Terbutaline Is a Developmental Neurotoxicant: Effects on Neuroproteins and Morphology in Cerebellum, Hippocampus, and Somatosensory Cortex

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ABSTRACT

β₂-Adrenoceptor agonists, especially terbutaline, are widely used to arrest preterm labor, but they also cross the placenta to stimulate fetal β-adrenoceptors that control neural cell differentiation. We evaluated the effects of terbutaline administration in neonatal rats, a stage of neurodevelopment corresponding to human fetal development. Terbutaline administered on postnatal days PN2 to 5 elicited neurochemical changes indicative of neuronal injury and reactive gliosis: immediate increases in glial fibrillary acidic protein and subsequent induction of the 68-kDa neurofilament protein. Quantitative morphological evaluations carried out on PN30 indicated structural abnormalities in the cerebellum, hippocampus, and somatosensory cortex. In the cerebellum, PN2 to 5 terbutaline treatment reduced the number of Purkinje cells and elicited thinning of the granular and molecular layers. The hippocampal CA3 region also displayed thinning, along with marked gliosis, effects that were restricted to females. In the somatosensory cortex, terbutaline evoked a reduction in the proportion of pyramidal cells and an increase in smaller, nonpyramidal cells; again, females were affected more than males. Although abnormalities were obtained with later terbutaline treatment (PN11 to 14), in general the effects were smaller than those seen with PN2 to 5 exposure. Our results indicate that terbutaline is a neurotoxicant that elicits biochemical alterations and structural damage in the immature brain during a critical period. These effects point to a causal relationship between fetal terbutaline exposure and the higher incidence of cognitive and neuropsychiatric disorders reported for the offspring of women receiving terbutaline therapy for preterm labor.

Preterm labor occurs in up to 20% of all pregnancies, with preterm delivery, a leading cause of neonatal morbidity and mortality, resulting in about one-half the cases (Berkowitz and Papiernik, 1993). β₂-Adrenoceptor (β₂AR) agonists, especially terbutaline, are frequently administered as tocolytics (Lam et al., 1998), although this use is not endorsed by the manufacturer and is specifically “off-label”. In addition to blocking uterine contractions, terbutaline penetrates the placenta to activate fetal βARs, eliciting a number of adverse neonatal effects, including alterations in glucose metabolism and tachycardia (for review, see Slotkin et al., 2003). In the long-term, offspring of women treated with terbutaline may show impaired school performance (Hadders-Algra et al., 1986; Feenstra, 1992), cognitive dysfunction, and increased incidence of psychiatric disorders (Pitzer et al., 2001). The neurobehavioral consequences of fetal exposure to terbutaline are obviously a major biomedical and societal concern and set the stage for the current study.

Terbutaline enters the fetal brain and activates β₂ARs present on developing neurons and glia, and neurochemical findings suggest that terbutaline alters patterns of neural cell replication and differentiation as well as later events such as synaptogenesis (Slotkin et al., 2003). Arrest of uterine contractions occurs because β₂ARs increase intracellular levels of cyclic AMP. However, cyclic AMP also initiates the switch from cell replication to differentiation (Slotkin et al., 2003), and excessive or inappropriate β₂AR stimulation can elicit cell injury and death (Joseph et al., 1983; Teerlink et al., 1994; Communal et al., 1998; Shizukuda et al., 1998; Gu

ABBREVIATIONS: βAR, β-adrenoceptor; PN, postnatal day; GFAP, glial fibrillary acidic protein; NF68, neurofilament protein 68 kDa; ANOVA, analysis of variance.
et al., 2000; Yan et al., 2000; Singh et al., 2001). Whereas mature cells are protected from prolonged βAR stimulation by their ability to uncouple receptors from the production of cyclic AMP, in the fetus or neonate, βARs are resistant to desensitization (Slotkin et al., 2003). In the case of terbutaline, we found that even repeated administration of high doses failed to desensitize βARs in the fetal/neonatal heart, liver, or brain, and instead elicited an increase in responsiveness, thus augmenting the net effect on cyclic AMP production (Slotkin et al., 2003).

