Maternal deprivation and milk replacement affect the integrity of gray and white matter in the developing lamb brain

Scott A. Love, Emmanuelle Haslin, Manon Bellardie, Frédéric Andersson, Laurent Barantin, Isabelle Filipiak, Hans Adriaensen, Csilla Lea Fazekas, Laurène Leroy, Dóra Zelena, et al.

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S. A. Love, E. Haslin, M. Bellardie, F. Andersson, L. Barantin, I. Filipiak, H. Adriaensen, , C. Fazekas, L. Leroy, D. Zelena, M. Morisse, F. Elleboudt, Chr. Moussu, F. Lévy, R. Nowak, E. Chaillou

Authors contribution:

S. Love: post-processing: statistical analysis, results discussion, secondary writer.
E. Haslin, L. Leroy, M. Bellardie, C. Fazekas: internship, segmentation, bibliography.
M. Morisse: technician, segmentation.
D. Zelena: PHC Balaton (research exchange with Csilla Fazekas)
F. Andersson, L. Barantin: MRI protocole, MRI acquisition.
I. Filipiak: Diffusion MRI sequences.
H. Adriaensen: MRI acquisition.
F. Elleboudt, Chr. Moussu: animal preparation, anaesthesia, caregivers.
F. Lévy: protocol discussion, interpretation, article reviewing.
R. Nowak: coordinator of the Ovin2A project, protocol discussion, interpretation, article reviewing.
E. Chaillou: coordinator of the "brain development and maturation" task in the project, supervisor of the study, lead author.

Abstract :

The psychoendocrine evaluation of lamb development has demonstrated that maternal deprivation and milk replacement alters health, behavior, and endocrine profiles. While lambs are able to discriminate familiar and non-familiar conspecifics (mother or lamb), only lambs reared with their mother develop such clear social discrimination or preference. Lambs reared without mother display no preference for a specific lamb from its own group. Differences in exploratory and emotional behaviors between mother-reared and mother-deprived lambs have also been reported. As these behavioural abilities are supported by the brain, we hypothesize that rearing with maternal deprivation and milk replacement leads to altered brain development and maturation. To test this hypothesis, we examined brain morphometric and microstructural variables extracted from in vivo T1-weighted and diffusion-weighted magnetic resonance images acquired longitudinally (1 week, 1.5 months, and 4.5 months of age) in mother-reared and mother-deprived lambs. From themorphometric variables the caudate nuclei volume was found to be smaller for mother-deprived than for mother-reared lambs. T1-weighted signal intensity and radial diffusivity were higher for mother-deprived than for mother-reared lambs in both the white and gray matters. The fractional anisotropy of the white matter was lower for mother-deprived than for mother-reared lambs. Based on these morphometric and microstructural characteristics we conclude that maternal deprivation delays and affects lamb brain

growth and maturation.

Introduction

In both human and non-human primates, the quality of motherhood is important for an infant's development, especially for social personality development (Pryce, 1995). In non-human primates, the lack of mothering from birth to adolescence leads to anxiousness for novelty exploration, increased aggressiveness at puberty and, peer-reared primiparous females display disturbance of maternal behaviour (Suomi, 1997). More generally, whether in pets or farm and zoo animals, maternal deprivation at birth or early weaning leads to behavioural problems such as reproductive or maternal deficits, aggressiveness, and stereotypic behaviours such as non-nutritive suckling, head-banging or self-harm in general (Rhesus macaque: (Liu et al., 2019); Lambs: (Gaudin et al., 2018); Piglets: (Rzezniczek et al., 2015); and for a review, (Latham and Mason, 2008)). These impacts of maternal deprivation can result simultaneously from the lack of a social model, i.e., the mother (Fleming et al., 2002) and from a lack of maternal milk as a source of bioactive factors (Bernstein and Hinde, 2016). However, the challenge is not to isolate the influence of only one factor (mother or milk) but to consider "the dynamic interactions among the multitude of equally important maternal and non-maternal variables" (Tang et al., 2014).

In sheep (*ovis aries*), a precocial species, it is difficult to dissociate the impact of maternal deprivation from that of formula-milk feeding; each affecting the infants' development. The reciprocal mother-infant bond between a ewe and her lambs occurs in the first 12 hours of life. It is based mainly on odorant cues and is characterized by an individual recognition of each partner and nursing exclusivity between a mother and her own infant (Nowak et al., 1997; Nowak and Poindron, 2006). In this context, it is not surprising that the mother is crucial for the development of the lamb. However, motherless rearing is commonly used in conventional dairy farming with very early separation of lambs from their mother, or in other situations if the mother is non-maternal, has too many lambs or if she has mastitis. But several studies reported deleterious impact of motherless rearing conditions with a level of mortality between 10 and 30% (Miller et al., 2010; Napolitano et al., 2008, 2002). According to the absence of mother, the milk composition and/or the age of weaning, the impact of early rearing conditions is very variable and affects a wide range of functions and behaviours.

At birth, colostrum is crucial to avoid mortality by the passive immune transfer from the mother to their lambs (Hernández-Castellano et al., 2015; Khan and Ahmad, 1997; Nowak and Poindron, 2006). The days after, the impact of mother deprivation on immune response is not so strong (Napolitano et al., 1995) especially if the lambs are fed with ewe's milk (Napolitano, 2003). However, there is an altered immune response in lambs fed with commercial milk in comparison with mothered lambs or lambs fed with a mix of maternal and commercial milks (Sevi et al., 1999).

During the weeks after birth, the mother plays a very strong role as a social demonstrator that influences the establishment of feeding and social preferences. For example, it has been reported that the influence of the mother for the feeding preferences is stronger than thus of a dry ewe (Thorhallsdottir et al., 1990) and could continue after weaning (Black-Rubio et al., 2007), this influence being reinforced by the access to the olfactory cues of the food (Saint-Dizier et al., 2007). Interestingly, the presence of the mother also influences the capacity of lambs to recognize partners or to create social bonds. For example, lambs express preference for their twin only if they are reared together by their mother, this capacity disappears if they are artificially reared in a group without their mother (Ligout and Porter, 2004). Moreover, the presence of the mother influences the development of social

attachment with new partners. For example, even if lambs have positive contacts with a stockperson who gives milk, they prefer their mother and are not appeased by the presence of the familiar human during isolation from conspecifics (Boivin et al., 2001). However, in absence of the mother, lambs have the capacity to create a very strong social bond with a familiar human caregiver, this bond presenting similar characteristics as those described in the mother-infant bond (Guesdon et al., 2016). The absence of the mother also has a negative impact on the sexual behaviour of male lambs reared in a uni-gender group from 24-36 hours after birth (Damián et al., 2018, 2015). Emotional reactivity, evaluated in a social isolation context by cortisol plasma and behavioural responses, is also impacted by maternal deprivation (Napolitano, 2003; Napolitano et al., 2002; Sevi et al., 1999). In addition to these behavioural alterations, endocrine troubles are also reported, in relationship with sexual behaviour (Damián et al., 2018, 2015), infant attachment (Gaudin et al., 2018) or nutrition (Berry et al., 2016).

Together, these studies demonstrate how maternal deprivation has wide ranging, deleterious consequences, affecting the development of the immune system, behavioural expression, endocrine development, growth and cognitive capacities of lambs. Whereas the benefits of breastfeeding for brain development have been reported in humans (Deoni et al., 2018, 2013; O'Muircheartaigh et al., 2014; Schack-Nielsen and Michaelsen, 2007) and non-human primates (Liu et al., 2019), sparse information has been reported in lambs. Mainly studied as a model of the developmental troubles posed by preterm birth (Castillo-Melendez et al., 2013; Chaillou et al., 2012; De Matteo et al., 2010; Dunlop et al., 1997; Huang et al., 2001; Malhotra et al., 2019), lamb brain development in relation to postnatal early experience is little understood. In relation to maternal deprivation, the only reported data concern a very short period of maternal deprivation (72h) at 2 weeks of age after birth (Polkowska and Wańkowska, 2010; Wańkowska et al., 2006). These studies examined the distribution of neuropeptide immunoreactivity in different hypothalamic areas in 3 mothered and 3 maternal deprived lambs. The authors noticed greater immunoreactivity in maternal deprived lambs for adrenocorticotropic hormone and gonadotrophin-releasing hormone (Wańkowska et al., 2006), for somatostatin in the periventricular zone and neuropeptide Y in the periventricular zone and arcuate nucleus (Polkowska and Wańkowska, 2010), consistent with the endocrine perturbations described in similar maternal deprivation contexts (Damián et al., 2018, 2015; Sevi et al., 1999).

