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Developmental neurotoxicity resulting from pharmacotherapy of preterm labor, modeled in vitro: Terbutaline and dexamethasone, separately and together

Theodore A. Slotkin*, Samantha Skavicus, Frederic J. Seidler

Department of Pharmacology & Cancer Biology, Duke University Medical Center, Durham, NC, 27710, USA

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ABSTRACT

Terbutaline and dexamethasone are used in the management of preterm labor, often for durations of treatment exceeding those recommended, and both have been implicated in increased risk of neurodevelopmental disorders. We used a variety of cell models to establish the critical stages at which neurodifferentiation is vulnerable to these agents and to determine whether combined exposures produce a worsened outcome. Terbutaline selectively promoted the initial emergence of glia from embryonic neural stem cells (NSCs). The target for terbutaline shifted with developmental stage: at later developmental stages modeled with C6 and PC12 cells, terbutaline had little effect on glial differentiation (C6 cells) but impaired the differentiation of neuronotypic PC12 cells into neurotransmitter phenotypes. In contrast to the specificity shown by terbutaline, dexamethasone affected both neuronal and glial differentiation at all stages, impairing the emergence of both cell types in NSCs but with a much greater impairment for glia. At later stages, dexamethasone promoted glial cell differentiation (C6 cells), while shifting neuronal cell differentiation so as to distort the balance of neurotransmitter phenotypes (PC12 cells). Finally, terbutaline and dexamethasone interacted synergistically at the level of late stage glial cell differentiation, with dexamethasone boosting the ability of terbutaline to enhance indices of glial cell growth and neurite formation while producing further decrements in glial cell numbers. Our results support the conclusion that terbutaline and dexamethasone are directly-acting neuroteratogens, and further indicate the potential for their combined use in preterm labor to worsen neurodevelopmental outcomes.

1. Introduction

Preterm delivery is a leading cause of perinatal morbidity and mortality, and of lasting neurobehavioral deficits. In the U.S., approximately 10% of live births occur preterm, and the rates are rising (Martin et al., 2017), reversing a longstanding trend. Glucocorticoids, notably dexamethasone, are the consensus treatment for preterm labor occurring between 24 and 34 weeks of gestation, so as to prevent neonatal respiratory distress syndrome (Gilstrap et al., 1995). β-Adrenergic receptor (βAR) agonists, particularly β2AR-selective drugs like terbutaline, are also typically given for up to 48 h, with the intent of inhibiting uterine contractions for a sufficient period to enable the glucocorticoids to act (Haas et al., 2014).

In clinical practice, both glucocorticoids and β2AR agonists are often given for much longer durations than those that are recommended, with dexamethasone administered in repeated courses (Dammann and Matthews, 2001) and terbutaline given continuously over a period of many weeks (Elliott and Morrison, 2013; Perna et al., 2014). It is increasingly clear that there are harmful consequences to these therapies. The developmental neurotoxicity of glucocorticoids is well established, as these agents disrupt neural cell replication and differentiation, producing synaptic deficiencies that ultimately result in neurobehavioral, endocrine and cardiovascular disorders (Cavalieri and Cohen, 2006; Drake et al., 2007; Meyer, 1985; Moritz et al., 2005; Pryce et al., 2011; Rokyta et al., 2008; Tegethoff et al., 2009); adverse outcomes have now been verified in children prenatally exposed to glucocorticoids (Crowther et al., 2007; Hirvikoski et al., 2007; Needelman et al., 2008; Newnham, 2001; Peltoniemi et al., 2011). However, the potential for neurobehavioral deficits resulting from terbutaline treatment are less well recognized. βARs, including the β2 subtype, are expressed prominently throughout the developing brain (Harden et al., 1977; Lorton et al., 1988) and control the balance between neural cell

Abbreviations: ANOVA, analysis of variance; βAR, β-adrenergic receptor; DAPI, 4′,6-diamidino-2-phenylindole; GFAP, glial fibrillary acidic protein; MAP2, microtubule-associated protein 2; NSC, neural stem cell

* Corresponding author at: Box 3813 DUMC, Duke Univ. Med. Ctr., Durham, NC, 27710, USA.
E-mail address: t.slotkin@duke.edu (T.A. Slotkin).