Despite these findings, to date there have been no studies assessing the morphological consequences of developmental exposure to terbutaline, nor have there been examinations of the types of neural cells that might be targeted by this agent. βARs are prominently expressed on both developing neurons and glia (Lorton et al., 1988; Hodges-Savola et al., 1996), so that terbutaline is likely to target either cell type. Accordingly, we conducted quantitative morphometric studies in the cerebellum, hippocampus, and somatosensory cortex after exposure of neonatal rats to terbutaline in two developmental periods, postnatal days PN2 to 5 and PN11 to 14. Because the rat is an altricial species, these windows correspond to human neurodevelopment in the mid- to late gestational periods. In addition, we assessed neuroprotein markers specific for astroglia (glial fibrillary acidic protein) and neurons (neurofilament protein 68-kDa, NF68) (O’Callaghan, 1988; Escurat et al., 1990).

Materials and Methods

Animals and Treatments. All experiments were carried out in accordance with the declaration of Helsinki and with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. Timed pregnant Sprague-Dawley rats (Charles River, Raleigh, NC) were housed in breeding cages with a 12-h light/dark cycle and free access to food and water. Pups from all litters were randomized on the day after birth and redistributed to the dams with litter sizes of 10 pups to ensure standardized nutrition and maternal care. Pups were then given daily subcutaneous injections of 10 mg/kg terbutaline sulfate (Sigma-Aldrich, St. Louis, MO) or equivalent volumes of saline on either PN2 to 5 or PN11 to 14. These regimens elicit robust βAR stimulation in the neonate (Slotkin et al., 2003) while retaining selectivity toward β2ARs (Slotkin et al., 2001). For tocolytic therapy in humans, doses typically lie in the range of 0.5 mg/kg/day but can also be as high as 1 to 2 mg/kg/day (Lam et al., 1998; Goldenberg, 2002). In light of the fact that terbutaline has a much shorter half-life in the light of the fact that terbutaline has a much shorter half-life in the neonate (Slotkin et al., 2003) while retaining selectivity toward β2ARs (Slotkin et al., 2001). For tocolytic therapy in humans, doses typically lie in the range of 0.5 mg/kg/day but can also be as high as 1 to 2 mg/kg/day (Lam et al., 1998; Goldenberg, 2002). In light of the fact that terbutaline has a much shorter half-life in the rat (Tegner et al., 1984), we used a proportionally higher dose. Within a given litter, all pups received the same treatment (saline or terbutaline) to avoid the possibility that the dams might discriminate between control and treated pups. Experiments were conducted using 6 animals in each treatment group at each age for morphometric studies, and 12 animals per group for neurospecific proteins, divided equally between males and females. No more than one male and one female were taken from the same litter. Animals were weaned on PN21.

Neurospecific Proteins. Twenty-four hours after the last terbutaline injection (PN6 or PN15 for the two regimens), animals were decapitated and brains were dissected into three regions: blunted cuts were made through the cerebellar peduncles, whereupon the cerebellum (including flocculi) was lifted from the underlying tissue; a cut was then made rostral to the thalamus to separate the forebrain from the midbrain + brainstem. This dissection, which follows the planes of the neonatal rat brain, includes the striatum, hippocampal formation, and neocortex within the area designated as forebrain, whereas the region designated as midbrain + brainstem includes the thalamus, hypothalamus, colliculi, pons, brainstem, and medulla oblongata (but not cervical spinal cord). For experiments on PN30, the forebrain was further divided into cerebral cortex, hippocampus, and striatum, and the midbrain and brainstem were separated from each other. Tissues were frozen immediately in liquid nitrogen and maintained at ~45°C.

Neurospecific proteins were assayed by dot-immunobinding (Gar-elia et al., 2002). Tissues were homogenized with a sonic probe (Heat Systems-Ultrasonics, Inc., Plainview NY) in 9 volumes of hot 1% sodium dodecyl sulfate (Bio-Rad, Hercules, CA) and were diluted in 120 mM KCl, 20 mM NaCl, 2 mM MgCl₂, 2 mM NaHCO₃, 0.7% Triton X-100, 0.2% NaN₃, and 5 mM HEPES (pH 7.4). Ten-microliter aliquots containing 0.75 to 7.5 μg of protein were blotted onto pre-washed nitrocellulose membranes (0.2 μm; Bio-Rad). Blots were dried and fixed in 25% isopropanol, 10% acetic acid, and 65% water, incubated for 5 min in Tris-buffered saline (200 mM NaCl, 50 mM Tris, pH 7.4) and treated for 1 h with a blocking solution of 0.5% gelatin (enzyme immunoasay grade; Bio-Rad) in Tris-buffered saline. Blots were then incubated for 2 h in blocking solution containing 0.1% Triton X-100 with addition of the appropriate antibodies: rabbit polyclonal anti-NF68 (diluted 1:4000; Chemicon International, Temecula, CA) or rabbit anti-bovine polyclonal anti-GFAP (diluted 1:500; DAKO, Carpinteria, CA). Blots were rinsed with blocking solution for 30 min, followed by addition of 30 μCi of 125I-