Using magnetic resonance imaging (MRI), it is possible to follow the growing brain and various indicators of brain maturation, especially with T1-weighted (T1w) signal intensity (Flood et al., 2019; Luby et al., 2013), diffusion MRI and diffusion tensor imaging (DTI) (Dubois et al., 2014, 2006; Hüppi and Dubois, 2006). Based on studies done in humans (Deoni et al., 2013; Luby et al., 2013) and non-human primates (Liu et al., 2019), we proposed to examine the impact of maternal deprivation on different morphometric and structural characteristics of the lamb brain. As the impact of early experience could affect the brain differently depending on the age of the subject (Bock et al., 2014), and because the myelination process is in progress for several months after birth (McINTOSH et al., 1979; Turley et al., 1996), we examined brain development from 1 to 19 weeks of age after birth in mothered and maternal deprived female lambs. In this context, we considered the mothered lambs as the control. In this aim, we investigated morphometric and structural parameters for the grey (GM) and white (WM) matters. For morphometric parameters, we measured, on T1w MRI, brain, GM, WM and cerebrospinal fluid (CSF) volumes as well as those of specific brain structures involved in socio-emotional behaviours. Here, our

hypothesis was that the absence of mother's milk would negatively affect the developmental increase of these morphometric parameters. For structural characteristics, we focused on T1w signal intensity and diffusion parameters extracted from DTI in GM, WM and specific WM areas. Here, our hypothesis was that maternal deprivation would affect the evolution of these structural parameters, especially in the WM.

Material and Methods

Ethics
All experimental procedures were approved (00821.03 and 02272.01) by the Val de Loire Ethics Committee (CEEA VdL, Nouzilly, France).

Experimental Animals
Twenty, Ile de France, female lambs (*Ovis aries*) took part in this study. The animals were born and reared at a French National Research Institute for Agriculture, Food and Environment experimental unit (PAO, INRAE, 2018. Animal Physiology Experimental Facility, DOI: 10.15454/1.5573896321728955E12). After parturition the lambs were allocated to one of two groups: 10 were kept with their mother for 24 hours after birth, which gave them access to colostrum, before being separated from their mothers (maternal deprived lambs, MD); the 10 others were kept with their mothers for the entire study (control mothered lambs, MC). The MD group had *ad libitum* access to water and formula-milk (Solvor 2015 Instant, composed of whey, whey protein concentrate, wheat gluten, palm oil, copra, rapeseed, flaxseed, soybeans, partially delactosed whey and extracts of yeast from inactive beer; 23% of crude protein and 25% of crude fat level). The formula-milk was distributed via an automatic milk feeder, which the lambs were trained to use by caregivers during the first 72-96h of life.
To follow lambs' growth they were weighed at birth, before each of 3 MRI acquisitions and at around 2 months of age (Figure 1).
During the experiment 3 lambs died in the MD group at 35, 42 and 45 days of age and 1 MC lamb was excluded because of mother's mastitis. Final group sizes were N=7 for MD and N=9 for MC.
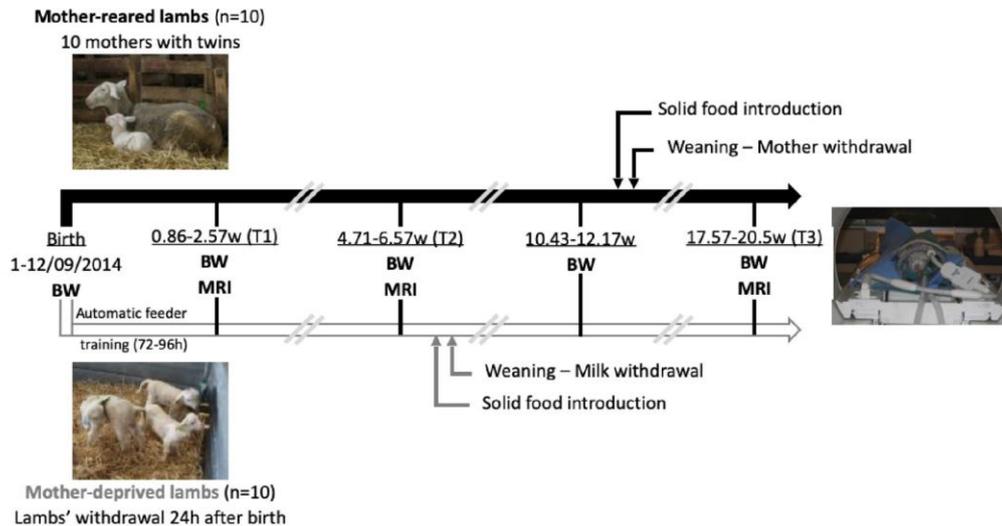
FIGURE 1 Timeline of the experimental design. (BW body weight; MRI magnetic resonance imaging; T1, T2, T3 timepoints of acquisition)

MRI Acquisition

MRI of the brain was performed with a 3T Siemens Magnetom Verio® whole body MR imaging system (Erlangen, Germany) located at the platform CIRE (Chirurgie et Imagerie pour la Recherche et l'Enseignement, INRAE Nouzilly, France). MRI was acquired from each lamb at three different time points. The first point of acquisition (PA) was collected around 1 week after birth (0.86-2.57 weeks; $PA_{0.86-2.57w}$), the second at around 1.5 months after birth (4.71-6.57 weeks, $PA_{4.71-6.57w}$) and the third at around 4.5 months after birth (17.57-20.5 weeks, $PA_{17.57-20.5w}$). Note that within each point of acquisition the individual ages are variable because lambs were born at term without induction of labour between the 1st and 12th of September 2014 and were submitted to MRI acquisitions at the rate of two animals per day. The MC lambs (n=9) were on average 1.6 (+/- 0.6), 5.5 (+/- 0.4) and 19.1 (+/- 1) weeks old and the MD lambs (n=7) were 1.6, 5.5 and 18.3 weeks old, respectively for the three points of acquisition.

Before each acquisition lambs were placed under general anaesthesia to prevent stress and avoid motion artefacts. To prevent nausea and the associated risks of food entering the lungs during anaesthesia, for the last 2 acquisitions ($PA_{4.71-6.57w}$ & $PA_{17.57-20.5w}$) lambs were fasted from the night before. Anaesthesia was induced by an intra-venous co-injection of rompun® and ketamine®. After this injection and the loss of muscular tonus, a tracheal tube was inserted to maintain the anaesthesia with Isoflurane®. The drug doses and tracheal tube sizes were adapted to each animal according to its age, body weight and size (Table 1). The duration of anaesthesia was limited to 2.5 hours and during that time cardiac rhythm and Sp02 levels were continuously monitored. Due to their young age, during the first PA ($PA_{0.86-2.27w}$), lambs were also perfused with a solution of Ringer-Glucose. At the end of the acquisition, Isoflurane® was stopped and the tracheal tube removed after the first signs of autonomous respiration.

