

Repeated courses of antenatal corticosteroids have adverse effects on aspects of brain development in naturally delivered baboon infants

Amy Shields¹, Merran Thomson², Vicki Winter³, Jacqueline Coalson³ and Sandra Rees¹

INTRODUCTION: Repeated courses of antenatal steroids in women at risk of preterm delivery have beneficial effects on lung maturation, but concern exists about the effects on brain development. We aimed to determine whether repeated courses of corticosteroids increased the risk of neuropathology as compared with single courses or no treatment.

METHODS: Single-course animals received a 6-mg dose of steroids at 123 and 124 d of gestation (dg; term, 185 dg; $n = 6$). Repeated-course animals received additional doses at 137 and 138 dg ($n = 7$). Controls received no steroids ($n = 5$). Baboons delivered naturally at term and necropsy was performed. Brains were assessed histologically for parameters of development and neuropathology.

RESULTS: Body weights did not differ between the groups ($P > 0.05$); neither did brain/body weight ratio. Density of glial fibrillary acidic protein (GFAP)-immunoreactive (IR) astrocytes in white matter (WM) was increased in the single- ($P < 0.05$) and repeated-course ($P < 0.01$) groups as compared with controls. Density of myelin basic protein (MBP)-IR oligodendrocytes was reduced in the repeated-course animals as compared with both the control and single-course groups ($P < 0.05$); oligodendrocyte transcription factor 2 (Olig2)-IR showed no difference between groups.

DISCUSSION: Repeated courses of antenatal corticosteroids have effects on myelination in the developing nonhuman primate brain, which should be taken into account when determining a dosing regimen.

Routine administration of antenatal steroids to mothers who are at risk of delivering prematurely has resulted in improved outcomes for preterm infants. Benefits to the newborn include a reduction of the risk of respiratory distress syndrome and intraventricular hemorrhage and reduced mortality and reliance on respiratory support (1). There is evidence that the effectiveness of antenatal corticosteroids diminishes with time (2), so if birth did not occur within 7 d of corticosteroid administration it was previously common practice to administer a second course of corticosteroids (3,4). Concerns about the safety of this practice were raised following the publication of cohort studies (5,6).

Randomized, controlled trials of repeated weekly or biweekly courses of antenatal steroids demonstrated improved neonatal respiratory outcomes (7); however, this was coupled with a reduction in some measures of weight and head circumference at birth (7,8). Several longer-term studies have shown no impact of repeated courses of corticosteroids on physical growth or neurodevelopmental outcome at 2 years of age (9–11); however, other studies suggest a higher rate of cerebral palsy (12), neurodevelopmental abnormalities (13), and behavioral effects (14) in infants exposed to repeated courses of corticosteroids.

Studies in sheep have shown a dose-dependent effect of corticosteroids on birth weight (15) and that a reduction in brain weight following antenatal corticosteroid administration persists to 3.5 years of age (adulthood in the sheep; ref. 16). Other animal studies have shown that repeated courses of antenatal steroids can have detrimental effects on the developing brain, delaying myelination (17–19) and causing cell death in the hippocampus (20); a single course has been reported to reduce neural cell proliferation in rats (21) and alter cytoskeletal protein and synaptophysin in baboon brains (22).

Therefore, in light of evidence of possible adverse effects on the developing brain, the question as to whether obstetricians should continue to give repeated courses of corticosteroids remains controversial (23–25). A recent meta-analysis (26) concludes that repeated betamethasone restricts intrauterine growth, with possible long-term consequences on neurodevelopment.

In this study, we had the opportunity to investigate the developmental sequelae of single or repeated courses of antenatal corticosteroids as compared with no treatment in a nonhuman primate model, the baboon. We have shown previously that this model is appropriate for studying neuropathology due to its similarity with human brain development although the baboon brain reaches a more mature state by birth (27–29). Our experiment was designed to assess the effects of antenatal corticosteroid treatments when gestation was not affected by confounding factors such as hypoxia or malnutrition and when offspring were delivered naturally at term (185 d of gestation (dg) in the baboon). Our aim was to determine whether a single course of corticosteroids at 123–124 dg (66% of gestation, equivalent to

¹Department of Anatomy and Cell Biology, University of Melbourne, Melbourne, Victoria, Australia; ²Division of Clinical Sciences, Imperial College, London, UK; ³Department of Pathology, University of Texas Health Science Center at San Antonio, San Antonio, Texas. Correspondence: Sandra Rees (s.rees@unimelb.edu.au)

~26 wk of gestation in the human; ref. 29) or repeated courses administered at 123–124 dg and 137–138 dg (74% of gestation, equivalent to ~30 wk of gestation in the human; ref. 29) increased the risk of altered brain development (including deficits in myelination) or neuropathology (including astrogliosis, microgliosis, apoptosis) as compared with no treatment.