 Protein A (specific activity, 370 Ci/mm; PerkinElmer Life Sciences, Boston, MA) and 0.1% Triton X-100 for 1 h. Blots were washed repeatedly, dried overnight, and counted for radioactivity. Each blot included serial dilutions of adult brainstem, which was then used to construct a standard curve to normalize the values across blots. Thus, although values are reported in relative units, quantitative

![Fig. 1. A representative hippocampal section at PN30, showing the segments sampled for CA1, CA3, and dentate gyrus (DG). Scale bar, 200 μm.](image-url)
comparisons across treatments, regions, and ages could be carried out.

Histopathological Tissue Processing. Animals were anesthetized with euthasol (1.25 ml/kg) and perfused through the heart with saline containing 0.1% heparin followed by 4% paraformaldehyde in Tris buffer. The brains were removed, postfixed in 4% paraformaldehyde for 24 h, and transferred to 70% ethanol. Brains were hemisected by a midline incision through the corpus callosum, and then a 4- to 5-mm-thick coronal slab of one side was obtained at the level of the rostral limit of the anterior commissure and the caudal end of the median eminence; each slab thus contained the parietal cortex and anterior portion of the dorsal hippocampus. Additionally, the cerebellum was removed from the rest of the brain. For sectioning, we maintained a coronal orientation, and cut 8-μm-thick sections which were mounted on glass slides and stained with hematoxylin and eosin. For each animal, two slides, each containing three tissue sections, were examined.

Morphometry. The slides were coded and the examiner was blinded to the animal number, sex, and treatment group. The validity of the morphometric measurements was confirmed by evaluating tissue shrinkage at the level of the anterior commissure; there were no treatment-related differences in the tissue weight, the number of sections obtained, or the overall thickness of the regions, nor were there any artifacts indicative of nonuniform shrinkage (data not shown). To ensure uniform sampling, we maintained the septotemporal and mediolateral orientations and used the positions of blood vessels as landmarks.

Morphometric measurements were carried out using an Olympus RO11 digital camera and BX51 microscope, selecting a field within

<table>
<thead>
<tr>
<th>Age</th>
<th>Region</th>
<th>GFAP</th>
<th>NF68</th>
</tr>
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<tbody>
<tr>
<td>PN6</td>
<td>Midbrain + brainstem</td>
<td>9.2 ± 0.3</td>
<td>47 ± 2</td>
</tr>
<tr>
<td></td>
<td>Forebrain</td>
<td>13 ± 1</td>
<td>34 ± 1</td>
</tr>
<tr>
<td></td>
<td>Cerebellum</td>
<td>40 ± 2</td>
<td>41 ± 1</td>
</tr>
<tr>
<td>PN15</td>
<td>Midbrain + brainstem</td>
<td>91 ± 3</td>
<td>86 ± 3</td>
</tr>
<tr>
<td></td>
<td>Forebrain</td>
<td>85 ± 4</td>
<td>39 ± 1</td>
</tr>
<tr>
<td></td>
<td>Cerebellum</td>
<td>31 ± 1</td>
<td>79 ± 3</td>
</tr>
<tr>
<td>PN30</td>
<td>Midbrain</td>
<td>72 ± 3</td>
<td>131 ± 3</td>
</tr>
<tr>
<td></td>
<td>Forebrain</td>
<td>24 ± 1</td>
<td>59 ± 2</td>
</tr>
<tr>
<td></td>
<td>Cerebellum</td>
<td>61 ± 2</td>
<td>50 ± 2</td>
</tr>
<tr>
<td></td>
<td>Striatum</td>
<td>21 ± 1</td>
<td>84 ± 2</td>
</tr>
<tr>
<td></td>
<td>Cerebellum</td>
<td>98 ± 7</td>
<td>56 ± 3</td>
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Fig. 2. Effects of terbutaline on neuroproteins assessed 24 h after the last terbutaline injection and on PN30. A, GFAP in animals treated on PN2 to 5. B, GFAP in animals treated on PN11 to 14. C, NF68 in animals treated on PN2 to 5. D, NF68 in animals treated on PN11 to 14. Values are reported for males and females combined because of the absence of interactions of treatment × sex or treatment × sex × region. ANOVA across regions is within each panel; separate statistical testing for each region was not carried out because of the absence of treatment × region interactions. Mbbs, midbrain + brainstem; fb, forebrain; cb, cerebellum; mb, midbrain; bs, brainstem; hp, hippocampus; st, striatum; cx, cerebral cortex.
the specified cell layer, and counting all the neuronal profiles shown on the monitor. Morphometry was conducted with NIH Image 1.62f software (http://rsb.info.nih.gov/nih-image; accessed 20 August 2003). The values obtained for each parameter in a given animal were then averaged to produce a single number, so that the “n” in each case represents the number of animals, not the number of cells or sections. For all measurements, we selected six sections 40 µm apart, making sure to choose the same location for each field, using 100× magnification for evaluations of layer parameters and 400× for cell profiles. Because the thickness of each section was 8 µm, this ensures that the same cell was not counted twice, given that the typical cell diameter is smaller than 25 µm. In addition to measurements of neuronal cell diameter, perimeter (minimum diameter and perimeter in the case of noncircular cells), and perikaryal area, we counted neurons and glia in each field of 0.09 mm². To standardize the measurements, only neurons exhibiting a hematoxylin-stained nucleus with a clear nucleolus were counted, and the values were corrected for the total area of each section. Measurements of cell size were restricted to the neuronal cell population, whereas glia were counted separately in each field; glia were readily distinguished from neurons by their size, nuclear shape, cytoplasm, location, and characteristic staining (Kaur et al., 1989).