During acquisition lambs lay on their stomachs (prone position) with a 4-channel Siemens FLEX coil wrapped around their head and, for their comfort and safety, were covered with a cloth and wore ear plugs (Figure 1). *T1-weighted images* were acquired with the 3D-

MPRAGE sequence with the following parameters at all points of acquisition: TR=2500ms, TE=3.44ms, TI=756ms, section thickness=0.4mm, FOV=152mm, matrix=384x384mm^2, 4NEX, TA=45min. *DTI-images* were acquired with a spin-echo echo-planar sequence with the following parameters at all points of acquisition: TR=13000ms, TE=80ms, FOV=152mm, matrix=128x128mm^2, TA=6-10min. Images were acquired in 30 directions with a b-value of 1000s/mm^2 (similar to those used in childhood studies, Mukherjee et al. 2002) and 6 images were acquired without diffusion weighting (b-value=0s/mm^2). *GRE field mapping* sequences were performed in magnitude and in phase with the following parameters: TR=500ms, TE short/long=4.92/7.38ms.

MRI Analysis
Native MR images were converted from DICOM to NIFTI with dcm2nii (mricron, http://www.mccauslandercenter.sc.edu/mricro/mricron).
Morphometric variables
T1w MRI for volume calculation of brain, gray and white matters and cerebrospinal fluid
T1-weighted images were first denoised using the *DenoiseImage* function of the Advanced Normalization Tools (ANTs, Avants et al., 2014; Manjon et al., 2008). They were then cropped in all three dimensions to isolate the cerebral tissue as closely as possible from all non-brain tissue. To reduce the time required for manual brain mask segmentation, the original resolution (0.4mm^3 isotropic) of the T1w images was linearly subsampled to 0.8mm^3 isotropic using the *resample-mm* function of the Convert3D (ITK-SNAP, www.itksnap.org, Yushkevich et al., 2006). The manual brain mask segmentation was performed with the ITK-SNAP software taking care to exclude the meninges, olfactory bulb and tentorium cerebelli (Figure 2). The segmented brain mask was then upsampled to the original T1w resolution of 0.4mm^3 isotropic before being manually inspected and corrected when necessary. Finally, all brain masks were controlled by experts in sheep neuroanatomy (EC, SAL). The automatic segmentation of gray matter (GM), white matter (WM) and cerebrospinal fluid (CSF) was conducted within each individual's brain mask using FAST (FMRIB Automated Segmentation Tool, https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/FAST, Zhang et al. 2001; Smith et al., 2004) (Figure 2).
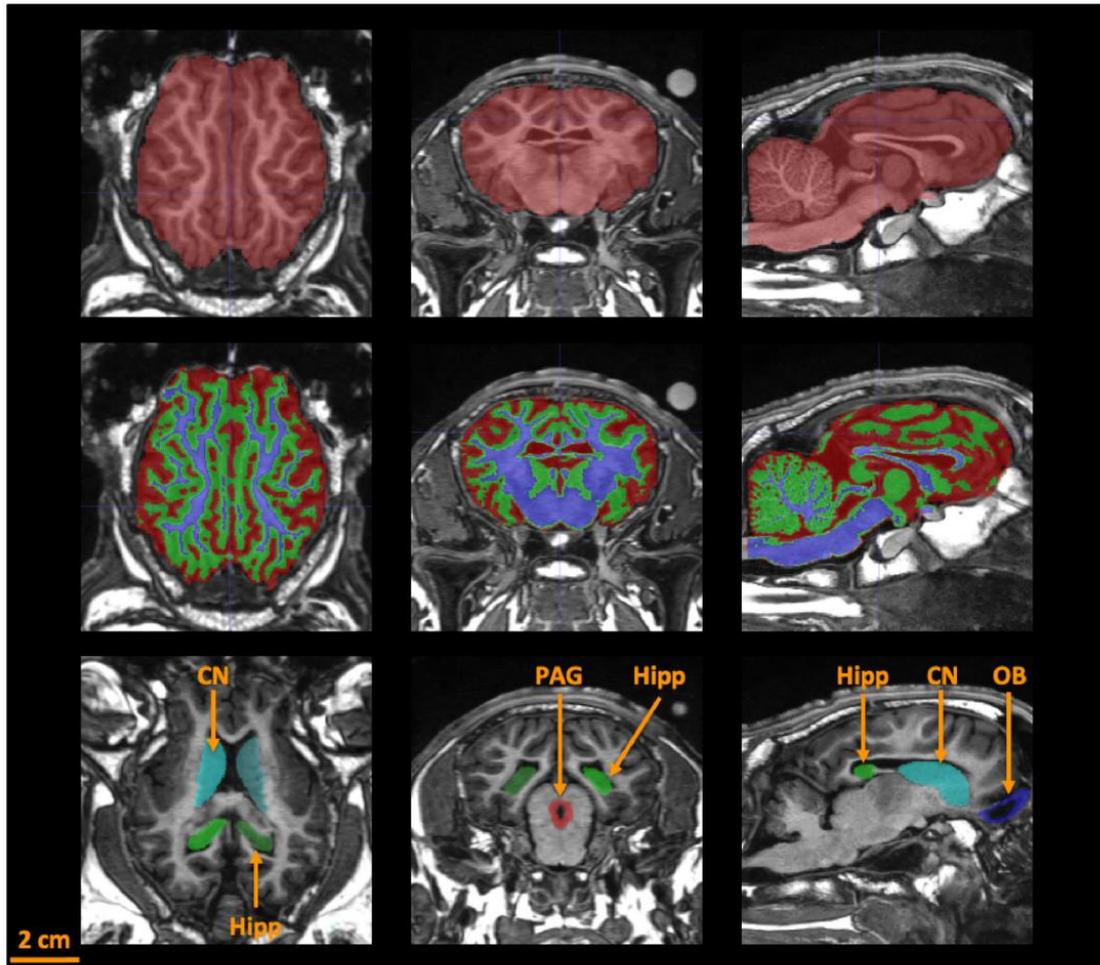
FIGURE 2 Axial (left), coronal (middle) and sagittal (right) slices of the T1w anatomical image of an individual lamb brain at T3 (around 4.5 months of age). The red overlay in the top row shows a manually segmented brain mask. The overlays in the middle row show the automatically segmented gray matter (green), white matter (blue) and cerebrospinal fluid (red). The overlays in the bottom row show the manually segmented regions-of-interest: olfactory bulbs (OB), periaqueductal gray (PAG), hippocampus (Hipp) and caudate nucleus (CN)

T1w MRI for volume calculation of specific brain structures

To calculate the volumes of specific brain structures involved in socioemotional behavior (olfactory bulbs [OB], periaqueductal gray [PAG], hippocampus [Hipp], caudate nucleus [CN], Figure 2c) regions-of-interest (ROI) were manually segmented on the denoised T1w images for $PA_{4.71-6.57w}$ and $PA_{17.57-20.5w}$ using ITK-SNAP. The OB, Hipp and CN were segmented bilaterally and the mean volume was calculated. All ROI were controlled by experts in sheep neuroanatomy.

Structural variables

T1w signal intensity

The mean T1w signal intensity was calculated for each GM, WM and CSF segmentation. As previously done in a pediatric study (Flood et al., 2019), the mean CSF value was used to normalise that of GM and WM.

Diffusion-parameters

The DTI-images were corrected for geometric deformations due to inhomogeneities in the magnetic field using the Fieldmap tool from the Statistical Parametric Mapping toolbox. Eddy-current correction was performed with FSL (FMRIB Software Library v.5.0.4). Using

