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Acute central effects of neonatal dexamethasone treatment: towards a rescue strategy

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Dexamethasone (DEX), a synthetic glucocorticoid, is widely used to wean preterm infants from the ventilator. Despite the important short-term benefit on lung function, there is growing concern about the long-term outcome of this treatment, since follow-up studies of preterm infants have shown pervasive adverse neurodevelopmental effects.

Since the mechanism underlying these neurodevelopmental impairments is largely unknown, the aim of the present study was (i) to investigate the acute effects of neonatal DEX treatment on the developing brain; and (ii) to block the effects of DEX in the brain by central administration of the glucocorticoid receptor (GR) antagonist mifepristone.

Long Evans rat pups were injected subcutaneously with tapering doses of DEX or saline (SAL) on postnatal days (pnd) 1, 2 and 3. Separate groups received intracerebroventricular injections with mifepristone prior to DEX treatment. On pnd 4 and 10 pups were sacrificed and brains collected for analysis of cell proliferation (Ki67) and gliosis (GFAP).

We report that neonatal DEX treatment reduced hippocampal cell proliferation on pnd 4 and caused a significant reduction in the number and density of astrocytes within various brain areas on pnd 10. These effects could be partially prevented by GR antagonist pre-treatment. These findings represent a proof of principle for the use of central GR antagonist pre-treatment as a potential intervention strategy to block postnatal DEX-induced alterations in brain development and function.
INTRODUCTION

Preterm birth is the leading cause of perinatal morbidity and mortality in developed countries (1). Although the prevalence of preterm birth has increased over time, survival rates are going up significantly because of technological advances (2). However, preterm infants are still at increased risk for neurodevelopmental impairments and other complications compared to their term counterparts. With most organs being immature in the preterm, lung development especially suffers from prematurity and is frequently associated with morbidity such as respiratory distress syndrome (RDS) and bronchopulmonary dysplasia (BPD). Glucocorticoids (GC) are the drug of choice to accelerate lung maturation and wean infants from the ventilator. Several studies have indeed shown beneficial effects of GC treatment (3) on acceleration of lung maturation and a decreased incidence and severity of BPD. However others fail to do so or show only modest effects (4).

Moreover, follow-up studies of prematurely born infants treated with GC have shown pervasive adverse neurodevelopmental effects (5, 6). Randomized placebo-controlled trials have shown that GC treatment leads to an increased incidence of cerebral palsy resulting in a higher level of neurodevelopmental impairment (5), poor motor skills and lower IQ scores compared to control preterm infants (7). Imaging studies have further revealed a significant reduction in total brain and cerebellar volume at term age in GC-treated premature infants when compared to non-treated preterm infants and control term infants (8, 9).

These alterations in cerebral volume might structurally underlie the cognitive and motor impairments reported in GC-treated infants. Therefore, there have been growing concerns as to whether the short-term benefits of GC treatment outweigh the adverse side effects leading to neurodevelopmental impairment (10). Supporting the evidence from human studies, data from rodent studies have now demonstrated that neonatal GC treatment results in long-lasting alterations in cognitive performance and hippocampal function (11-14), social behaviour (15), stress responsiveness (16, 17) and eventually may even lead to a significant shortening of the lifespan (14, 18).

Here, we aim to investigate the acute impact of neonatal GC treatment on the developing brain in rats. We describe its effects on markers for glial activation and cell proliferation at two different time points after administration, 24 hours and 7 days post-treatment respectively. Additionally we propose a potential intervention strategy to block the acute effects of GC treatment on the developing brain.

METHODS

Animals

Adult female and male Long Evans rats from our breeding population were used as breeders. Two females were mated with one male for 10 days in type 4 polycarbonate cages (59x38x20cm) containing sawdust bedding and tissues. Food (RM3, Special Diet Services, Witham, Essex, UK) and water (8 ml 25% HCl /10 L tap...
water) were provided ad libitum. Animals were maintained on a 11-h light : 13-h dark cycle with lights on at 08.30h, in a temperature (21± 2°C) and humidity (55 ± 5%) controlled room. After breeding, pregnant females were housed individually. Females were checked daily for presence of pups. If pups were present, the day of birth for that particular litter was defined as postnatal day 0 (pnd 0). On pnd 1, litters consisting of 8-12 pups with an appropriate gender distribution (40-60 % male) were selected. If a litter did not meet these criteria, the litter was excluded from the study; no culling was performed. Cages were not cleaned until time of sacrifice. Animal experiments were approved by the Local Committee for Animal Health, Ethics and Research of Leiden University and carried out in accordance with European Communities Council Directive (86/609/EEC).

