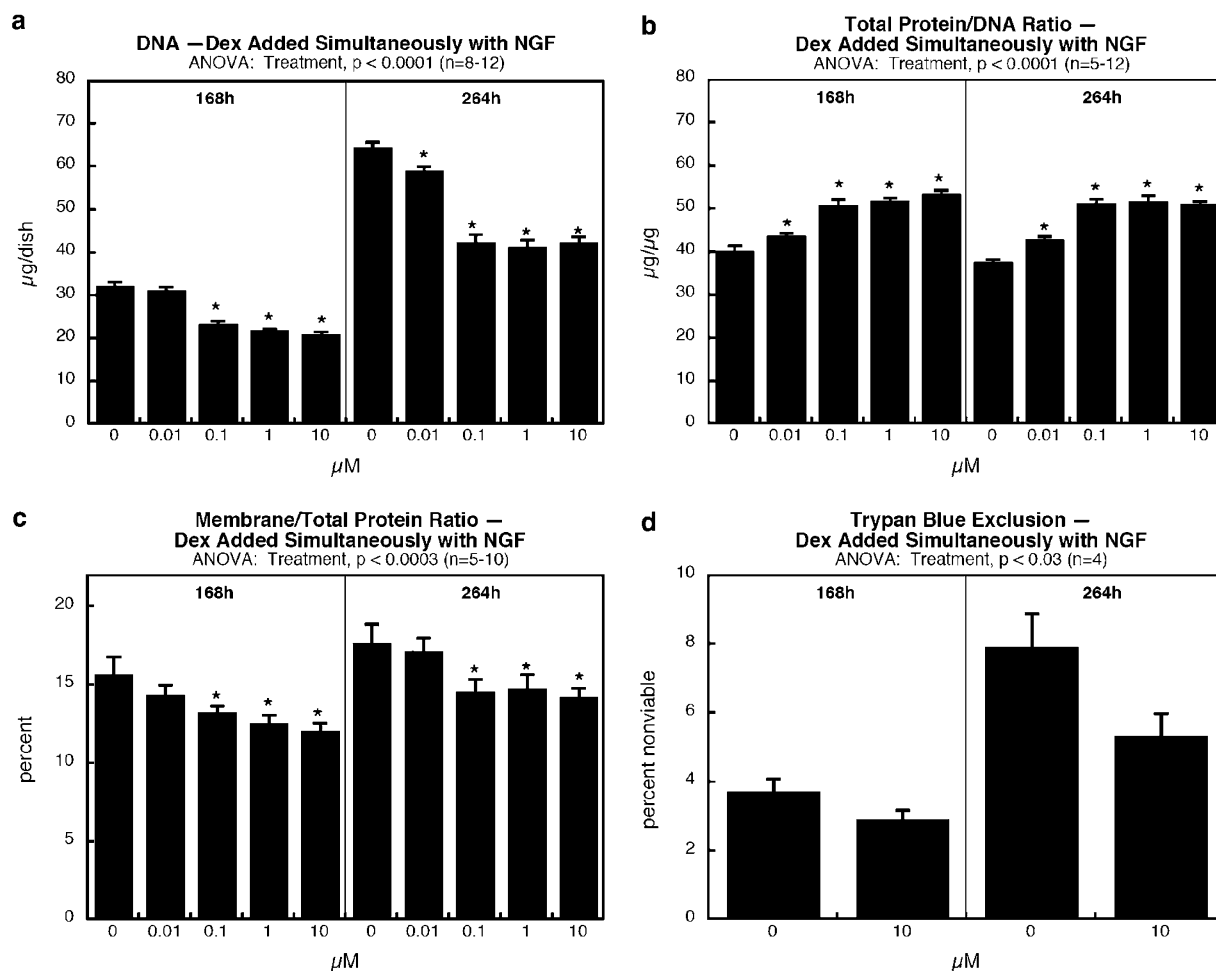
### Cell Acquisition, Growth, and Viability at Mid-Differentiation