Figure 1 shows the loci examined for hippocampal CA1, CA3, and dentate gyrus. For the cerebellum, lobule 5 was examined and for the somatosensory cortex, a random field was chosen within layer 5, which was identified by the size and packing density of the constituent cells, and its characteristic, large pyramidal neurons (Paxinos and Watson, 1998). In addition to measurements of cellular parameters, at three locations for each slide (a total of six measurements per animal), we measured the thickness of the pyramidal cell layers of hippocampal CA1 and CA3, the granule cell layer of the dentate gyrus, and the cerebellar granular and molecular layers (Vaudry et al., 1999).

**Statistical Analysis.** Data were evaluated as means and standard errors, considering each animal as an experimental subject. For convenience, some results are shown as the percentage of change from control values but statistical evaluations were always conducted on the original data. To avoid type I statistical errors in subdividing the data into the different measures, brain regions, ages, and sexes, we first performed global analyses of variance (ANOVAs) incorporating all factors (treatment, age, sex, and region) on data groupings corresponding to the four classes of measurements: neuroproteins, hippocampal morphology, cerebellar morphology, and somatosensory cortex morphology. Because each animal contributed multiple assessments in each category, the various determinations were treated as repeated measures. This initial test indicated treatment effects that differed among the different measures, so data were then examined separately for each measure, again using a multivariate ANOVA, followed by lower order ANOVAs as dictated by the interactions of treatment with the other variables. In addition, the frequency distribution of cell sizes in layer 5 of the somatosensory cortex was evaluated using χ² for observed versus expected frequencies. Significance was evaluated at the level of p < 0.05 for all main effects; however, for interactions at p < 0.1, we also examined whether lower order main effects were detectable after subdivision of the interactive variables.

**Results**

Neither terbutaline regimen elicited significant deficits in body or brain region weights (data not shown). Nevertheless, there were significant alterations in neuroproteins and in morphology in every brain region that was evaluated, as detailed below.

**Neuroproteins.** In accordance with earlier results (Garcia et al., 2002, 2003), GFAP and NF68 expression in the developing brain increased markedly between PN6 and PN15 (Table 1). On PN30, there were large disparities among the various brain regions, with highest GFAP expression in the brainstem and hippocampus. In contrast, for NF68, the rank order was brainstem > striatum > midbrain > cerebral cortex ≧ cerebellum ≧ hippocampus. None of the regions showed significant overall sex differences or interactions of sex × other variables.