**Drug treatment**

Pups were randomly assigned to one of six treatment groups (see table 1) according to a within-litter design. Males and females were equally distributed over the treatment groups. Pups in the DEX groups were subcutaneously (SC) injected with dexamethasone-21-phosphate (Sigma Aldrich, Zwijndrecht, The Netherlands) on pnd 1 (0,5 µg/g body weight), pnd 2 (0,3 µg/g) and pnd 3 (0,1 µg/g). Pups in the SAL groups were injected with equivalent volumes of sterile and pyrogen free saline (SAL). Groups 3-6 received bilateral intracerebroventricular (ICV) injections with either vehicle (VEH: 0.4% Tween 80 (Sigma) in SAL) or the GR antagonist mifepristone (50 ng/µl VEH, Corcept Therapeutics, Menlo Park, USA) 30 minutes prior to DEX/SAL administration. Since mifepristone was administered bilaterally, the total dose per day was 100 ng dissolved in 2 µl VEH (1 µl per hemisphere). ICV injections were performed according to (19) reaching a success rate of > 90%. Custom made syringes and needles were used for both SC and ICV injections (Hamilton, Germany). All pups were daily marked using a non-toxic, odourless marker in order to distinguish the littermates assigned to different treatment groups. Daily marking was essential since marks did not last long due to maternal licking and grooming of the pups.

**Table 1. Description of experimental groups.**

<table>
<thead>
<tr>
<th>Group</th>
<th>SC</th>
<th>ICV</th>
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<tbody>
<tr>
<td>1</td>
<td>SAL</td>
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<td>2</td>
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<td>12</td>
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<tr>
<td>6</td>
<td>DEX</td>
<td>MIF</td>
<td>13</td>
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**Procedure**

Pnd 1-3: The daily procedure consisted of removing all pups from the nest followed by transfer to an adjacent room, where the cage was placed on a heating pad. If applicable, first the VEH/mifepristone ICV injection was given. Then body weights (BW) were recorded, followed by marking of the pups and administration of the SC injection. After the procedure (which took ~ 35 minutes per litter) all pups were immediately returned to the home cage.

Pnd 4-10: The daily procedure consisted of removing all pups from the nest followed by transfer to an adjacent room. BWs were recorded, followed by marking of the pups. After the procedure (which took ~ 8 minutes per litter) all pups were immediately returned to the home cage. Besides these manipulations (between 9.00 and 13.00h) litters were left undisturbed.

**Tissue preparation**

To determine effects of neonatal DEX treatment on glial activity and cell proliferation, pups were sacrificed on pnd 4 or 10 (24h and 7 days post-treatment respectively). On the day of sacrifice animals were anesthetized by an intraperitoneal injection with pentobarbital sodium salt (Nembutal, A.U.V., Cuijk, The Netherlands; 1 ml/kg BW) and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde (PFA) in phosphate buffer (PB, 0.1M, pH 7.4). Brains were post-fixed overnight in the skull at 4°C. The next day they were carefully removed from the skull, washed and cryoprotected by equilibration in a buffered 30% sucrose solution. Afterwards brains were snap frozen in ice-cold isopentane and stored at -80°C until further processing.

Brains were sectioned coronally (30 μm) using a cryostat and collected and stored in an antifreeze solution (30% ethylene glycol, 20% glycerol, 0.02 M Na₂HPO₄, 6.6 mM NaH₂PO₄) at -20°C until further processing.