To pursue the issue of critical periods of vulnerability, we next allowed cells to undergo NGF-induced differentiation for 4 days before beginning treatment with DEX; accordingly, the 24, 72 or 168 h time points for DEX treatment reflect 5, 7, or 11 days' total exposure to NGF, respectively. Under these conditions, new cells continued to be formed, as evidenced by the progressive rise in DNA content under control conditions (Figure 4a). DEX administration still interfered with cell acquisition with a low concentration threshold (significant effects even at 0.01  $\mu\text{M}$ ) and a maximum deficit of about 30%, approximately the same

profile as had been seen when DEX was included at the initiation of differentiation. Similarly, DEX was still promotional for cell growth as indicated by significant increases in the total protein/DNA ratio (Figure 4b). The later introduction of DEX also interfered with the subsequent rise in membrane/total protein ratio, consistent with inhibitory actions directed toward neurite formation (Figure 4c), although the effects were somewhat smaller in magnitude than had been seen when DEX was included from the outset of NGF-induced differentiation (15% maximum deficit compared to 25%). Notably, this DEX regimen failed to produce an improvement in cell viability, as there were no significant reductions in Trypan blue staining (Figure 4d).

### Cholinergic vs Catecholaminergic Phenotype

To determine effects on phenotype, we turned to enzymatic measures characterizing cholinergic and catecholaminergic subpopulations, ChAT and TH, respectively. Since TH is known to have a glucocorticoid response element in its promoter region (Hagerty *et al*, 2001), we first concentrated on the effects of DEX on ChAT activity in undifferentiated



**Figure 3** Concentration–response and time–response relationships for the effects of DEX on cell number, cell size and viability in cells undergoing NGF-induced differentiation, with DEX added simultaneously with NGF: (a) DNA concentration, (b) total protein/DNA ratio, (c) membrane/total protein ratio, and (d) Trypan blue exclusion. Cells were plated at the same initial density for each time point (see Methods). ANOVA across all concentrations and time points is shown at the top of each panel and the number of determinations at each time and concentration is shown in parentheses. \* Denotes individual values that differ from the corresponding control ( $0 \mu\text{M}$ ).