Before evaluating the effects of treatment on each individual neuroprotein marker, we performed a global ANOVA on the entire data set. ANOVA indicated a main treatment effect (p < 0.003) that differed between GFAP and NF68 and that depended upon the brain region (treatment × neuroprotein × region interaction; p < 0.008). Accordingly, the effects on GFAP and NF68 were assessed separately for the two different regional groupings (midbrain + brainstem, forebrain, cerebellum 24 h after the last injection; six subregions on PN30). GFAP was significantly elevated immediately after the early postnatal treatment regimen (PN2–5), displaying a main treatment effect without interactions with sex or brain region 24 h after the last injection (Fig. 2A); by PN30, significant differences could no longer be detected. In contrast, no immediate post-treatment increase was seen at the comparable point (PN15) in animals receiving terbutaline on PN11 to 14, nor were there any significant alterations on PN30 (Fig. 2B). For NF68, we did not observe any significant
effects 24 h after the last terbutaline injection with either regimen but on PN30 there were overall increases across all brain regions with both treatment regimens, again without interactions with sex or region (Fig. 2, C and D).

**Cerebellar Morphology.** All morphometric evaluations were conducted on PN30, a point at which neurogenesis and synaptogenesis are largely completed in most brain regions (Rodier, 1988). Across all Purkinje cell parameters (perikaryal area, perimeter, diameter, and number of cells), ANOVA indicated a significant effect of terbutaline treatment that differed according to whether exposure occurred on PN2 to 5 or PN11 to 14, differed among the various measures, and differed between males and females ($p < 0.07$ for treatment × regimen, $p < 0.03$ for treatment × regimen × measure, $p < 0.08$ for treatment × sex × measure). The PN2 to 5 regimen elicited greater damage (treatment, $p < 0.04$; treatment × measure, $p < 0.1$), whereas there were no significant differences with the PN11 to 14 regimen. Within the PN2 to 5 treatment group, the significant differences were confined to males (treatment × measure, $p < 0.007$; females, not significant). The statistical differences reflected a substantial decrease (44% ± 5%) in the number of Purkinje cells per unit area, with small increases in perikaryal area (7.3% ± 2.8%). Although the overall analysis across all Purkinje cell parameters did not indicate significant effects in females, they also showed a reduction in the number of Purkinje cells (29% ± 15%) that could not be distinguished statistically from the significant effect found in males. Representative photomicrographs (Fig. 3) show the decrease in Purkinje cell numbers in males from the PN2 to 5 treatment group.

Terbutaline treatment also altered the thickness of the...
cerebellar lobules, as assessed by measurements conducted on lobule 5 at a standard distance from the apex. Across the two regimens, there was a significant treatment × regimen interaction (p < 0.02), necessitating separation of the values according to the terbutaline treatment period. For the PN2 to 5 treatment group, there was a robust reduction (26%) in net lobule thickness, as well as reductions across both the granular and molecular layers (p < 0.02 for the main treatment effect; Fig. 4A); although the effect tended to be larger in the molecular layer, there was no statistical distinction between the layers (no treatment × layer interaction). Representative photomicrographs readily illustrate this effect (Fig. 4, B and C). In contrast, the PN11 to 14 treatment did not show significant alterations.

Hippocampal Morphology. Repeated measures ANOVA incorporating all three hippocampal regions (CA1, CA3, and dentate gyrus), all perikaryal parameters (area, perimeter, and diameter), the number of neurons and glia, and layer thickness, indicated treatment effects that depended on the region of the hippocampus (treatment × region; p < 0.05) as well as sex and measure (treatment × sex × measure, p < 0.1; treatment × sex × measure, p < 0.08). Accordingly, we examined each hippocampal region separately, looking for treatment main effects and interactions with other variables. Across all measures, there were no significant effects in CA1 (Fig. 5A) or the dentate gyrus (Fig. 5B), but there were statistically robust alterations in CA3 (p < 0.0003 for treatment × measure, p < 0.02 for treatment × sex × measure). Separating these values by sex, we found significant differences only in females (p < 0.0003 for treatment × measure).