**Immunohistochemistry**

Free-floating immunohistochemistry for Glial Fibrillary Acidic Protein (GFAP, polyclonal rabbit anti-GFAP, DAKO, 1:1000) was used to determine glial activity in the corpus callosum (CC) and dentate gyrus (DG). The primary antibody was amplified by biotinylated goat anti-rabbit (Santa Cruz, 1:1500), and avidin-biotin enzyme complex (ABC-Kit, Elite Vectastain, Brunschwig Chemie, 1:200). Chromogen development was done with diaminobenzidine (DAB; 0.05% in 0.1M PBS, 0.06% H₂O₂), after which sections were carefully mounted on Superfrost Plus slides (Menzel-Gläser, Germany).

Immunohistochemistry for Ki-67 (polyclonal rabbit anti-Ki67; Novocastra, 1:2000) was used to determine cell proliferation in the DG of the hippocampus as described before (Oomen et al., 2010). The primary antibody was amplified by biotinylated goat anti-rabbit (Vector Laboratories, 1:200), avidin-biotin enzyme complex (Elite Vectastain ABC kit, Brunschwig Chemie, 1:1000) and tyramide (1:500; 0.01% H₂O₂). Subsequent chromogen development was done with diaminobenzidine (20 mg/100 ml Tris buffer, 0.01% H₂O₂).
Stereological quantification

For quantification of the number of GFAP positive cells and density of GFAP staining in CC and hilus of the DG, pictures from these brain areas were taken using a light microscope (Leica DM6000B). Photoshop (Adobe) was used to convert pictures to grayscale. Grayscale pictures were then analyzed with ImageJ (Version 1.41) for optical density of GFAP (correcting for corresponding background optical density) and for quantification of GFAP positive cells. Pictures showing substantial tissue damage were removed from the analysis. On average, 3 sections per animal were used for CC quantification and 7 for DG quantification. Due to technical limitations it was not possible to discriminate between individual astrocytes in the pnd 4 sections. Therefore, for this time point only optical density measurements are shown. However, in the pnd 10 sections both the number of GFAP positive cells and optical density of GFAP were measured.

For quantification of Ki67 positive cells in the DG of the hippocampus, a stereological quantification procedure was performed in every 6th coronal section along the rostrocaudal axis, i.e. a total of 8 sections per animal. Total numbers of Ki67 positive cells were quantified by systematic random sampling performed with the Stereo Investigator system (MicroBrightField, Germany). As Optical Fractionator settings for sampling of pnd 10 sections a grid size of 125 x 125 µm and a counting frame of 30 x 30 µm were used, which resulted in an average count of 300 markers per animal. For analysis of pnd 4 sections a grid size of 75 x 75 µm and a counting frame of 30 x 30 µm were used, which resulted in an average count of 200 markers per animal. DG surface area and volume was determined according to Cavalieri’s principle using the Stereo Investigator system.

Data Analysis

All statistics were performed using SPSS 17.0 for Windows. Data are presented as mean ± SEM. Since the data did not show a significant effect of gender, data from males and females were pooled. Due to the limited capacity of our immunohistochemistry setup, analysis of the samples occurred over multiple experimental runs. To correct for inter-run variation we normalized all output against the mean of either the SAL or the VEH SAL samples in the relevant run. This way, for each experimental group, the difference with its relevant control group (either SAL or VEH SAL) is shown in percentages.

Differences between SAL and DEX were analyzed using T-test. Differences between the ICV treated groups were analyzed using two-way ANOVA with ICV treatment (VEH or mifepristone) and peripheral SC treatment (SAL or DEX) as between-subjects factors.

Body weight data were analyzed using Repeated Measures ANOVA with ‘pnd’ as within-subjects effect and ‘treatment’ as between-subjects effect.
RESULTS

Body weight pnd 1-10
Neonatal DEX treatment significantly reduces BW gain during the early postnatal period (p < 0.001; fig. 1A). This reduction is not normalized by central mifepristone pre-treatment (fig. 1B).

![Graph A](image1)

**Fig 1.** Body weight on pnd 1-10 of SAL and DEX treated animals without (A) or with (B) ICV treatment. DEX treatment significantly reduced body weight; this effect is not prevented by central mifepristone pre-treatment. **p < .001

Glial activity pnd 4
24h post treatment, DEX treatment did not affect optical density of GFAP expression in the CC (fig. 2A). Also in the ICV treated groups (fig. 2B) there are no significant differences between treatment groups. However, there is a tendency towards reduction of GFAP expression in DEX compared to SAL treated groups (p = 0.072). Interestingly, mifepristone pre-treatment appears to result in a slight (not statistically significant) increase in GFAP expression compared to VEH treatment (p = 0.096). No significant interaction between the SC and ICV treatment was found.