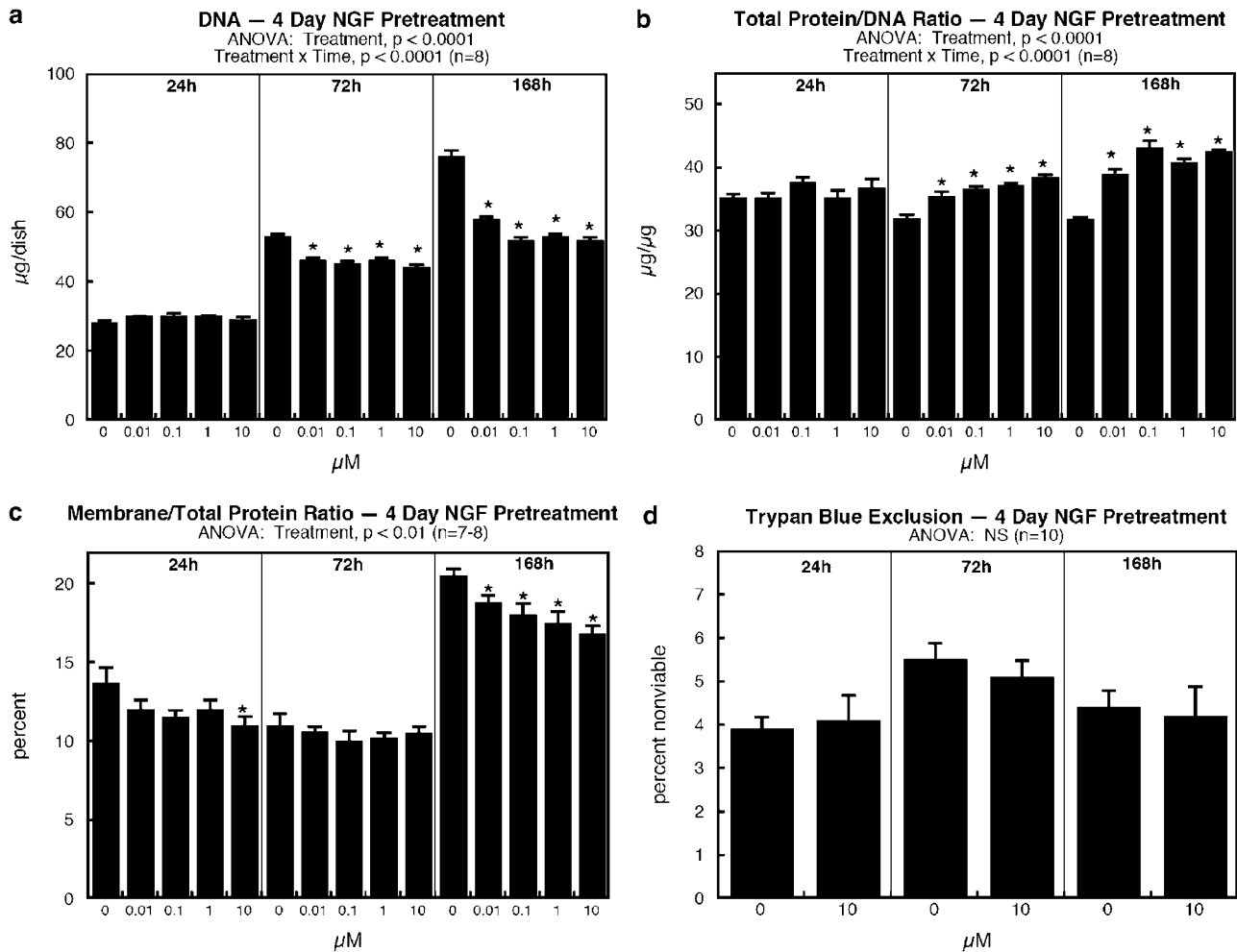
vs differentiating PC12 cells. Considering ChAT activity per cell (ie per unit DNA), 72 h of DEX exposure evoked a significant increase but only at relatively high concentrations (Figure 5a). The addition of NGF evoked a much larger increase in ChAT/DNA (note different scale for the right panel in Figure 5a). When DEX was included from the onset of differentiation (added simultaneously with NGF), it interfered with the promotional effect of NGF, evoking significant decrements in ChAT even at  $0.01 \mu\text{M}$ , with a maximal reduction of about 40% compared to control values. Nevertheless, it should be noted that DEX did not totally block the increase in ChAT caused by NGF-induced differentiation, since the values in the DEX groups were still more than twice those of the starting point in undifferentiated cells.

As both NGF and DEX alter cell size, it is important to consider the effects on ChAT expression relative to changes in other proteins. In undifferentiated cells, in contrast to the increase in ChAT seen on a per cell basis, DEX treatment reduced ChAT per unit protein (Figure 5b), indicating that, although it is promotional for ChAT, the enzyme is increased to a lesser extent than are the other proteins

associated with cell enlargement. NGF still increased ChAT robustly when considered as activity per unit protein (note different scale for the right panel in Figure 5b) and DEX remained strongly inhibitory for the expression of ChAT in the differentiating cells.