FSL, the FA- and RD-maps were calculated. The mean FA- and RD-values were calculated for GM, WM, and for specific WM-areas sampled by the segmentation of 3 (corpus callosum, optic chiasm and cerebellum; see Figure 3) or 4 (frontal, parietal, temporal and occipital lobes; see Figure 3) successive sections. This segmentation was done manually on the T1w images and controlled by experts in sheep neuroanatomy. The ROI were then re-sampled to the DTI-image resolution ($2x2x2mm^3$) and the mean FA- and RD-values were calculated.
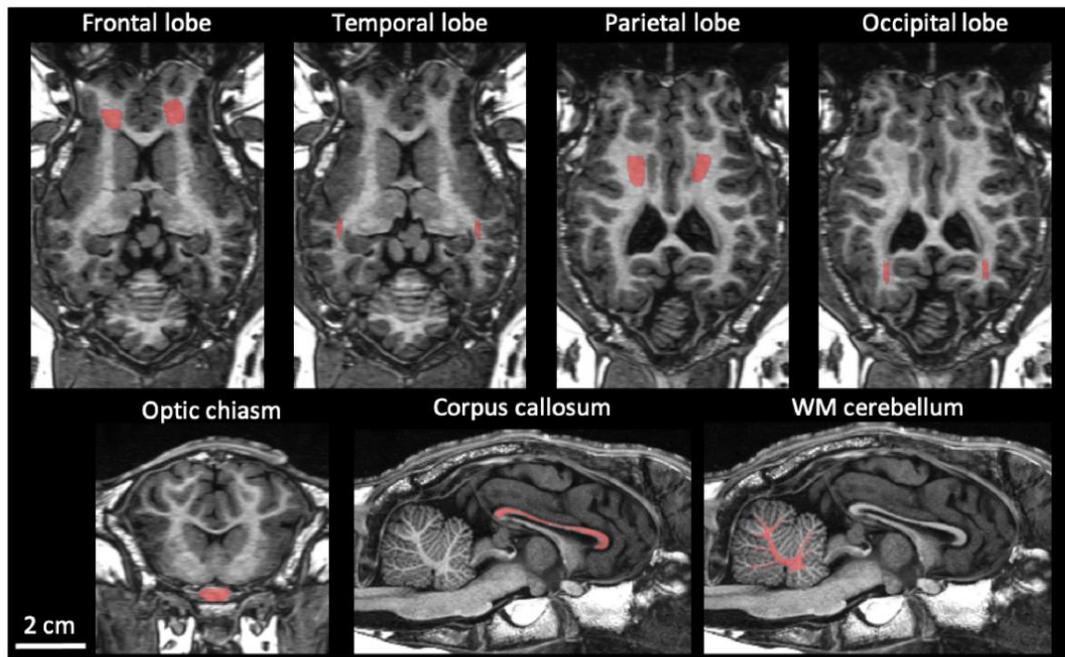

FIGURE 3 Regions-of-interest (red overlay) manually segmented in the white matter

Statistical Analysis

Missing data
Body weight data was not collected for 3 MC $PA_{0.86-2.57w}$ lambs. Due to a lack of T1w MR image contrast in a few individuals, the olfactory bulbs were only segmented for n=6 MC and n=6 MD lambs and the hippocampus for n=7 MC lambs. At the resolution of the DTI-images, some specific WM-areas, that had been segmented on T1w images, contained very few or even zero voxels for some individuals. We only include WM-areas of 3 voxels or more in the statistical analysis.

Statistical analyses
To investigate the impact of early experience (EE) on lamb growth, the coefficient of correlation between age (in days) and body weight (in kg), measured 4 times during the experiment (Figure 1), were compared between the groups using a t-test (JASP version 0.13.0, https://jasp-stats.org/, JASP Team, 2020).

ANCOVA analyses with the covariate age at acquisition
To investigate the impact of early experience (EE) and/or the point of acquisition (PA), each measure (the different morphological and structural parameters and body weight) was analysed independently using an ANCOVA with age at acquisition as a covariate of no interest and Type II Sum of Squares (JASP). Post-hoc analyses were conducted with

Bonferroni correction. Summary statistics for the different measures are presented as means ± standard deviation.

Results
Body weight
Body weight and age of animals are positively correlated in MC (r2=0.995±0.005, n=9) and in MD (r2=0.993±0.003, n=7). T-test analysis highlighted no significant effect of EE on the coefficient of correlation (t=-0.969, p=0.381).

The ANCOVA investigating the impact of EE & PA on body weight after controlling for age at acquisition highlighted no significant effect of EE ($F_{(1,52)}$ = 0.81, p = 0.37, η^2 = 0.013), PA ($F_{(3,52)}$ = 2.20, p = 0.10, η^2 = 0.108) or their interaction ($F_{(3,52)}$ = 0.63, p = 0.6, η^2 = 0.031) (Figure 4).
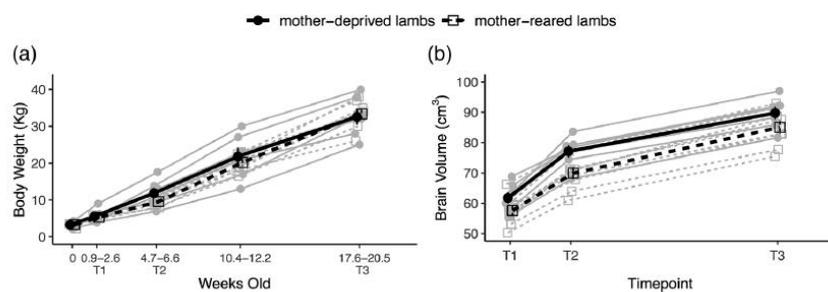

FIGURE 4 Body weights (a) and total brain volumes (b) are presented as group means ± standard error in black and as individual data in gray

Morphometric data
Brain volume

The total brain volume ANCOVA highlighted effects of EE ($F_{(1,41)}$ = 12.20, p = 0.001, η^2 = 0.161) and PA ($F_{(2,41)}$ = 10.85, p < 0.001, η^2 = 0.287) but not for their interaction ($F_{(2,41)}$ = 0.38, p = 0.69, η^2 = 0.01). Brain volume increased with the PA and MD lamb brain volume was higher than that of MC lambs: $PA_{0.86-2.57w}$ MD=61.79±4.57cm^3, MC=57.63±4.57cm^3; $PA_{4.71-6.57w}$ MD=77.08±4.84cm^3, MC=69.95±5.32cm^3; $PA_{17.57-20.5w}$ MD=89.75±5.01cm^3, MC=85.05±5.89cm^3 (Figure 5).
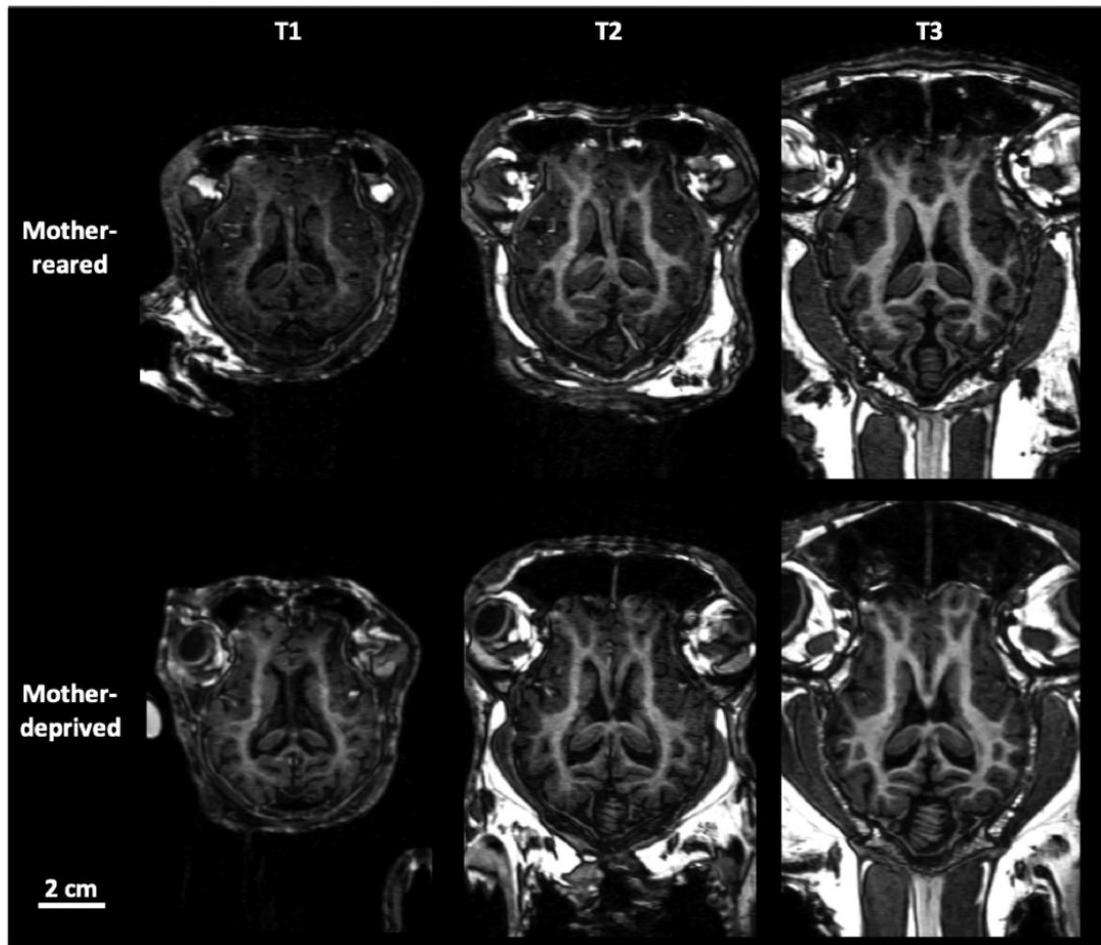
FIGURE 5 Representative horizontal slices of T1-weighted images at each timepoint (T1, T2, T3) of one mother-reared lamb (#14738) and one mother-deprived lamb (#14627)