In the hilus of the hippocampus, no main effects of, or interaction between, SC and ICV treatment were observed (fig. 2C & D).

Glial number and activity pnd 10
Although we did not observe an acute effect of DEX treatment (under basal, non-ICV conditions) on GFAP expression, 1 week post treatment DEX resulted in reductions in both density of GFAP expression (p = 0.023, data not shown) and number of GFAP positive cells in the CC (p = 0.003; fig. 3A). In the hilus, DEX treatment resulted in a trend towards reduced density (p = 0.068, data not shown) and significantly reduced the number of GFAP positive cells (p = 0.002; fig. 3B).

In the ICV treated groups, we observed a significant interaction between SC and ICV treatment in CC (p = 0.001) indicating that the observed reduction in the VEH DEX group is fully normalized by central mifepristone pre-treatment (fig. 3C). There is a trend towards an interaction between SC and ICV treatment in the hilus (p = 0.080, fig. 3D). Due to enhanced variation these interactions do not reach statistical significance in the optical density measurements (data not shown).
Fig 2. Optical density of Glial Fibrillary Acidic Protein (GFAP) staining in Corpus Callosum (A and B) and hilus of the hippocampus (C and D) 24h post treatment, i.e. on pnd 4.

Fig 3. Number of Glial Fibrillary Acidic Protein (GFAP) positive cells in the Corpus Callosum (A and C) and Hilus of the hippocampus (B and D) 7 days post treatment, i.e. on pnd 10 in SAL and DEX treated animals with (C and D) or without (A and B) ICV treatment. ** in A and B: DEX compared to SAL p < .01. ** in C: interaction between SC and ICV treatment p < .01.
Cell proliferation pnd 4
DEX treatment significantly reduced the total number of proliferating cells in the DG of the hippocampus 24h post treatment (p = 0.007, fig. 4A) as well as the volume of the DG (p = 0.001, fig. 4B). Additionally, after correction for differences in volume (total number of Ki67+ cells/DG volume) we observed a trend (p = 0.10, fig. 4C) towards a reduction in density of proliferating cells in the DG in the DEX group.

In the ICV treated groups, DEX also significantly reduced the total number of proliferating cells in the DG of the hippocampus (p < 0.001, fig. 5A) and the volume of the DG (p = 0.001, fig. 5B). These reductions were not normalized by central mifepristone pre-treatment. Additionally, the density of proliferating cells was lower in all DEX treated groups (p = 0.016, fig. 5C). Although the interaction between ICV and SC treatment does not reach statistical significance there appears to be a tendency towards normalization by central mifepristone pre-treatment (fig. 5C).

Cell proliferation pnd 10
Seven days post treatment the reduction in total number of proliferating cells in the DG (as was observed 24h post treatment) is normalized in the DEX treated group towards control (SAL) levels (fig. 6A). Although the volume of the DG appears smaller in the DEX group (fig. 6B), and the density of proliferating cells higher (fig. 6C), these effects do not reach statistical significance.

Also in the ICV treated groups, group differences that were observed 24h post treatment had normalized to control levels 1 week post treatment (fig. 7A, B & C).

Fig 4. Total number of Ki67 positive cells in (A), volume of (B) and density of Ki67 positive cells (C) in the dentate gyrus of the hippocampus 24h post treatment, i.e. on pnd 4 in SAL and DEX treated animals. ** p < .01.
Fig 5. Total number of Ki67 positive cells in (A), volume of (B) and density of Ki67 positive cells (C) in the dentate gyrus of the hippocampus 24h post treatment, i.e. on pnd 4 in SAL and DEX treated animals with ICV pre-treatment. ** DEX vs SAL p < .01; * DEX vs SAL p < .05.