Given the known ability of high concentrations of DEX to increase TH in undifferentiated PC12 cells (Hagerty *et al*, 2001; Lucas and Thoenen, 1977), we evaluated the threshold for these effects by assessing TH after 72 h of exposure to the lowest DEX concentration,  $0.01 \mu\text{M}$ . There was unequivocal TH induction, whether assessed as activity per cell or per unit protein: control ( $n=11$ ),  $354 \pm 13$  pmol/h/ $\mu\text{g}$  DNA,  $18.5 \pm 0.7$  pmol/h/ $\mu\text{g}$  protein; DEX  $0.01 \mu\text{M}$  ( $n=10$ ),  $428 \pm 17$  ( $F_{1,19}=12.5$ ,  $p < 0.003$ ), and  $23.6 \pm 0.8$  ( $F_{1,19}=23.6$ ,  $p < 0.0001$ ), respectively.

Finally, we explored the dichotomy of DEX effects targeting the cholinergic and catecholaminergic phenotypes during differentiation by evaluating TH and ChAT activities in cells pretreated with NGF for 4 days, followed by the introduction of DEX. Again, we focused on the lowest DEX concentration ( $0.01 \mu\text{M}$ ), well below those achieved in therapeutic settings. NGF by itself evoked robust increases in



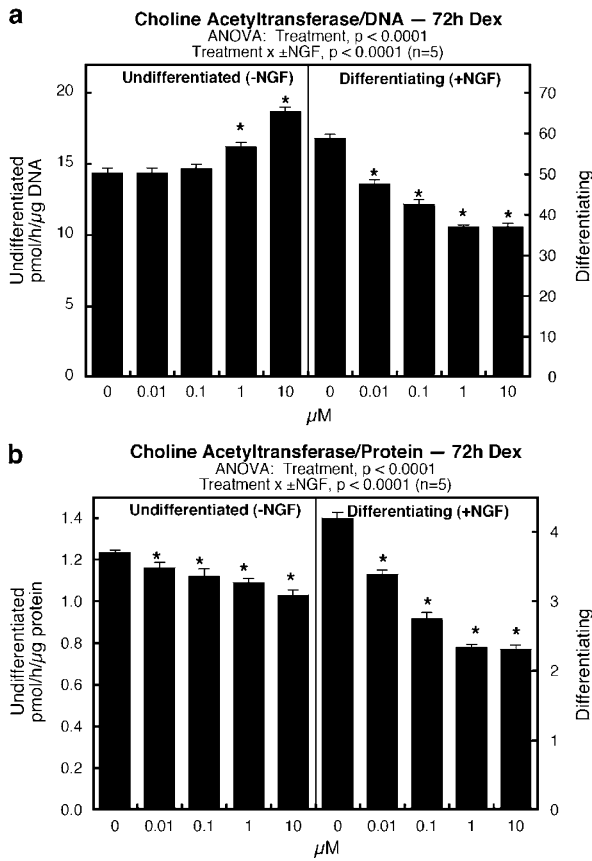
**Figure 4** Concentration–response and time–response relationships for the effects of DEX on cell number, cell size, and viability in cells undergoing NGF-induced differentiation, with DEX added after 4 days of NGF pretreatment: (a) DNA concentration, (b) total protein/DNA ratio, (c) membrane/total protein ratio, and (d) Trypan blue exclusion. Cells were plated at the same initial density for each time point (see Methods). The indicated times refer to DEX treatment, so that the 24 h point represents 5 days' total NGF exposure with inclusion of DEX just for the last 24 h. ANOVA across all concentrations and time points is shown at the top of each panel and the number of determinations at each time and concentration is shown in parentheses. \* Denotes individual values that differ from the corresponding control (0  $\mu\text{M}$ ). NS, not significant.

TH as compared to undifferentiated cells: ca. 900 pmol/h/ $\mu\text{g}$  DNA (Figure 6a) or 32 pmol/h/ $\mu\text{g}$  protein, as compared to 350 and 19 units, respectively. After 72 h exposure to DEX, TH was increased even further, whether evaluated per cell (Figure 6a) or per unit protein (Figure 6b). Notably, the effect on TH represented a 30% increase above that seen with NGF alone, a larger proportional increase than was obtained with the same DEX treatment in undifferentiated cells. In contrast, DEX treatment in mid-differentiation reduced ChAT only by about 10% (Figure 6a and b). For ChAT, then, the effect of introducing DEX in mid-differentiation was less deleterious than when it was included from the start of differentiation.