RESULTS

Group Characteristics

There was no difference between groups ($P > 0.05$) in the age at delivery (control: 186.0 ± 0.71 ; single course: 186.2 ± 0.17 ; repeated course: 186.4 ± 0.37) or the ratios of males to females (control: 2/3; single course: 4/2; repeated course: 4/3).

Brain Growth and Development

Body weights at necropsy did not differ between the groups ($P > 0.05$; **Table 1**). Total brain and cerebellar weights were increased in the single-course group as compared with both controls and the repeated-course group ($P < 0.05$; **Table 1**); there was no difference between groups in either brain or cerebellar weights when expressed in relation to body weight ($P > 0.05$; **Table 1**).

The volume of deep gray matter was reduced in the repeated-course group as compared with the control group ($P < 0.05$; **Table 2**), but this was not significant when expressed relative to total forebrain volume. There were no differences in other volumetric parameters between the groups (**Table 2**). The overall surface folding index between groups was not different ($P > 0.05$; **Table 2**).

Neuropathology

There was no evidence of cerebral infarction, cystic lesions, or intraventricular hemorrhage in the cerebral hemispheres of any animal.

There was no difference between groups in the density of glial fibrillary acidic protein (GFAP)-immunoreactive (IR) astrocytes in the neocortex ($P > 0.05$; **Table 3**); however, in the deep and subcortical white matter (WM), the density of astrocytes was increased in both the single- and the repeated-course groups as compared with controls ($P < 0.05$; **Table 3**). This is illustrated for the deep WM by comparing GFAP-IR from the

single- (**Figure 1b**) and repeated-course (**Figure 1c**) groups with controls (**Figure 1a**).

There was no difference in the areal density of oligodendrocyte transcription factor 2 (Olig2)-IR cells between groups in either region. This is illustrated by comparing the control group (**Figure 1d**) with the single-course (**Figure 1e**) and repeated-course (**Figure 1f**) groups. Myelin basic protein (MBP)-IR cell density in repeated-course animals was lower than controls and single-course animals in both the deep and subcortical WM ($P < 0.05$; **Table 3**); this is illustrated for the subcortical WM by comparing MBP-IR from repeated-course animals (**Figure 1i**) with controls (**Figure 1g**) and single-course animals (**Figure 1h**). The intensity of MBP-IR, as assessed by dark-field illumination in the corpus callosum, was reduced markedly in the repeated-course group (**Figure 1j**), and was reduced mildly in the single-course group (**Figure 1k**) as compared with controls (**Figure 1j**).

There was no difference between groups in the density of ionized calcium-binding adapter molecule 1-IR cells in cortex, deep, or subcortical WM ($P > 0.05$; **Table 3**).

Histological analysis revealed no overt damage and no consistent abnormalities in the morphology of CA1-3 pyramidal

Table 2. Forebrain volumetric parameters

	Term control (n = 5)	Single course (n = 6)	Repeated course (n = 7)
Volume of the (right) forebrain (mm ³)	35,338 ± 2,068	34,957 ± 1,719	31,328 ± 1,370
Neocortical volume (mm ³)	21,240 ± 1,283	21,277 ± 993	19,787 ± 1,032
White matter volume (mm ³)	9,415 ± 735	9,351 ± 419	8,342 ± 593
Deep gray matter volume (mm ³)	3,884 ± 205	3,558 ± 158	3,202 ± 176*
Ventricular volume (mm ³)	658 ± 93	640 ± 55	564 ± 70
Neocortex/total hemispheric volume (%)	61.2 ± 0.7	62.1 ± 1.5	64.3 ± 1.3
White matter/total hemispheric volume (%)	27.1 ± 1.0	27.3 ± 0.5	27.0 ± 1.1
Deep gray matter/total hemispheric volume (%)	11.2 ± 0.3	10.4 ± 0.5	10.5 ± 0.6
Ventricular volume/total hemispheric volume (%)	1.9 ± 0.2	1.9 ± 0.1	1.8 ± 0.2
Neocortical volume/white matter volume	2.3 ± 0.1	2.3 ± 0.1	2.4 ± 0.1
SFI	82.0 ± 2.3	87.2 ± 3.1	79.0 ± 1.9

Data are presented as mean ± SEM.