Fig 6. Total number of Ki67 positive cells in (A), volume of (B) and density of Ki67 positive cells (C) in the dentate gyrus of the hippocampus 7 days post treatment, i.e. on pnd 10 in SAL and DEX treated animals.
DISCUSSION

We report that neonatal DEX treatment had differential effects on GFAP expression 24h and 7 days post treatment which could be (partially) prevented by central GR antagonist pre-treatment. Additionally, we report that neonatal DEX treatment acutely reduced DG cell proliferation and volume. These effects had normalized 1 week post treatment, and were not affected by central GR antagonist pre-treatment. These findings indicate that neonatal DEX treatment exerts acute and delayed as well as transient effects on the developing brain. Additionally, central anti-glucocorticoid pre-treatment may serve as a potential early intervention strategy, partially normalizing DEX-induced changes in the developing brain.

Differential effects of DEX on glial number and activity

The effect of DEX on GFAP expression was two-fold. First, the density of GFAP expression tended to be acutely reduced in the CC by DEX treatment in animals that received ICV injections, whereas DEX treatment did not acutely affect GFAP expression in non-ICV injected animals. Interestingly, the overall expression of GFAP was higher in the ICV injected groups as compared to the non-ICV injected groups (data not shown). A diffuse glial scar comprising reactive astrocytes is likely to form in response to 3 consecutive days of bilateral ICV injections (20, 21). This reactive gliosis tended to be responsive to DEX treatment. GCs have been reported before to regulate the number of GFAP positive astrocytes (22), the production of GFAP in astrocytes (23) and their differentiation (24). Additionally,
steroid hormones have been shown to reduce reactive gliosis upon injury (25, 26). Since the glial scar has characteristics leading to inhibition of neuronal growth and regeneration (27), strategies to reduce these characteristics are used as treatment for brain injury (28). It has been reported that the gliosis-reducing effect of steroid hormones is associated with enhanced functional recovery after injury (25) and can therefore be considered a beneficial effect.

Secondly, DEX treatment led to a significant reduction in the number of GFAP positive cells 7 days post treatment, both in animals with and without ICV treatment, which could be fully restored by central GR antagonist pre-treatment. Several studies have shown GC-induced reductions in GFAP expression both in terms of RNA or protein levels (23, 29, 30) and number of GFAP positive cells (22). Unfortunately we were not able to quantify cell numbers on pnd 4. Therefore we cannot rule out whether an acute DEX-induced reduction in glial cell number on day 4 was maintained until day 10, or whether this effect developed progressively during maturation of the brain.

Whereas the gliosis-suppressing effect of DEX could have a beneficial effect, the reduction in cell number, present in all DEX treated animals, appears detrimental considering the supportive role of astrocytes in the brain especially during development (31). Huang and colleagues have reported a delayed maturation of astrocytes in CC after GC treatment, leading to a delayed tightening of the blood-brain barrier, which is suggested to have detrimental effects on CNS development (30). Additionally, GC exposure leads to delayed myelination of callosal fibers (32) and disturbances in long-term maintenance of myelin (33). These disturbances led to decreased conduction velocity potentially affecting information processing and cognitive performance (34). A reduction in astrocyte number during development is likely to mediate and/or contribute to this phenomenon and might underlie the frequently reported cognitive impairments after neonatal synthetic GC exposure (7, 12).

**DEX temporarily suppresses hippocampal cell proliferation**

We report that neonatal exposure to DEX acutely reduced DG cell proliferation, an effect that had normalized by pnd 10. The inhibitory effect of this GC on cell proliferation has been shown frequently, with some studies reporting a transient nature (35, 36) and others showing lasting effects of early GC or stress treatment (14, 37). The current findings are in line with previous findings from Tauber and colleagues showing that early exposure to DEX resulted in reduced cell proliferation in the newborn marmoset (38) which was restored to control levels at 2 year of age (39) indicating that the decreased proliferation rate in the newborn is transient.

From these, and the current findings it should not be concluded that perinatal GC treatment does not have long-lasting consequences and that its use can be considered safe. In rodents, the development of the hippocampus, especially the DG, happens largely during the early postnatal period (40-43). It is believed that the extensive plasticity that characterizes normal early development is necessary to
set the stage for proper adult functioning of the hippocampus, especially in terms of adult neurogenesis (43-45). This indicates that, even a temporary reduction in cell proliferation during a developmental stage that is normally characterized by high levels of proliferation, can exert long-lasting effects on the functioning of the hippocampus and other connected brain structures. These alterations could contribute to the frequently reported cognitive impairments in both animals and humans neonatally exposed to synthetic GCs (7, 12).