## DISCUSSION

Our results indicate that DEX directly targets neurodevelopment in stages ranging from cell replication and growth, through early differentiation, neurite formation, and

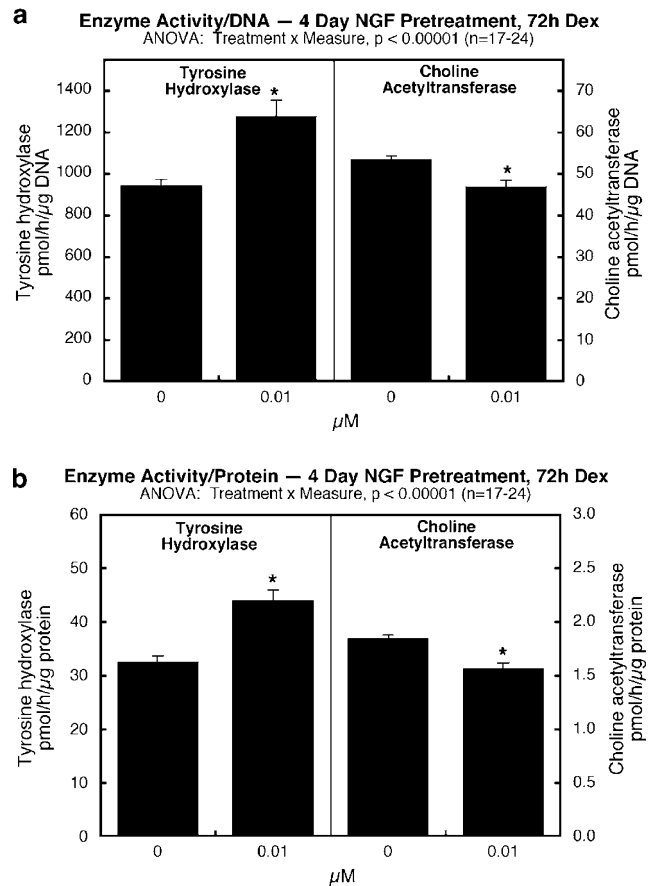
phenotypic specialization, and does so at concentrations well below those required for therapeutic intervention in preterm infants. There are a number of important ramifications of this basic finding. First, glucocorticoid use in preterm labor could contribute directly to adverse neurobehavioral outcomes, over and above the confounds of preterm delivery or of secondary effects on the maternal-fetal unit, maternal-neonatal interactions, or maternal or offspring neuroendocrine function. Second, the wide window of vulnerability of neurodevelopment to disruption by DEX means that adverse effects, such as those already noted for glucocorticoid administration *in vivo* (Bohn, 1984; Fuxe *et al*, 1994, 1996; Gilad *et al*, 1998; Gould *et al*, 1997; Kreider *et al*, 2005a, b, 2006; Maccari *et al*, 2003; Matthews, 2000; Matthews *et al*, 2002; McEwen, 1992; Meaney *et al*, 1996; Weinstock, 2001; Welberg and Seckl, 2001), are likely to be exerted at any stage in the period of 24–34 weeks of gestation in which these agents are recommended for use (Gilstrap *et al*, 1995). Third, the targeting of multiple stages of neurodevelopment means that the net outcome will differ



**Figure 5** Concentration–response relationships for the effects of DEX on choline acetyltransferase activity: (a) activity relative to the total number of cells (activity per unit DNA) or (b) relative to total protein (activity per  $\mu$ g protein). Each panel shows the effects of 72 h of DEX treatment in undifferentiated cells (left) and in cells treated simultaneously with NGF and DEX (right). ANOVA across all concentrations and both differentiation states ( $\pm$ NGF) is shown at the top of each panel and the number of determinations at each time and concentration is shown in parentheses. \* Denotes individual values that differ from the corresponding control (0  $\mu$ M). Note the different scales for undifferentiated and differentiating cells.