SFI, surface folding index.

* $P < 0.05$ as compared with controls.

Table 1. Effects of prenatal steroids on body and brain weights

	Term control (n = 5)	Single course (n = 6)	Repeated course (n = 7)
Body weight at postmortem (g)	846 ± 68	991 ± 74	864 ± 44
Total brain weight (g)	78.1 ± 1.7	94.5 ± 4.7***	81.2 ± 2.2
Brain/body weight (%)	9.7 ± 0.8	9.5 ± 0.3	9.2 ± 0.3
Cerebellar weight (g)	5.5 ± 0.4	7.0 ± 0.3***	5.6 ± 0.2
Cerebellar/body weight (%)	0.70 ± 0.03	0.72 ± 0.03	0.64 ± 0.03
Cerebellar/brain weight (%)	7.2 ± 0.4	7.6 ± 0.3	7.1 ± 0.2

Data are presented as mean ± SEM.

* $P < 0.05$ as compared with controls; ** $P < 0.01$ as compared with controls; *** $P < 0.05$ as compared with repeated course.

Table 3. Forebrain neuroglial cell densities

	Term control (n = 5)	Single course (n = 6)	Repeated course (n = 7)
GFAP-IR cells			
Cortex	5 ± 2	6 ± 2	3 ± 1
Subcortical WM	305 ± 23	480 ± 31*	473 ± 27*
Deep WM	277 ± 18	445 ± 35*	548 ± 55**
Olig2-IR cells			
Subcortical WM	311 ± 16	309 ± 14	285 ± 25
Deep WM	356 ± 13	358 ± 11	355 ± 32
MBP-IR cells			
Subcortical WM	126 ± 15	136 ± 5	83 ± 9***
Deep WM	179 ± 12	170 ± 12	136 ± 6***
Iba1-IR cells			
Cortex	66 ± 3	67 ± 2	60 ± 3
Subcortical WM	103 ± 6	113 ± 6	104 ± 6
Deep WM	148 ± 8	165 ± 10	148 ± 14

All measurements cells/mm². Data are presented as mean ± SEM.

GFAP, glial fibrillary acidic protein; Iba1, ionized calcium-binding adapter molecule 1; IR, immunoreactive; MBP, myelin basic protein; Olig2, oligodendrocyte transcription factor 2; WM, white matter.

* $P < 0.05$ as compared with controls; ** $P < 0.01$ as compared with controls; *** $P < 0.05$ as compared with single course.

cells or of dentate granule cells in corticosteroid-treated animals as compared with controls. Apoptotic cells were observed infrequently (~1.4 cells/mm²) in the CA1-3 region of controls (Figure 2a) and corticosteroid-treated animals (Figure 2b,c).

Relationship Between Myelination and Brain Volumes

There were positive correlations between the density of MBP-IR cells in the WM and the volume of the right hemisphere ($r^2 = 0.31$; $P < 0.03$), the deep gray matter ($r^2 = 0.31$; $P < 0.03$), and the neocortex ($r^2 = 0.23$; $P < 0.03$). These correlations occurred across all groups, and are consistent with normal brain growth. There were no other correlations across all parameters tested.

DISCUSSION

These studies were designed to determine whether single or repeated courses of antenatal corticosteroids in fetuses not exposed to any other factors such as hypoxia or infection affect brain development at normal term delivery. The main finding was that repeated courses have a greater effect on the brain than a single course, delaying myelination and causing a reduction in the volume of the deep gray matter; this reduction, however, was not reflected in an altered brain/body weight ratio. Both single and repeated courses of corticosteroids led to astrogliosis in the deep and subcortical WM, suggesting that steroids might be causing some brain injury although there were no overt signs of cell death or infarction at birth. Alterations could be present at the microstructural and/or neurochemical level, but we were unable to address these issues in this study. A strength of the study is that it was performed with nonhuman primates where, as in humans, major events in brain development occur *in utero* and the brain has similar proportions of gray and WM. The course