Mifepristone and anti-progesterone activity in proliferation
One of the aims of this study was to investigate the possibility to prevent DEX-induced alterations in the developing brain by blocking the GR during DEX treatment. We report that the DEX-induced reduction in glial cell number observed on pnd 10 was fully normalized by central mifepristone pre-treatment. However, the substantial reduction in hippocampal cell proliferation, observed on pnd 4 was not prevented by mifepristone pre-treatment. Blocking the GC-induced reduction in cell proliferation by mifepristone was expected to induce a proliferation-enhancing effect (14, 35, 38, 46) by promoting the protective function of the mineralocorticoid receptor (MR) (47). However, we did not observe this effect in the current study.

The role of mifepristone in cell proliferation is however multidimensional. Besides blocking GR mediated effects and enhancing the role of MR (altogether enhancing proliferation), mifepristone also has intrinsic anti-proliferative effects (48). These effects are likely the result of its anti-progesterone activity (49). Since we did not observe anti-proliferative effects in the MIF SAL animals or investigate the role of progesterone activity in this experiment, we cannot conclude that progesterone receptor-mediated effects overruled those mediated via GR. Altogether, these findings suggest that in future studies, the possibility of blocking DEX-induced, GR-dependent effects should be investigated using a selective GR antagonist.

Factors mediating function of GR ligands
There are additional factors that mediate the outcome of treatment with GR ligands. Under certain conditions mifepristone can exert effects similar to those induced by DEX (50) and act as a full GR agonist with respect to GR-mediated transrepression (51). Therefore, DEX-induced alterations depending on GR-mediated transrepression are less prone to be prevented by mifepristone pre-treatment.

Additionally, alterations in GR density determine the degree of (ant)agonistic activity of GR ligands in transrepression as well as the efficacy and potency of DEX (52). GR expression is known to be affected by neonatal GC exposure (53) and since these effects appear rather acute (within days) (54), differences in central GR density between the treatment groups may develop during the 3 days of drug treatment. This variation might lead to group-specific alterations in the behaviour of the GR ligands (both agonist and antagonist) in terms of GR-mediated transrepression.
MR mediated effects
The finding that not all DEX-induced effects can be normalized using GR antagonist pre-treatment could also indicate that DEX exerts its function partially via another receptor or mechanism. For example, it has been suggested that DEX, despite a substantially higher affinity for GR, binds to MR as well (55-57). Since the blood-brain barrier is not fully developed in the neonate (58, 59) GCs may readily enter the brain (60, 61) at these life stages, where they may be able to target central MR in addition to GR, which are both expressed in the developing brain (61, 62). Certain DEX-induced alterations can be counteracted if there is sufficient MR activation (63).

The daily procedure of removing pups from the nest followed by handling and injections can be considered an experience disrupting the neonate’s stress hyporesponsive period, potentially leading to an inappropriate rise in endogenous GC levels (64, 65). This potential rise in corticosterone can be modulated by DEX (66) and mifepristone leading to a complex interplay between endogenous and exogenous (anti-) glucocorticoids, resulting in differential MR activation profiles in the different experimental groups.

CONCLUDING REMARKS
The aim of this study was to investigate the possibility to block DEX-induced changes on the developing brain using GR antagonist pre-treatment. To summarize, DEX treatment exerts acute, delayed as well as transient effects, which might contribute to the frequently reported long-term functional alterations reported following neonatal GC treatment. DEX-induced alterations can be partially prevented by central mifepristone pre-treatment. Therefore we suggest that these findings represent a proof of principle for the use of central GR antagonist pre-treatment as a potential intervention strategy to block DEX-induced alterations. It has to be noted however, that there is only partial normalization and that the route of mifepristone administration is relatively invasive, resulting in an additional impact (reactive gliosis) on the developing brain. Future studies are therefore needed to investigate alternative routes of administration (such as intrathecal therapy) to determine the clinical potential of this intervention strategy.

ACKNOWLEDGEMENT
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