Gray, white matters and CSF volumes

The GM volume ANCOVA highlighted effects of EE ($F_{(1,41)}$ = 9.05, p = 0.004, η^2 = 0.143) and PA ($F_{(2,41)}$ = 6.15, p = 0.005, η^2 = 0.194) but not for their interaction ($F_{(2,41)}$ = 0.29, p = 0.747, η^2 = 0.009). GM volume increased with the PA and MD lamb GM volume was higher than that of MC lambs: $PA_{0.86-2.57w}$ MD=30.15±2.05cm^3, MC=28.60±2.09cm^3; $PA_{4.71-6.57w}$ MD=35.36±2.43cm^3, MC=32.48±2.84cm^3; $PA_{17.57-20.5w}$ MD=39.59±2.32cm^3, MC=36.59±2.88cm^3 (Figure 6a).
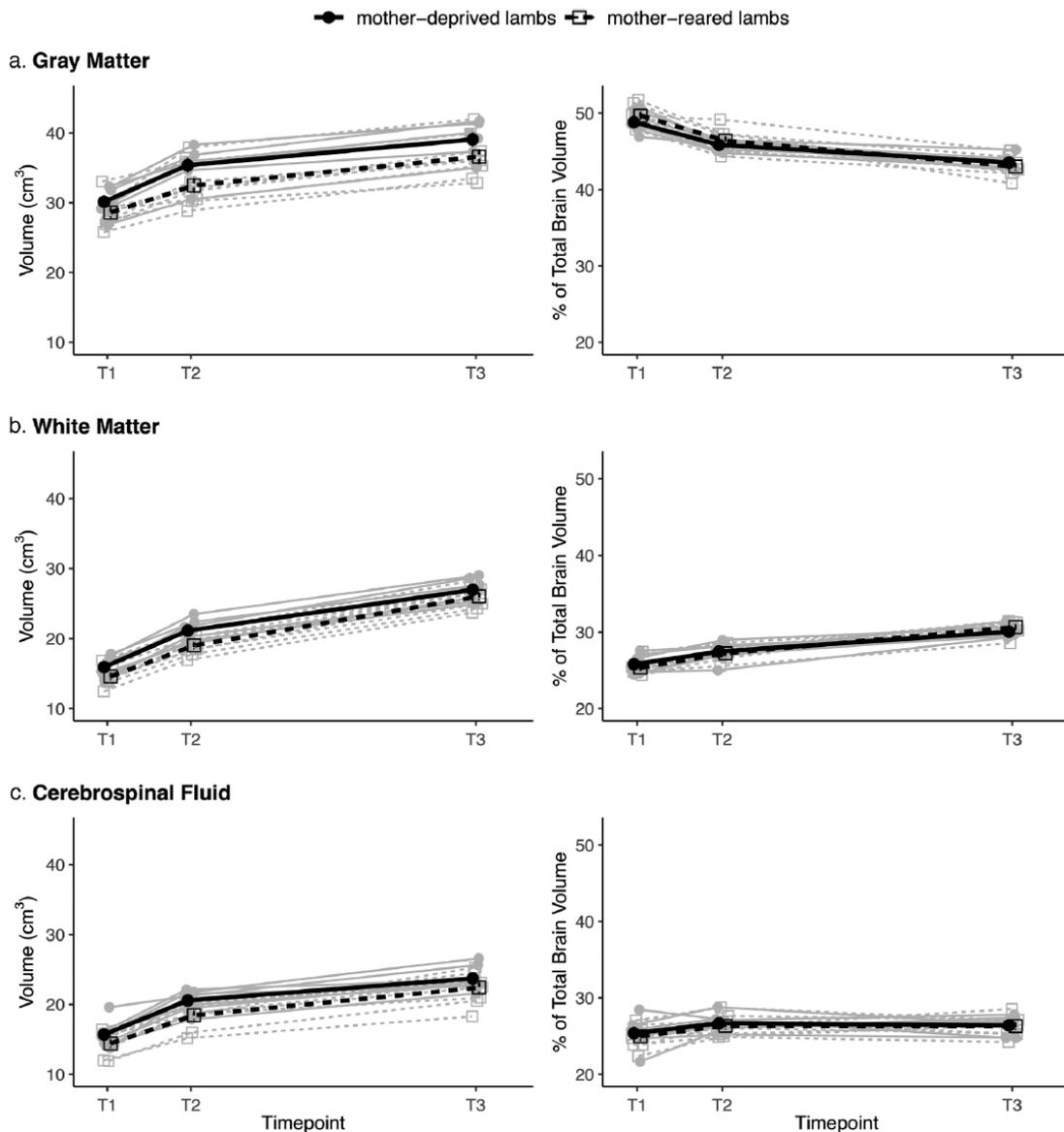
FIGURE 6 Volumes (left) and proportion of total brain volumes (right) of the gray matter (a), white matter (b), and cerebrospinal fluid (c) are presented as group means ± standard error in black and as individual data in gray

The WM volume ANCOVA highlighted effects of EE ($F_{(1,41)}$ = 13.66, p = < 0.001, η^2 = 0.169) and PA ($F_{(2,41)}$ = 11.98, p < 0.001, η^2 = 0.296) but not for their interaction ($F_{(2,41)}$ = 0.52, p = 0.60, η^2 = 0.013). WM volume increased with the PA and MD lamb WM volume was higher than that of MC lambs: $PA_{0.86-2.57w}$ MD=15.94±1.46cm^3, MC=14.61±1.32cm^3; $PA_{4.71-6.57w}$ MD=21.15±1.53cm^3, MC=19.03±1.26cm^3; $PA_{17.57-20.5w}$ MD=26.98±1.61cm^3, MC=26.05±1.46cm^3 (Figure 6b).

The CSF volume ANCOVA highlighted effects of EE ($F_{(1,41)}$ = 8.1 p = 0.007, η^2 = 0.114) and PA ($F_{(2,41)}$ = 10.67, p < 0.001, η^2 = 0.3) but not for their interaction ($F_{(2,41)}$ = 0.31, p = 0.74, η^2 = 0.009). CSF volume increased with the PA and MD lamb CSF volume was higher than that of MC lambs: $PA_{0.86-2.57w}$ MD=15.70±1.96cm^3, MC=14.41±1.56cm^3; $PA_{4.71-6.57w}$ MD=20.58±1.53cm^3, MC=18.45±1.77cm^3; $PA_{17.57-20.5w}$ MD=23.71±1.78cm^3, MC=22.41±2.16cm^3 (Figure 6c).

However, the examination of the GM, WM and CSF percentages in the brain showed that they evolved in the same way in MC and MD lambs during their growth (Figure 6).