of antenatal steroids used in this study is identical to that used in previous studies of the 125 dg baboon model (31). The dose is half that used in humans to reflect the differing body weight of a pregnant baboon and human. The dose exposure was, therefore, similar to that in humans and given in an appropriate time frame to observe any intrauterine developmental alterations. We acknowledge that the baboon brain is more developed than the human brain at birth (29). In a companion study on the lung by our group, it has been shown that lung volumes are increased and there was a decrease in mesenchyme in the alveolar walls, suggesting antenatal corticosteroids may have an impact on the connective tissue matrix (unpublished data).

Overall Brain Growth Was Not Affected by Single- or Repeated-Course Corticosteroids

The body weight of animals at postmortem was not different between single-course, repeated-course, and control groups. Whole-brain and cerebellar weights of the single-course infants were significantly greater than both control and repeated-course infants, although this was not significant when expressed in relation to body weight. Volumetric measurements did not exhibit any differences between groups apart from a reduction in the deep gray matter volume in the repeated-course group as compared with controls. Again, this was not significant when expressed in relation to body weight. Furthermore, there was no difference in surface folding index between groups, indicating that gyral formation was not affected. Taken together, it would appear that these regimens of antenatal steroid treatment did not have a marked effect on overall brain growth. Other animal studies have found differences in body and brain weight in the short (32) and long term (16), but these involved different dosing regimens and species from those used here. Intrauterine growth restriction can result from antenatal corticosteroid treatment in humans, but this usually follows several courses of corticosteroids (26) as compared with the two courses administered in this study.

Repeated Courses of Corticosteroids Delay Myelination

We identified that repeated but not single courses of antenatal steroids affected myelination in the developing subcortical and deep WM, as indicated by lower numbers of MBP-IR cells as compared with controls. Furthermore, qualitative assessment of MBP-IR in the corpus callosum revealed a marked reduction in the repeated-course group and a mild reduction in the single-course group as compared with controls. As it was only possible to examine the effects of steroids at one time point, we are unable to determine whether myelination recovers to control levels postnatally. We do know, however, that the density of Olig2-positive cells was not different between groups at term. As Olig2 is a transcription factor that marks all cells of the oligodendrocyte lineage (33), we suggest that myelination is most likely to be delayed rather than permanently reduced; long-term studies will be required to confirm this.

Although this is the first study to describe alterations to myelination in a nonhuman primate as a result of repeated exposure to antenatal corticosteroids, previous studies have shown that repeated courses administered to fetal sheep result in delayed