according to the maturational timetable for neurogenesis and differentiation in each brain region (Bayer *et al*, 1993). Finally, the promotion of the catecholaminergic phenotype at the expense of the cholinergic phenotype raises the possibility that, even in an anatomically ‘normal’ region, there may be functional miswiring, with inappropriate innervation of target cells by presynaptic terminals manufacturing the ‘wrong’ neurotransmitter. Accordingly, the results with the PC12 cell model will be discussed below in light of the potential contributions of each type of effect to these outcomes.

A direct comparison of *in vitro* concentrations to those achieved *in vivo* is inevitably complicated by the inherent differences in time course of action, the superimposition of diurnal changes in the intact animal but not the culture system, and the catabolism of glucocorticoids *in vivo* but not *in vitro*. Nevertheless, in undifferentiated PC12 cells undergoing active mitosis, we obtained clear inhibition of cell replication at DEX concentrations as low as 0.01  $\mu$ M, more than an order of magnitude below the concentrations



**Figure 6** Effects of 0.01  $\mu$ M DEX on TH and choline acetyltransferase activities in differentiating cells: (a) activity relative to the total number of cells (activity per unit DNA) or (b) relative to total protein (activity per  $\mu$ g protein). NGF was added for 4 days prior to the introduction of DEX, so that the 72 h point represents 7 days’ total NGF exposure with inclusion of DEX just for the last 72 h. ANOVA across treatments for both measures is shown at the top of each panel and the number of determinations for each measure is shown in parentheses. \* Denotes individual values that differ from the corresponding control (0  $\mu$ M). Note the different scales for TH (left) and choline acetyltransferase (right).

expected from typical therapy (Charles *et al*, 1993; Gilstrap *et al*, 1995; Osathanondh *et al*, 1977). The effect did not wane with prolonged exposure, in keeping with the situation in the developing fetus, where glucocorticoid receptors typically do not downregulate or desensitize, whereas they do in the adult (Ghosh *et al*, 2000). However, inhibition of DNA synthesis will have the greatest impact on final cell number when it occurs at high mitotic rates, when most of the cells are being acquired, whereas the same degree of inhibition will have less impact after mitosis has slowed. Again, this matches the regional specification of deficits of neural cells after DEX administration as reported for treatment *in vivo* (Bohn, 1984; Kreider *et al*, 2005a, 2006), reinforcing the likelihood that these, too, represent direct effects of DEX on the developing brain. Furthermore, DEX arrests mitotic activity at the  $G_0/G_1$  phase (Greenberg *et al*, 2002), which contributes to its therapeutic effect in the immature lung, switching pneumocytes from replication to differentiation and thus promoting surfactant production (Gross, 1990). Given the same mechanism, but a lower



concentration threshold for effects on neural cell division, this finding reinforces the idea that therapeutic use of glucocorticoids aimed at lung maturation will also target the developing brain.

We also observed cumulative deficits, as much as 50%, in the DNA content. The reduction in mitotic index thus contributes to an actual decline in the number of neural cells, just as observed anatomically with the use of antenatal steroids in animals and humans (Bohn, 1984; Kreider *et al*, 2005a, 2006; Murphy *et al*, 2001). Furthermore, we did not observe any decrease in cell viability, effectively ruling out the possibility that DEX reduces cell number through cytotoxic actions. In fact, prolonged exposure to high concentrations of DEX actually improved cell viability, a factor which may be important in considering its interaction with other events relevant to preterm infants, as will be discussed later. Further evidence of the specific targeting of cell replication in undifferentiated cells was provided by the fact that DEX did not inhibit cell growth and in fact, promoted perikaryal enlargement as indicated by a rise in the protein/DNA ratio and a decline in the membrane/total protein ratio.