For the proportion ofWMin the brain, the ANCOVA highlighted an effect of timepoint (F(2,41) = 4.64, p = .015, η2 = 0.153) but not of early experience (F(1,41) = 0.37 p = .55, η2 = 0.006) or their interaction (F(2,41) = 0.73, p = .49, η2 = 0.024). Regardless of early experience the proportion of WM in the brain increased with timepoint.

For the proportion of CSF in the, brain the ANCOVA highlighted no significant effects: early experience (F(1,41) = 0.35 p = .56, η2 = 0.007), timepoint (F(2,41) = 2.89, p = .067, η2 = 0.122), or their interaction (F(2,41) = 0.07, p = .93, η2 = 0.003).

GM-brain structures
For the caudate nucleus volume, the ANCOVA highlighted effects of EE ($F_{(1,27)}$ = 14.86, p < 0.001, η^2 = 0.276) and PA ($F_{(1,27)}$ = 6.97, p = 0.014, η^2 = 0.129) but not for their interaction ($F_{(1,27)}$ = 0.69, p = 0.413, η^2 = 0.013). The volume of the caudate nucleus increased with PA and was larger for MC than MD lambs.

For the hippocampal volume, the ANCOVA highlighted a significant effect of PA ($F_{(1,23)}$ = 39.09, p = 0.001, η^2 = 0.422) but not of EE ($F_{(1,23)}$ = 0.02, p = 0.891, η^2 < 0.001) or their interaction ($F_{(1,23)}$ = 0.35, p = 0.562, η^2 = 0.004). The volume of the hippocampus increased with PA.

For the olfactory bulb volume, the ANCOVA highlighted no significant effects: EE ($F_{(1,19)}$ = 0.68, p = 0.42, η^2 = 0.034), PA ($F_{(1,19)}$ = 0.02, p = 0.885, η^2 = 0.001) and their interaction ($F_{(1,19)}$ = 0.12, p = 0.73, η^2 = 0.006).

For the PAG volume, the ANCOVA highlighted no significant effects: EE ($F_{(1,27)}$ = 2.45, p = 0.129, η^2 = 0.077), PA ($F_{(1,27)}$ = 0.07, p = 0.79, η^2 = 0.002) and their interaction ($F_{(1,27)}$ = 1.36, p = 0.249, η^2 = 0.044).

Microstructural data

Examination of the microstructural data highlighted an impact of early experience in both gray and white matter, with more parameters being impacted in white matter (Figure 7). Indeed, T1w signal intensity and RD were higher in the gray and white matter of mother-deprived lambs than in mother-reared lambs. FA was also impacted in white matter with higher values observed in mother-reared than in mother-deprived lambs. Moreover, the microstructural data changed between the first two timepoints in white matter (increased T1w signal intensity and FA) and in gray matter (increased FA). FA and RD in different white matter areas indicate an impact of early experience and/or timepoint in a restricted number of areas. In the frontal white matter, there was higher FA in mother-reared than in mother-deprived lambs, and in parietal white matter, there was increased FA throughout the experiment. Detailed statistical results are described below.
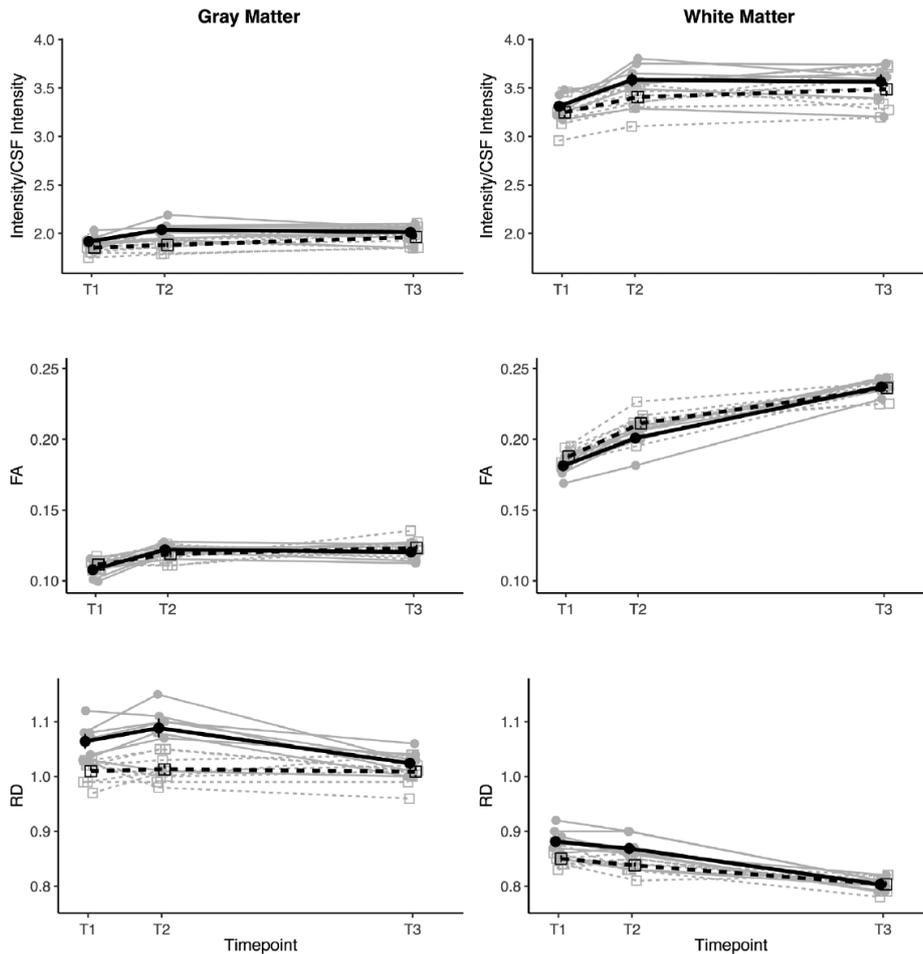
FIGURE 7 T1w signal intensity (top), fractional anisotropy (FA, middle) and radial diffusivity (RD, bottom) are presented as group means ± standard error in black and as individual data in gray for both the gray (left column) and white (right column) matters

Gray matter

For the T1w signal intensity of GM relative to CSF, the ANCOVA highlighted a significant effect of EE ($F_{(1,41)} = 20.885$, $p < 0.001$, $\eta^2 = 0.034$) but not of PA ($F_{(2,41)} = 2.09$, $p = 0.136$, $\eta^2 = 0.058$) or their interaction ($F_{(2,41)} = 2.89$, $p = 0.067$, $\eta^2 = 0.08$). GM intensity was higher for MD compared to MC lambs.

For FA in GM, the ANCOVA highlighted a significant effect of PA ($F_{(2,41)} = 10.72$, $p < 0.001$, $\eta^2 = 0.319$) but not of EE ($F_{(1,41)} = 0.588$, $p = 0.448$, $\eta^2 = 0.009$) or of their interaction ($F_{(2,41)} = 1.914$, $p = 0.16$, $\eta^2 = 0.057$). The FA values in GM increased significantly only between $PA_{0.86-2.57w}$ and $PA_{4.71-6.57w}$ ($T = -2.53$, $p = 0.046$).

For RD in GM, the ANCOVA highlighted a significant interaction between EE and PA ($F_{(2,41)} = 4.41$, $p = 0.018$, $\eta^2 = 0.103$) and a significant effect of EE ($F_{(1,41)} = 32.68$, $p < 0.001$, $\eta^2 = 0.38$) but not of PA ($F_{(2,41)} = 1.68$, $p = 0.198$, $\eta^2 = 0.039$). The main effect of EE was driven by higher RD values for MD lambs than MC lambs. Investigating the interaction effect by observing the data however, showed that the higher RD values for MD lambs were present at $PA_{0.86-2.57w}$ and $PA_{4.71-6.57w}$ but not $PA_{17.57-20.5w}$; however, none of the Bonferroni corrected post-hoc tests to investigate this interaction were significant.