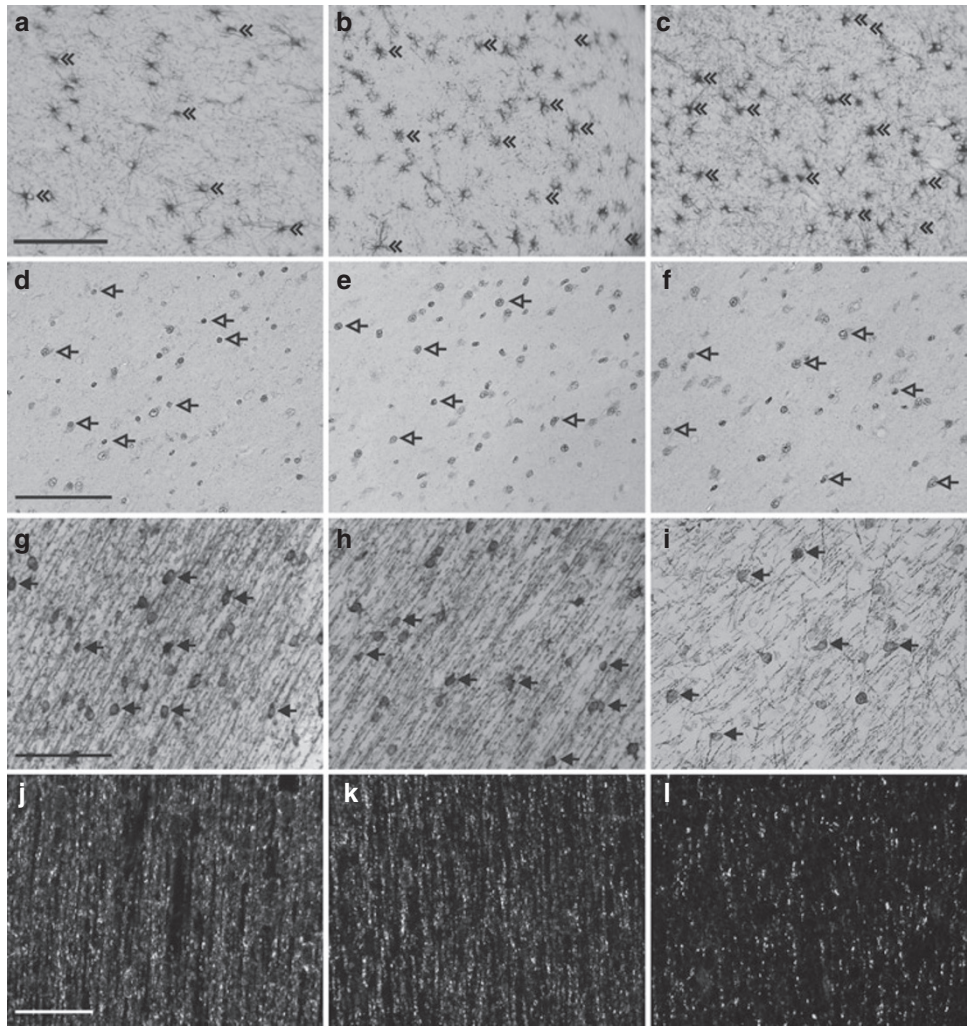


Figure 1. Effect of steroids on neuroglia in WM. Areal density of GFAP-IR astrocytes (arrowheads) in deep WM as compared with (a) controls was increased in both (b) single- and (c) repeated-course groups. Olig2-IR oligodendrocytes (open arrows) did not differ between (d) control, and (e) single- and (f) repeated-course groups (subcortical WM). Areal density of MBP-IR cells (filled arrows, subcortical WM) was reduced in the (i) repeated-course group as compared with both (g) controls and the (h) single-course group. MBP-IR in the corpus callosum, viewed under dark-field illumination, was markedly reduced in the (l) repeated-course group and mildly reduced in the (k) single-course group as compared with (j) controls. Bar = a–f, 100 μm; g–i, 50 μm. GFAP, glial fibrillary acidic protein; IR, immunoreactive; MBP, myelin basic protein; Olig2, oligodendrocyte transcription factor 2; WM, white matter.

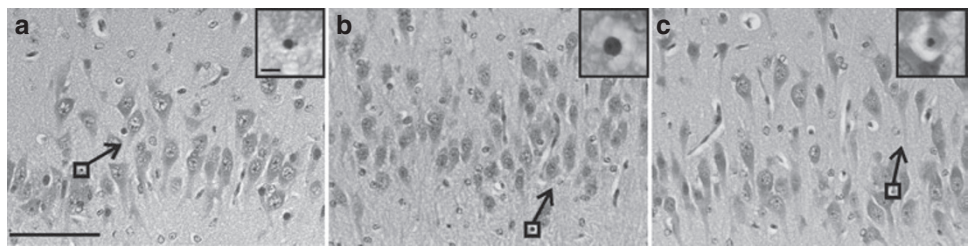


Figure 2. Hematoxylin and eosin staining. Similar levels of apoptosis are seen in the hippocampus across (a) control, and (b) single- and (c) repeated-course groups. Inset shows apoptotic cells (from boxed region) at higher magnification. Bar = a–c, 100 μm; a–c inset, 5 μm.

myelination in the corpus callosum at term (17). A similar regimen delays myelination in the optic nerve during gestation (19), but recovery occurs by term. The authors suggest that regional differences in the effects of corticosteroids on the central nervous system might be due to the greater maturity of the

optic nerve, where 10% of the fibers are myelinated at the time of the first injection of corticosteroids (17). Myelination in the baboon has already begun in the cerebral WM but not in the corpus callosum at the time of the first course of corticosteroids (29). With a single course of antenatal steroids, myelination in

the deep and subcortical WM is not different from controls at term; however, there is a mild reduction in MBP-IR in the corpus callosum. We suggest that the more mature oligodendrocytes in the deep and subcortical WM recover from any adverse effects by term but that the less mature oligodendrocyte lineage in the corpus callosum sustains a mild deficit in myelination. In preterm baboons exposed to an additional course of steroids, the pool of vulnerable pre-oligodendrocytes is further affected, delaying their maturation and resulting in reduced myelination at term in all regions. Oligodendrocytes are known to be at particular risk of damage or altered function during the peak period of myelination (34).