Terbutaline exposure had a significant effect on the relative numbers of neurons and glia and on layer thickness in female CA3 (Fig. 5C). In contrast to the alterations seen in the cerebellum, all the changes in CA3 showed only main treatment effects without specificity for a specific regimen (no treatment × regimen interaction). Across both regimens, terbutaline caused a small reduction in the number of neurons that did not achieve significance but for glia, there were robust, statistically significant increases. The combination of reduced neurons and increased glia evoked an even greater change in the glia/neuron ratio. The thickness of the CA3 layer was significantly reduced by about 10%. It should be noted that, although males did not show a statistically significant overall effect of terbutaline (data not shown), one of the biomarkers (number of glial cells) showed an increase of about 20% for the PN11 to 14 regimen, about one-half the increase seen in females. There were no significant alterations in the three parameters of perikaryal size (data not shown).

Cell Size Distribution in the Somatosensory Cortex.

The somatosensory cortex contains two major classes of neurons: pyramidal neurons and smaller, nonpyramidal neurons (Miller, 1986). Thus, comparing a simple average of perikaryal dimensions is not appropriate in this region. Instead, we examined the distribution of cell sizes to look for shifts in the proportions of pyramidal and nonpyramidal neurons (Roy et al., 2002). Across both treatment regimens and both sexes, we found a significant alteration in the size distribution using cell perimeter, diameter, or perikaryal area (p < 0.0001 for each by $\chi^2$ analysis), with the net shift from larger to smaller cells. Accordingly, one of the parameters (perikaryal area) was chosen to examine effects for both regimens and both sexes.

Male rats exposed to terbutaline on PN2 to 5 showed a shift in the distribution of somatosensory cortical neurons toward...
smaller cells, evidenced by elevations in the proportions of cells with areas <128 μm² and a reduction in cells with areas >128 μm² (Fig. 6A). The shift was much more robust in females (p < 0.0001 versus effect in males). For the PN11 to 14 treatment regimen, there were significant alterations in males but without a consistent shift in the distribution toward smaller cells; values tended to spread over a wider distribution to either side of the average. Females showed a consistent shift toward smaller cells but the effect was far less notable than with the PN2 to 5 treatment (p < 0.001 for differences in effect between the two regimens).

**Discussion**

The current results indicate that, during stages of brain development in the rat that correspond to the second to third trimester of human neurodevelopment (Rodier, 1988), terbutaline is a neurotoxicant that disrupts biochemical and morphological targets in multiple brain regions. Although we did not perform dose-response or antagonist studies to provide definitive demonstration that the effects are mediated through β2ARs within the developing brain, the fact that the pattern of alterations mirrors the regional and temporal distribution of the receptors, incorporates the known consequences of βAR overstimulation on cell replication and cell death, and parallels the effects of terbutaline on cell signaling all point to a receptor-mediated origin (Joseph et al., 1983; Lorton et al., 1988; Erdtsieck-Ernste et al., 1991; Teerlink et al., 1994; Hodges-Savola et al., 1996; Communal et al., 1998; Shizukuda et al., 1998; Gu et al., 2000; Yan et al., 2000; Singh et al., 2001; Slotkin et al., 2001, 2003; Garofolo et al., 2003). Using neuroprotein biomarkers for astroglia (GFAP) and proximal neuronal projections (NF68), we obtained patterns that are typical of neuronal damage and reactive gliosis: initial increases in GFAP that eventually resolve and are replaced by elevated NF68, which is associated with reactive sprouting in response to injury (O’Callaghan, 1988; Schroeder et al., 2003). There are two important features of the alterations in neuroproteins. First, the effects seem to be fairly widespread rather than focusing on a single region. Second, effects were seen for both treatment regimens but the early exposure paradigm elicited changes in both GFAP and NF68, whereas the later exposure affected only the latter. A major limitation of biochemical approaches is that brain regions contain separate neuronal groupings, so that...
robust subregional effects are offset by inclusion of unaffected areas. Thus, the alterations seen here for effects on GFAP and NF68 are small in magnitude but suggest that there are actually much larger changes in more defined loci.