White matter:

For the T1w signal intensity of WM relative to CSF, the ANCOVA highlighted significant effects of EE ($F_{(1,41)}$ = 4.67, p = 0.037, η^2 = 0.086), PA ($F_{(2,41)}$ = 3.92, p = 0.028, η^2 = 0.144) but not their interaction ($F_{(2,41)}$ = 0.535, p = 0.59, η^2 = 0.02). The T1w signal intensity in WM was higher for MD than MC lambs. Observation of the data showed that the T1w signal intensity in WM increased between $PA_{0.86-2.57w}$ and $PA_{4.71-6.57w}$ but not between $PA_{4.71-6.57w}$ and $PA_{17.57-20.5w}$; however, none of the Bonferroni corrected post-hoc tests to investigate the effect of PA were significant.

For FA in WM, the ANCOVA highlighted significant effects of EE ($F_{(1,41)}$ = 5.78, p = 0.021, η^2 = 0.065), PA ($F_{(2,41)}$ = 12.02, p < 0.001, η^2 = 0.271) and their interaction ($F_{(2,41)}$ = 4.47, p = 0.018, η^2 = 0.101). Observation of the data showed that MC lambs had higher FA values in WM than MD lambs for $PA_{0.86-2.57w}$ and $PA_{4.71-6.57w}$ but not $PA_{17.57-20.5w}$; however, none of the Bonferroni corrected post-hoc tests to investigate this interaction were significant.

For RD in WM, the ANCOVA highlighted a significant interaction between EE and PA ($F_{(2,41)}$ = 5.68, p = 0.007, η^2 = 0.157) and a significant effect of EE ($F_{(1,41)}$ = 14.9, p < 0.001, η^2 = 0.206) but not of PA ($F_{(2,41)}$ = 0.27, p = 0.764, η^2 = 0.007). Observation of the data showed that MD lambs had higher RD values in WM than MC lambs for $PA_{0.86-2.57w}$ and $PA_{4.71-6.57w}$ but not $PA_{17.57-20.5w}$; however, none of the Bonferroni corrected post-hoc tests to investigate this interaction were significant.

WM-brain areas
For FA in the WM-brain area of the frontal lobe, the ANCOVA highlighted a significant effect of EE ($F_{(1,36)}$ = 5.05, p = 0.031, η^2 = 0.117) but not of PA ($F_{(2,36)}$ = 0.80, p = 0.456, η^2 = 0.037) or their interaction ($F_{(2,36)}$ = 0.26, p = 0.771, η^2 = 0.012). MC lambs had higher FA values than MD lambs in the WM-brain area of the frontal lobe. For RD in the WM-brain area of the frontal lobe, the ANCOVA….

For FA in the WM-brain area of the parietal lobe, the ANCOVA highlighted a significant effect of PA ($F_{(2,41)}$ = 4.91, p = 0.031, η^2 = 0.012) but not of EE ($F_{(1,41)}$ = 0.02, p = 0.889, η^2 = 0.002) or their interaction ($F_{(2,41)}$ = 0.85, p = 0.436, η^2 = 0.025). Observation of the data showed that the FA values in this area were higher at $PA_{4.71-6.57w}$ than at $PA_{0.86-2.57w}$ and $PA_{17.57-20.5w}$; however, none of the Bonferroni corrected post-hoc tests to investigate the effect were significant. For RD in the WM-brain area of the parietal lobe, the ANCOVA highlighted no significant effects: EE ($F_{(1,41)}$ = 0.83, p = 0.368, η^2 = 0.016), PA ($F_{(2,41)}$ = 2.74, p = 0.077, η^2 = 0.104) and their interaction ($F_{(2,41)}$ = 0.79, p = 0.458, η^2 = 0.03).

For FA in the WM-brain area of the temporal lobe, the ANCOVA highlighted no significant effects: EE ($F_{(1,41)}$ = 3.78, p = 0.059, η^2 = 0.08), PA ($F_{(2,41)}$ = 0.37, p = 0.70, η^2 = 0.016) and their interaction ($F_{(2,41)}$ = 0.58, p = 0.566, η^2 = 0.025). Similarly, for RD in the WM-brain area of the temporal lobe, the ANCOVA highlighted no significant effects: EE ($F_{(1,41)}$ = 3.51, p = 0.068, η^2 = 0.07), PA ($F_{(2,41)}$ = 0.57, p = 0.58, η^2 = 0.022) and their interaction ($F_{(2,41)}$ = 1.57, p = 0.220, η^2 = 0.063).

For FA in the WM-brain area of the occipital lobe, the ANCOVA highlighted no significant effects: EE ($F_{(1,41)}$ = 0.69, p = 0.410, η^2 = 0.015), PA ($F_{(2,41)}$ = 1.64, p = 0.207, η^2 = 0.07) and their interaction ($F_{(2,41)}$ = 0.12, p = 0.89, η^2 = 0.005). Similarly, for RD in the WM-brain area of

the occipital lobe, the ANCOVA highlighted no significant effects: EE ($F_{(1,41)}$ = 1.15, p = 0.29, η^2 = 0.023), PA ($F_{(2,41)}$ = 2.94, p = 0.06, η^2 = 0.118) and their interaction (F(2,41) = 0.07, p = 0.928, η^2 = 0.003).

For FA in the corpus callosum, the ANCOVA highlighted a significant effect of PA ($F_{(2,32)}$ = 3.29, p = 0.05, η^2 = 0.157) but not of EE ($F_{(1,32)}$ = 0.47, p = 0.498, η^2 = 0.011) or their interaction ($F_{(2,32)}$ = 1.28, p = 0.293, η^2 = 0.061). None of the Bonferroni corrected post-hoc tests investigating the effect of PA were significant. For RD in the corpus callosum, the ANCOVA highlighted no significant effects: EE ($F_{(1,32)}$ = 0.44, p = 0.513, η^2 = 0.012), PA ($F_{(2,32)}$ = 1.12, p = 0.339, η^2 = 0.059) and their interaction ($F_{(2,32)}$ = 0.37, p = 0.695, η^2 = 0.02).

For FA in the optic chiasm, the ANCOVA highlighted no significant effects: EE ($F_{(1,29)}$ = 2.96, p = 0.096, η^2 = 0.077), PA ($F_{(2,29)}$ = 0.46, p = 0.639, η^2 = 0.024) and their interaction ($F_{(2,29)}$ = 2.86, p = 0.074, η^2 = 0.148). Similarly, for FA in the optic chiasm, the ANCOVA highlighted no significant effects: EE ($F_{(1,29)}$ = 2.40, p = 0.132, η^2 = 0.067), PA ($F_{(2,29)}$ = 0.56, p = 0.579, η^2 = 0.031) and their interaction ($F_{(2,29)}$ = 1.44, p = 0.252, η^2 = 0.081).

For FA in the cerebellum, the ANCOVA highlighted no significant effects: EE ($F_{(1,41)}$ = 0.10, p = 0.749, η^2 = 0.002), PA ($F_{(2,41)}$ = 3.07, p = 0.057, η^2 = 0.119) and their interaction ($F_{(2,41)}$ = 0.12, p = 0.892, η^2 = 0.004). Similarly for RD in the cerebellum, the ANCOVA highlighted no significant effects: EE ($F_{(1,41)}$ = 0.10, p = 0.756, η^2 = 0.002), PA ($F_{(2,41)}$ = 2.17, p = 0.127, η^2 = 0.089) and their interaction ($F_{(2,41)}$ = 0.25, p = 0.783, η^2 = 0.01).

Discussion
Brain development and maturation in mothered control lambs
Considering the mothered lambs as the healthy condition of rearing, we expected an increase of brain volume as well as of grey and white matter volumes throughout the experiment. In humans, typical paediatric development is characterized by an increased WM volume and an inverted U-shaped trajectory for the total cerebral and GM volumes in developing children and adolescents (Giedd and Rapoport, 2010). Increased total brain volume and percentages of WM and GM have also been observed in rhesus macaques imaged between 2 and 6 months of age (Liu et al., 2019). In our study, we observed the increase of the brain, GM, WM and CSF volumes with similar trajectory-shapes, and that the increase appears less important after 1.5 month especially for the CSF. According to the age of puberty for Ile-de-France ewes, that occurs between 8-10 months depending on the rearing conditions (Perret, 1986), the development of the brain, GM and WM are probably unfinished at the end of our experiment, around 4.5 months. However, focusing on specific brain structures, we noticed that the volumes of olfactory bulbs and PAG are not statistically different between the two last points of acquisition (around 1.5 and 4.5 months) suggesting that the growth of these structures may be almost complete.