Even if myelination reaches normal levels postnatally, it is possible that maturational delays might influence functional outcomes. Myelin thickness affects axonal conduction velocity (35) and, thus, influences latency of action potentials in cerebral fiber tracts; this could affect synchrony of neuronal firing. Synchronous activity of neurons is thought to be important for refinement of connections (36). A recent study of developing human brain using magnetic resonance imaging tractography supports the concept that appropriate development of myelinated axons plays an important role in optimizing connectivity (37). A delay in myelination at critical periods of brain development could conceivably affect connectivity and underlie long-term deficits in function.

As indicated earlier, it seems most likely that myelination is affected via effects of glucocorticoids on myelin synthesis rather than the death of oligodendrocytes. Glucocorticoids can act via genomic or nongenomic mechanisms, or alternatively by inducing effects on membranes (18). Glucocorticoids have been shown to act on post-transcriptional processes influencing the production of specific components of myelin, including glycerol phosphate dehydrogenase, MBP, and proteolipid protein (38).

Neuropathology

Both single and repeated courses of corticosteroids caused astrogliosis in the subcortical and deep WM as compared with controls, suggesting that exogenous steroids might trigger an underlying adverse event that results in a gliotic response; cellular damage can cause sustained astrogliosis in developing brain (39). However, there was no evidence of any infarction, hemorrhage, overt signs of neuronal or axonal damage, or increased microglial invasion when assessed in the hemispheres at postmortem. Qualitative examination of the hippocampus showed that apoptosis was not increased in corticosteroid-treated animals as compared with controls; we acknowledge that unbiased stereological counting is required to unequivocally determine whether neuronal death has occurred. A study of repeated corticosteroid administration in macaques demonstrated death of hippocampal pyramidal cells in animals delivered prematurely (20); in animals delivered at term alterations in the hippocampus were still present, but to a lesser degree (20). It is possible that in our study, injury had occurred earlier in gestation in response to exogenous steroids but cellular debris had been removed prior to postmortem; this might also explain the lack of persistent microgliosis.

Conclusion

In conclusion, we have shown that, in a nonhuman primate model at term, repeated courses of antenatal corticosteroids affect brain development more significantly than a single course, particularly in relation to myelination. Whether myelination recovers postnatally with further development is unknown; however, the integrity of the oligodendrocyte lineage would provide a basis for this to occur. The functional significance of a maturational delay in myelination is not known but could have long-lasting effects on brain function. Overall, brain growth in relation to body growth was not affected by corticosteroid treatment, but there was evidence of astrogliosis in both groups of treated animals, suggesting that there might be some underlying brain injury. In relating our findings to the human infant, we acknowledge that there are limitations of our study, including the small number of animals and the short duration of the study. However, our results suggest that repeated courses of corticosteroids are associated with a higher risk of brain alterations than a single course. Adverse effects on brain development will need to be balanced against the requirement to promote lung development when deciding whether to give repeated courses.

METHODS

All animal studies were performed at the Southwest Foundation for Biomedical Research in San Antonio, Texas. All animal husbandry, animal handling, and procedures were reviewed and approved by the institutional animal use and care committees of the Southwest Foundation for Biomedical Research and the University of Texas Health Science Center of San Antonio and conformed to American Association for Accreditation of Laboratory Animal Care guidelines.

Timed gestations were determined by observing characteristic sex skin changes and confirmed by serial fetal ultrasound examinations. Pregnant baboon dams (*Papio papio*) were randomly assigned to one of the following groups: the single-course group ($n = 6$) received a dose of antenatal steroids (6 mg betamethasone intramuscularly or 6 mg dexamethasone intramuscularly), at 123 and 124 dg; the repeated-course group ($n = 7$) received an additional two doses, one at 137 dg and one at 138 dg; a third, control, group received no placebo injections or sham handling procedures and served as the no-antenatal-steroid-treatment group ($n = 5$). Betamethasone was administered as a mixture of 3 mg/ml betamethasone acetate and 3 mg/ml betamethasone phosphate. Access to betamethasone was stopped by the supplier over a period of time during this study; dexamethasone had to be substituted, but this resulted in no discernible differences in outcome. All dams delivered air-breathing infants naturally at term (185 d). Necropsy was performed 1–2 d after delivery.