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replication and apoptosis (Garofolo et al., 2003; Hodges-Savola et al., 1996; Slotkin et al., 1988; Zhu et al., 1998, 1999). In animal models, developmental exposure to terbutaline leads to glial activation (Rhodes et al., 2004; Zerrate et al., 2007), a hallmark of neurotoxicity (O'Callaghan, 1993), culminating in abnormalities of brain structure, impaired synaptic function and behavioral deficits (Aldridge et al., 2005; Meyer et al., 2005; Rhodes et al., 2004; Slotkin et al., 1989; Slotkin and Seidler, 2007; Zerrate et al., 2007). In clinical studies, prolonged use of terbutaline to prevent preterm delivery increases the risk of autism, learning disabilities and neuropsychiatric disorders in the offspring (Connors et al., 2005; Croen et al., 2011; Perna et al., 2014; Pitzer et al., 2001; Witter et al., 2009), particularly in individuals with β2AR polymorphisms that impair the ability to desensitize the receptors (Connors et al., 2005). Indeed, many of the structural, synaptic functional and behavioral features of autism are recapitulated by developmental exposure to terbutaline in animal models (Bercum et al., 2015; Slotkin, 2008; Slotkin and Seidler, 2013; Zerrate et al., 2007). In acknowledgment of these and other problems, and in light of the lack of efficacy of terbutaline for maintenance tocolysis, the U.S. Food and Drug Administration issued a “black box” warning against terbutaline use beyond an initial 48 h period (U.S. Food and Drug Administration, 2011a,b).

In the current study, we explored two aspects of the developmental neurotoxicity resulting from pharmacotherapies of preterm labor: (1) the mechanism of action and targets for adverse effects of terbutaline compared to dexamethasone, and (2) the effects of combination therapy, specifically the potential for these two agents to produce synergistic effects on neural cell development. We made use of in vitro models that encompass two “decision nodes” that define critical stages of neurodifferentiation that are vulnerable to toxicant injury (Slotkin et al., 2016, 2017a). First, we used neural stem cells (NSCs) derived from rat neuroepithelium on embryonic day 14, when separation into neurons and glia is determined, so as to examine how the treatments divert neural fate toward or away from neuronal and glial phenotypes (Slotkin et al., 2016, 2017a). In addition to this early decision node, we also examined a later node after the commitment to glial or neuronal phenotypes, characterized by effects on neuronotypic PC12 cells or gliotypic C6 cells, both of which are derived from the same species (rat) as the NSCs. PC12 cells are already committed to a neuronal phenotype and their subsequent differentiation involves selection of one of two neurotransmitters, dopamine or acetylcholine (Teng and Greene, 1994). C6 cells closely mimic later stage differentiation of astroglia, including the transition from cell replication to cell enlargement and neurite formation (Garcia et al., 2005).

Importantly, βARs, the target for terbutaline, are present on neural precursor cells and regulate their proliferation and differentiation (Ishizuka et al., 2012; Jhaveri et al., 2010, 2014; Masuda et al., 2012). Likewise, βARs, including the β2AR subtype, are present in both PC12 and C6 cells (Haack et al., 2010; Jia et al., 2015; Neve and Molinoff, 1986; Zhong and Minneman, 1993). We have already used these cell models to elucidate the mechanism of action and critical stages of vulnerability to a variety of developmental neurotoxicants (Qiao et al., 2001; Slotkin et al., 2016, 2017a,b) and here, we used the same approach for terbutaline alone and in combination with dexamethasone.