In light of the limitations of the neurochemical approach, we then used quantitative morphology to characterize terbutaline-induced damage. Specifically, we first examined the cerebellum, which showed only modest changes in neuroproteins; other biomarkers suggest that the cerebellum is, in fact, a prominent target for developmental disruption by terbutaline (Slotkin et al., 1989, 1990, 2003; Garofolo et al., 2003) and this region contains the highest proportion of the β2 subtype (Lorton et al., 1988; Erdtsieck-Ernste et al., 1991). We noted a decreased number of Purkinje cells and thinning of the cerebellar lobules, with reductions in the width of both molecular and granular layers. Importantly, these effects occurred with the early treatment only, because no significant differences were obtained with exposure on PN11 to 14. The early neonatal period occupies the time frame where the majority of cerebellar granule cells are generated and during which architectural remodeling occurs through apoptosis (Rodier, 1988; Wood et al., 1993). Inappropriate or excessive βAR stimulation is known to impair cell replication during development (Slotkin et al., 2003), and in an earlier study, we identified adverse effects of terbutaline on cerebellar DNA content after early neonatal exposure (Garofolo et al., 2003), a result in keeping with the critical period identified in the present study. In addition, βAR overstimulation can enhance cell death (Communal et al., 1998; Slotkin et al., 2003). Finally, reductions in the granular layer may also proceed secondarily from the reductions in Purkinje cells (Smyene et al., 1995).

In contrast to the effects seen in the cerebellum, hippocampal morphology was affected with either of the terbutaline regimens and showed distinct sex preference, targeting females. Within the hippocampus, significant deficits were limited to CA3, again demonstrating how effects on neurochemical markers, which look at the entire hippocampus, underestimate the degree of focal damage. There were two notable characteristics, a thinning of the CA3 region and reactive gliosis, with a 40% increase in the glia/neuron ratio. Vulnerability of the hippocampus can be expected to occupy a fairly wide developmental window, given the fact that neurogenesis and synaptic remodeling continue into adolescence in this region (McWilliams and Lynch, 1983; Altman and Bayer, 1990). It is of particular importance that the human hippocampus has a high concentration of β2ARs, the specific subtype mediating the effects of terbutaline (Duncan et al., 1991).

We similarly observed sex-dependent effects in the somatosensory cortex, characterized by a shift from the larger, pyramidal cells, to smaller, nonpyramidal cells. As was true for the cerebellum, the effects were much more notable with exposure on PN2 to 5 than on PN11 to 14, a time course in keeping with the observation that the proportion of β2ARs is high in the neonate and falls substantially with postnatal development (Lorton et al., 1988; Erdtsieck-Ernste et al., 1991). Changes in the proportions of these two cell types are known to influence the excitability of neurons within the somatosensory cortex (DeFelipe, 1999), and further studies are thus warranted to examine the functional consequences of the morphological changes noted here.

Sex selectivity for the effects of terbutaline on brain development thus falls into two distinct patterns. In the cerebellum, although both sexes were affected, males tended to show greater alterations. This is consistent with our previous biochemical observations on biochemical markers of cell number (Garofolo et al., 2003). βAR stimulation reduces testosterone levels (Selvage and Rivier, 2003), which may provide a component of the sex-selective actions on this region. In contrast, females were targeted for effects on the hippocampus and somatosensory cortex. Again, this is consistent with previous biochemical evaluations of effects on cell replication (Garofolo et al., 2003). Estrogen receptors are highly expressed in these two regions, where they influence the rate of neural cell turnover, cell replication, and apoptosis (Tanapat et al., 1999; Osterlund and Hurd, 2001). The net impact of terbutaline on brain development is thus likely to depend highly on sex and hormonal status, superimposed on the critical windows of vulnerability dictated by the maturational timetable of specific regions. Although our results do not pinpoint a specific mechanism for the sex dependence of terbutaline’s effects, they are consistent with other findings of differential coupling of βARs to cell signaling and to pathologic changes from βAR overstimulation in males and females (Vizgirda et al., 2002; Gao et al., 2003).

In conclusion, terbutaline is a neuroteratogen that alters neuroprotein markers, architectural organization, and neuronal and glial cell distributions in the cerebellum, hippocampus, and somatosensory cortex. Because the rat is an altricial species (Rodier, 1988), a critical window of vulnerability during the early neonatal period corresponds to stages of neurodevelopment that are relevant to the use of terbutaline for preterm labor in humans. Although the adverse effects of terbutaline are expressed in multiple regions, the effects are qualitatively subtle, detectable only with quantitative morphological characterization in combination with sensitive neurochemical biomarkers. Nevertheless, these morphological changes are likely to underlie many of the synaptic deficits evoked by terbutaline in animal studies (Slotkin et al., 1989, 1990, 2003; Garofolo et al., 2003). In turn, our results point to a causative link between βAR tocolytic treatment for preterm labor and subsequent adverse neurobehavioral outcomes in the offspring (Hadders-Algra et al., 1986; Feenstra, 1992; Pitzer et al., 2001).

References


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