To a limited extent, then, the actions of DEX share some of the differentiation characteristics of NGF, namely the promotion of cell growth at the expense of mitotic activity. However, the effects on differentiating cells indicate that the similarities end there. As a primary differentiation signal, NGF promotes neurite formation and membrane complexity, evidenced by the sharp increase in membrane/total protein ratio over and above the rise in total proteins reflecting overall cell growth. In contrast, DEX administration begun at the onset of differentiation reduced the membrane/total protein ratio, implying an inhibitory effect on neuritic outgrowth. Indeed, this effect showed a different concentration-response relationship from those on perikaryal growth or cell number, reinforcing the concept that it represents a separable outcome. Our results thus are in agreement with impaired neurite formation reported earlier at much higher concentrations (Unsicker *et al*, 1978) and as found more recently in our studies with DEX treatment *in vivo*, including doses below those in therapeutic use (Kreider *et al*, 2005a, 2006). Effects on this aspect of cell differentiation are likely to contribute to the observation that, even in brain regions that are largely finished with neurogenesis at the time of glucocorticoid therapy, DEX administration elicits reductions in volume (Murphy *et al*, 2001). Also, in this phase, as was true for undifferentiated cells, high DEX concentrations did not decrease cell viability and actually reduced the proportion of cells showing trypan blue staining.

With the initiation of differentiation, a number of the DEX effects showed a shift in sensitivity, magnitude, and direction of change, supporting the concept that the actions during phases in which neural cells are dividing differ substantially from those that occur during specialization. This conclusion is reinforced by our findings with the third treatment model, where DEX was introduced in mid-differentiation, after the cells had already undergone 4 days' NGF pretreatment. We still observed a cumulative deficit in cell number but to a smaller extent than in undifferentiated cells; again, this reflects the fact that the compromise of cell acquisition occurs at all stages, with the

greatest effect reflecting high rates of mitosis rather than a higher inherent sensitivity to DEX. However, both the inhibition of neurite outgrowth and the improvement in cell viability were less evident in differentiating cells, implying that these effects wane in mid-differentiation. Again, the results in the PC12 model recapitulate our findings for *in vivo* DEX treatments (Kreider *et al*, 2005a, 2006).

The results obtained for expression of the cholinergic *vs* catecholaminergic phenotype provide the strongest evidence for a critical period of vulnerability centered around early as opposed to later stages of differentiation. In undifferentiated cells, DEX increases TH activity because of the presence of a glucocorticoid response element in the promoter of the TH gene (Hagerty *et al*, 2001). Here, we found that DEX also increased ChAT expression on the basis of activity per cell but not on the basis of activity per unit protein. Again, this is consistent with a prodifferentiation signal from DEX administration, but one where the effects on the cholinergic phenotype are smaller than the promotional effects on growth and the consequent rise in total protein per cell. In turn, this dichotomy points out the importance of considering the effects of DEX when superimposed on large changes in cell number and size that have different concentration-response relationships, factors that have not been considered heretofore (Berse and Blusztajn, 1997; Lucas and Thoenen, 1977); obviously, these are critical to determining whether there are direct effects of DEX on different neuronal phenotypes when used in a therapeutic setting, over and above those that might be secondary to neural cell acquisition or growth. These considerations become more important with the rise in activity seen as differentiating cells specialize into the two different phenotypes. As expected (Teng and Greene, 1994), NGF-induced differentiation produced a large increment in both ChAT and TH activities, whether assessed on a per cell basis or relative to total protein. However, whereas DEX increased TH activity, it decreased ChAT, again at concentrations orders of magnitude below those required for clinical utility. Furthermore, the effects were greater when DEX was included from the start of differentiation than when cells were allowed to reach mid-differentiation before the beginning of DEX treatment. Accordingly, it is highly likely that DEX acts at the point at which cells first determine their phenotypic fate, to a much greater extent than it does once that decision has been made. For the cholinergic phenotype, the effect in differentiating cells is thus completely opposite to that in undifferentiated cells, where ChAT per cell was actually increased by DEX; the critical period thus involves a transition from promotional effects to inhibitory effects that then wane as differentiation proceeds. Furthermore, the fact that suppression of the cholinergic phenotype is achieved with promotion of the catecholaminergic phenotype raises the possibility of developing neurons making the 'wrong' phenotypic decision, thus producing a mismatch between the neurotransmitter contained within a presynaptic terminal and the receptor population at the postsynaptic site. Our results thus match closely the suppression of the enzymes responsible for acetylcholine biosynthesis and promotion of those responsible for catecholamine biosynthesis, and for the enhanced expression of adrenergic receptors noted previously for glucocorticoid effects on developing neurons