2. Materials & methods

2.1. NSC cultures and treatments

The techniques for NSC preparation, culturing and assays have all appeared previously (Slotkin et al., 2016). Primary neural stem cells (passage zero; MTI-GlobalStem, Gaithersburg, MD) were isolated from rat cortical neuroepithelium on embryonic day 14 and were frozen in DMEM/F-12 medium with N2 supplement (MTI-GlobalStem) and 10% dimethylsulfoxide. Cells were thawed and plated at 35,000 cells/cm^2 on 12 mm coverslips pre-coated with poly-L-ornithine, contained in 24-well culture plates. The culture medium consisted of DMEM/F-12, GlutaMAX™ with N2 Supplement, 20 ng/ml human fibroblast growth factor and 20 ng/ml epidermal growth factor (all from MTI-GlobalStem). Cultures were maintained in a humidified incubator at 37° C with 5% CO_2. Twenty-four hours later, the medium was changed to initiate spontaneous differentiation by eliminating the two growth factors, with the addition of 200 μM ascorbic acid and the test compounds, terbutaline hemisulfate, dexamethasone phosphate and *d,l*-propranolol HCl (all from Sigma-Aldrich, St. Louis, MO). After 3 days, half the medium was replaced, including the indicated treatment agents, and the exposures were continued for another 3 days (6 days total exposure). Concentration ranges for all agents were chosen based on prior work with in vitro models (Haack et al., 2010; Jameson et al., 2006a; Jia et al., 2015; Lemmens et al., 2017; Markus et al., 2010; Slotkin et al., 2016; Zhong and Minneman, 1993).

2.2. NSC assays

At the end of the exposure period, the medium was removed and the coverslips washed with Dulbecco's phosphate-buffered saline, fixed with 4% paraformaldehyde and washed three times with Dulbecco's phosphate-buffered saline containing additional Ca^{2+} and Mg^{2+}. Cells were permeabilized for 30 min in phosphate buffered saline containing 0.2% Triton X-100, washed three times with phosphate buffered saline (without Triton), followed by a 30 min incubation in BlockAid™ solution. Cells expressing a neuronal or astroglial phenotype were identified by immunocytochemistry according to manufacturers' instructions, using microtubule-associated protein 2 (MAP2) for neurons and glial fibrillary acidic protein (GFAP) for astroglia. After permeabilization, the coverslips were incubated for 1 h at room temperature using rabbit anti-MAP2 (1:200) and rat anti-GFAP (1:20) in BlockAid™. Coverslips were rinsed four times with phosphate-buffered saline and then incubated for 1 h at room temperature with the appropriate fluor-conjugated secondary antibodies (donkey anti-rabbit IgG Alexa Fluor 647 and goat anti-rat IgG Alexa Fluor 555) diluted 1:400 in BlockAid™. After an additional five rinses with phosphate buffered saline, coverslips were incubated for 5 min with 300 nM 4′,6-diamidino-2-phenylindole (DAPI) nucleic acid stain to label individual cells. Coverslips were rinsed three times with phosphate buffered saline and mounted onto glass slides using ProLong Diamond Antifade mountant. All reagents for the NSC assays were obtained from MTI-GlobalStem except for rabbit anti-MAP2 (EMD Millipore, Billerica MA) and antifade mountant (Thermo Fisher Scientific, Waltham, MA).

Images of 3 to 4 fields/slide (each field = 3.22×10^5 μm^2) were captured using a Zeiss Axio Imager widefield fluorescence microscope with 200× magnification and quantified for total cells (DAPI-positive stain for nuclei); across the multiple fields in a given culture, thousands of cells were counted. Each cell was then examined to see if it expressed a neuronal phenotype (MAP2-positive) or a glial phenotype (GFAP-positive). A cell was counted as positive only when the stain for a given phenotype coincided with a DAPI-stained nucleus. Values were averaged across the fields to render a single value for each culture.

2.3. PC12 cells

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. As described previously (Qiao et al., 2003; Song et al., 1998), PC12 cells (American Type Culture Collection CRL-1721, obtained from the Duke Comprehensive Cancer Center, Durham, NC) were seeded (3×10^6 cells) onto 100 mm poly-D-lysine-coated plates in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% horse serum (Sigma-Aldrich), 5% fetal bovine serum (Sigma-Aldrich), and 50 μg/ml penicillin streptomycin (Invitrogen, Carlsbad, CA). Incubations were carried out with 5% CO_2 at 37 °C, standard conditions for PC12 cells. Twenty-four hours after plating, we initiated