Histology and Immunohistochemistry

Brains were weighed, immersed in 4% paraformaldehyde or 10% formalin in 0.1 mol/l phosphate buffer and 14–15 blocks from the right forebrain (5-mm intervals) were processed to paraffin. Ten coronal sections (8- μ m thick) were cut from the rostral surface of each block. One section per block was stained with hematoxylin and eosin and assessed for gross morphologic changes, including the presence of hemorrhages, lesions or infarcts, neuronal death, axonal injury, gliosis, and perivascular cuffing.

Immunohistochemistry for rabbit anti-cow-GFAP (1:500; Sigma, St Louis, MO) was used to identify astrocytes, and rabbit anti-ionized calcium-binding adapter molecule 1 (1:1,500; Wako, Richmond, VA) was used to identify microglia/macrophages. To identify cells in the oligodendrocyte lineage, mouse anti-chicken MBP (1:100; Chemicon, Temecula, CA) and rabbit anti-Olig2 polyclonal antibody (1:200; Chemicon) were used. Analyses were performed on all brains in

the study. Qualitative and quantitative measurements were made on coded slides blinded to the observer.

Analysis

For each animal, all quantitative measurements were made using an image analysis system (Image Pro v4.1; Media Cybernetics, Bethesda, MD). Areas analyzed for density measurements within specific regions were randomly selected by an independent observer. Values were calculated as mean of means for each group; measurements of cell numbers are expressed as cells/mm².

The cross-sectional area of the forebrain in each coronal section (right hemisphere, 14–15 sections/brain) was assessed by tracing the outline in thionin-stained sections and the total forebrain volume estimated using the Cavalieri principle (volume = \sum Area \times section thickness \times inverse of sampling interval; ref. 30). The total volume of WM, deep gray matter (basal ganglia, thalamus, and hippocampus), ventricle, and neocortex were also assessed in this manner for each animal. The surface folding index, which gives an estimation of the expansion of the surface area relative to volume, was then determined (29).

For each animal in the study, five sections were taken at comparable levels of the rostrocaudal extent of the forebrain; in each section GFAP-IR cells were counted ($\times 300$; sample area 0.02 mm²) in two randomly selected regions of the deep and subcortical WM and one region in each of the dorsal, lateral, and ventral neocortex (layers 2–4).

For each animal in the study, five sections were taken at comparable levels of the rostrocaudal extent of the forebrain and MBP-IR and Olig2-IR oligodendrocytes counted ($\times 300$) in two randomly selected areas (0.02 mm²) in both the deep and subcortical WM. Dark-field images were taken of the corpus callosum in MPB-IR sections to qualitatively assess the extent of myelination within this structure.

Ionized calcium-binding adapter molecule 1-IR cells were counted in the WM and neocortex of five sections (selected as described earlier) from each animal. In each section, cells were counted in three randomly selected regions in the neocortex (dorsal, lateral, and ventral; $\times 300$; sample area 0.02 mm²) and two randomly selected regions of each of the deep and subcortical WM ($\times 300$; sample area 0.02 mm²).

For each animal, two hematoxylin and eosin-stained sections at comparable levels of the hippocampus were selected and a qualitative morphological analysis performed to assess the presence of any overt damage, to assess the morphology of hippocampal pyramidal and dentate granule cells, and to identify the presence of apoptotic cells.

Statistical Analysis

Differences between single-, repeated-course, and control animals were assessed using a one-way ANOVA with *post hoc* test (Tukey's test) for histologic parameters; a probability of $P < 0.05$ was considered significant. Linear regression analysis was carried out to determine whether there was a correlation between histologic parameters (astrocyte, oligodendrocyte, and microglia/macrophage cell densities) and volumetric measurements; a probability of $P < 0.05$ was considered significant. Results are expressed as mean \pm SEM (weights and areas) and mean of means \pm SEM (histologic parameters).

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