*in vivo* and *in vitro* (Berse and Blusztajn, 1997; Black, 1978; Hu *et al*, 1996; Kreider *et al*, 2005a, 2006). Again, these are likely to contribute to the long-term alterations in cholinergic synaptic proteins, synaptic activity and related behaviors seen in animal models of perinatal DEX administration (Kreider *et al*, 2005a, b, 2006) and cognitive dysfunction in humans (Yeh *et al*, 2004).

Finally, DEX evoked a significant improvement in cell viability in undifferentiated cells or at early stages of differentiation, although we evaluated only the highest DEX concentration in the present study. In contrast, NGF by itself reduced viability, a finding in keeping with the rise in oxidative stress that attends early stages of differentiation (Kato *et al*, 1997; Qiao *et al*, 2005). One possibility, then, is that DEX reduces oxidative stress in differentiating neural cells, an effect that could contribute to apparent protective actions of this treatment toward hypoxic-ischemic brain damage (Barks *et al*, 1991). On the other hand, a moderate degree of oxidative stress actually provides one of the normal ontogenetic signals that initiates differentiation and is also required to prevent apoptotic cell death (Ikeda *et al*, 2002; Kato *et al*, 1997; McCollum *et al*, 2004). The key factor may then be both the dose and timing of DEX administration. In fact, depending on the dose, treatment period, and brain region, DEX administration can also worsen hypoxia-induced damage (Carlos *et al*, 1991). Here, the improvement in cell viability was noted at a concentration well above those at which DEX interfered with neurogenesis and differentiation, suggesting that the protective effect may require doses far in excess of those that are themselves damaging to brain development. Clearly, future studies will need to address the dichotomies in vulnerable periods and dose-effect relationships for these competing outcomes.

There is increasing evidence that the benefits of glucocorticoid therapy of preterm infants comes at significant risk to future health (Blackmon *et al*, 2002; Coe and Lubach, 2005; Newnham, 2001; Raff, 2004; Seckl, 2004). Our results reinforce the fact that brain development is among the processes most vulnerable to glucocorticoids, with disruption of cell acquisition and differentiation achieved at concentrations well below those required for therapeutic interventions. The demonstration of these actions in neural cell cultures, reproducing all the essential findings from glucocorticoid treatment *in vivo* (Bohn, 1984; Fuxe *et al*, 1994, 1996; Gilad *et al*, 1998; Gould *et al*, 1997; Kreider *et al*, 2005a, b, 2006; Maccari *et al*, 2003; Matthews, 2000; Matthews *et al*, 2002; McEwen, 1992; Meaney *et al*, 1996; Weinstock, 2001; Welberg and Seckl, 2001), indicates that disrupted neurodevelopment is a direct glucocorticoid effect, not secondary to growth impairment, neuroendocrine disruption or other confounding actions in the fetus, neonate or mother. The successful prevention of respiratory distress in tens of thousands of preterm infants annually in the USA needs to be balanced against the adverse effects on brain development in the hundreds of thousands of individuals that receive the treatment.

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