The examination of GM, WM and CSF percentages relative to the total brain volume revealed that the trajectory for the CSF is stable whereas the trajectories of GM and WM percentages are in mirror. This finding could suggest that GM percentage decreases in favour of an increase in WM percentage. In our study, these values have been evaluated after automatic segmentation based on the MRI T1w signal intensity and contrast, which varied during brain development. These changes of signal intensity are explained by the decrease of water content in GM and WM, and the increase of myelin (Barkovich et al.,

1988) which can result in mirror evolutions of GM and WM. Thus, structural parameters may be more informative than the total or percentage volume of brain regions.

As suggested by several studies performed in primates, it is relevant to study the brain development/maturation by examining MRI parameters extracted from different types of MRI sequences. First, with the examination of the T1w signal intensity, we hypothesized higher values in WM than GM, and decreasing values in both brain tissues throughout infant development. We found the highest intensity values in the WM that increased between the first two points of acquisition before decreasing. The shape of T1w signal intensity in the GM followed the same shape as that of the WM but with an overall lower intensity. Second, we examined the FA- and RD-values in GM and WM, hypothesizing an increased FA- and decreasing RD-values in both GM and WM. Moreover, we predicted higher FA-values and smaller RD-values in the WM than in the GM. As expected, the evolution of diffusion parameters in WM is coherent with reported data in children (Dubois et al., 2014, 2006; Hüppi and Dubois, 2006) and non-human primate infants (Liu et al., 2019). The FA increased from the first to the last point of acquisition, whereas the RD decreased. These concomitant evolutions in the WM could be the consequences of the myelination of the brain, whereas in the GM the increased FA could be due to a decreasing of free water (Mukherjee et al., 2002).

In summary, our study described for the first time the brain development and maturation of mothered lambs, showing that the most evident modifications were found between about 1-2 weeks and 1.5 months. Moreover, our observations asked the question of the best method to evaluate brain development and maturation. In our opinion, it is better to follow the percentage of different brain matter since the brain volume is tightly linked to the body weight in children (Giedd and Rapoport, 2010), even if the question of the best method to evaluate the brain size could be always questioned (Hasboun et al., 1996). Interestingly, our results demonstrated that brain maturation is still in progress after 4 months of age, whatever the MRI parameters. Indeed, the T1w-signal intensity value decreased in both grey and white matter, suggesting a modification of brain structure. This idea is also supported by the increased FA in both grey and white matter.

Impact of maternal deprivation
Our study showed for the first time, in lambs, the impact of maternal deprivation on different parameters of brain growth and maturation.

Total brain, WM, GM and CSF volumes were all lower in MC than in MD lambs. This is in contrast to study of Rhesus macaques in which no impact of maternal deprivation on brain volume was found at 2, 4 or 6 months of age (Liu et al., 2019). However, we noticed that early experience did not affect the percentages of GM, WM and CSF, which were modified throughout the study. This observation confirmed our question concerning the best indicator of whole brain growth, or even its relevance.

Because of their place in the socio-emotional neuronal network (Grahn et al., 2008; Menant et al., 2016; White, 2009) (Zelena et al., 2018; Nowak et al., 2016), we examined the impact of early experience on OB, CN, Hipp and PAG volumes evaluated at 1 and 4 months of age. We highlighted two relevant information according to our observations. First, the growth of brain structures was not the same for all of them since increased volumes were found only for the CN and Hipp, suggesting that brain development is heterogeneous and not finished at 4 months. Second, we discovered an impact of maternal deprivation on the CN, which was smaller in MD than in MC lambs. These observations were coherent with our hypothesis

of a negative impact of maternal deprivation on these brain structures development. For the CN, regarding its role in motor processes and cognitive functions (Grahn et al., 2008; White, 2009), we expected differences between MC and MD lambs because of the role of mother in lambs' cognitive development and activities. This effect of maternal deprivation on CN development may result from the nutrition in early life, since maternal milk is more adapted than formula-milk. This kind of effect was reported in adolescents of preterm birth who were exposed to high- or standard-nutrient regimes (Isaacs et al., 2008) and in early-life stressed humans (Cohen et al., 2006). In our study, the lack of impact of maternal deprivation on the Hipp volumes is similar to previous data reported in rhesus monkeys reared with their mother or in a maternal deprivation condition (Sánchez et al., 1998; Spinelli et al., 2009) or in humans submitted to early-life stress (Cohen et al., 2006), whereas lower hippocampal volumes have previously been reported in children exposed to poverty (Luby et al., 2013).

Moreover, we hypothesized that maternal deprivation leads to delay or affect brain maturation. This effect may result from psychological stress with the absence of maternal care and/or from formula-milk being less nutritious than breast milk. Our results are consistent with our hypotheses for the T1w-signal intensity and the diffusion parameters, and with reported data obtained in primates.

The global view of GM and WM allowed to identify a negative impact of maternal deprivation on brain maturation that may be more important between about 1.5 week and 1.5 month, several differences no longer existed at about 4.5 months of age. In GM, we noticed higher T1w-signal intensity and higher RD-values in maternal deprived lambs than in mothered lambs especially before 4.5 months. These higher values suggest less maturation, since this process is associated with decreased T1-w signal intensity and decreased RD-values induced by the incorporation of macromolecules into membranes of neural cells and the decrease of free water (Geeraert et al., 2019; Mukherjee et al., 2002). The WM seems to be the most impacted part of the brain since the evolution of all structural parameters showed a delay of maturation in maternal deprived lambs. Indeed, the T1w-signal intensity and RD-values were higher and FA-values were smaller in MD than in MC lambs. Similar results have been reported many times whether in formula-fed children (Deoni et al., 2018, 2013); in bonnet macaques born from stressed mother (Coplan et al., 2010) or in formula-fed rhesus macaques (Liu et al., 2019). Based on description of T1w-signal intensity, FA- and RD-values evolution in normal brain (Barkovich et al., 1988; Flood et al., 2019; Mukherjee et al., 2002; Westlye et al., 2010), we conclude that maternal deprivation leads to delay in brain maturation in lambs. Even if the impact we observed on white matter may result from less nutritious milk, it may also result from stress. Indeed, whereas no data has described the impact of early life stress on brain development in lambs, a negative impact of gestational stress on neural cells at birth (Petit et al., 2015) and a negative impact of gestational corticosteroid exposure by increasing the number of unmyelinated neurons in corpus callosum (Huang et al., 2001) have been reported. Moreover, our results in specific WM-area showed that they were not impacted by the point of acquisition and/or the early experience in the same manners. These differences suggest that it may exist a gradient of WM maturation in sheep as it is described in humans (Dubois et al., 2014). This idea needs to be further explored to better understand the impact of maternal deprivation in lambs since our results allow to conclude a lesser maturation in the WM of the frontal lobe and tendency in the temporal lobe and optic chiasm. A second point needs to be further explored. It concerns the duration of the effect of maternal deprivation, since some

difference between MD and MC lambs disappeared at 4.5 months of age. However, the lesser brain maturation seen in maternal deprived lambs before 4.5 months of age could have long-term effects on animal's life.

Conclusions

We described for the first time the brain development and maturation in mothered lambs. These present the same evolution as thus of primates: increased volumes of brain, gray and white matter, increased values of FA and decreased values of RD. The modifications observed for brain development and maturation are more important before 4.5 months of age. Interestingly, we demonstrated that maternal deprivation, currently used in dairy production, leads to strong modification of brain maturation delaying it from as early as 1.5 weeks of age. These alterations observed may help to explain the behavioural, endocrine and health alterations reported in artificial reared lambs.

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