Psychoneuroendocrinology (2013) 38, 1112—1121

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Early adverse experience alters dendritic spine density and gene expression in prefrontal cortex and hippocampus in lambs

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Received 19 July 2012; received in revised form 22 October 2012; accepted 22 October 2012

KEYWORDS
Prenatal stress;
Glucocorticoids;
Spine density and morphology;
Gene expression;
Hippocampus;
Prefrontal cortex;
Lambs

Summary In the laboratory, prenatal stress produces alterations in the structure and function of corticolimbic neurons. Here we report changes in gene expression and corticolimbic dendritic spine morphology in the offspring of pregnant ewes subjected to aversive interactions with human handlers during the last five weeks of pregnancy (AVS) compared to control dams that received gentle handling (GEN). AVS lambs had higher spine density on pyramidal neurons in area CA1 of the hippocampus and in medial prefrontal cortex compared to GEN lambs, as well as a lower ratio of mushroom spines to stubby and thin spines in area CA1. Expression of genes involved in brain development and spine morphogenesis was decreased in hippocampus and prefrontal cortex in AVS compared to GEN lambs. This study is the first demonstration that an ecologically relevant aversive experience in a field setting alters neuronal structure similarly to previous reports from laboratory settings and that even for animals domesticated over 12,000 years ago, an apparently mild stressor, resulting from human—animal interactions, can have similarly profound impacts on corticolimbic morphology.

1. Introduction

Prenatal stress is associated with the development of childhood and adult onset psychiatric disorders such as depression (Van den Bergh et al., 2007), post-traumatic stress disorder (Seckl and Meaney, 2006), and schizophrenia (Koenig et al., 2002). A large body of laboratory experiments demonstrates

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http://dx.doi.org/10.1016/j.psyneuen.2012.10.018

that stressful events experienced by a pregnant female produce a variety of negative outcomes on emotionality and its neural substrates in the offspring (Glover, 2011). For instance, prenatal stress in rodents alters prepulse inhibition (Koenig et al., 2005), acquisition (Lee et al., 2011) and extinction of conditioned fear (Green et al., 2011), and anxiety-like behaviors (Vallée et al., 1997) in adults. These cognitive and behavioral alterations are associated with alterations in corticolimbic structures involved in regulation of emotion (prefrontal cortex and hippocampus) and hypothalamic-pituitary adrenal (HPA) axis function, including alterations in dendritic morphology and spine density in the hippocampus (Mychasiuk et al., 2012) and in the prefrontal cortex (Murmu et al., 2006; Mychasiuk et al., 2012).

Aversive handling and negative human–animal interactions are correlated with increased fear of humans in domestic animals and have a number of undesirable consequences for animal welfare and productivity (Rushen et al., 1999). In contrast, the development of a positive human–animal relationship, involving regular and long-term contact with humans, reduces stress and the risk of injury to animals and humans (Waiblinger et al., 2006). Thus, variations in the quality of these interactions are potential sources of perinatal stress, which could have important consequences for offspring. Recent studies in several species of farm animals have shown effects of prenatal stress on emotional reactivity of the young (e.g., sheep and goats (Roussel et al., 2005, 2006); cattle (Lay et al., 1997); pigs (Jarvis et al., 2006)). These studies typically used stressors such as isolation, repeated transportation, or 'weekly' social disruption events that are unlikely to happen on farms. Furthermore, very few studies in farm animals have included brain measures in the offspring to assess the consequences of prenatal stress (Otten et al., 2010). Previously, we showed that aversive handling stressed pregnant ewes (AVS), increased their grooming behavior toward their offspring (Hild et al., 2011) and increased fearfulness in their progeny (Coulon et al., 2011) compared to gentle treated ewes (GEN). Therefore, the goal of this study was to provide the first assessment of changes in glucocorticoids, gene expression and corticolimbic morphology in offspring associated with an ecologically valid prenatal aversive treatment in a field setting studying domestic sheep.

For this purpose, we first analyzed the concentration of cortisol and cortisone in the placenta of AVS and GEN ewes after lambing to investigate the effect of the aversive treatment on lambs' exposure to glucocorticoids. We previously reported a tendency toward higher concentration of salivary cortisol in the aversively treated ewes after the treatment sessions compared to the gently treated ewes (Hild et al., 2011). Glucocorticoids easily cross the placenta during the last third of pregnancy in ewes (Wood and Rudolph, 1984) and excess glucocorticoid exposure may alter brain development in fetal lambs (Brunton and Russell, 2011). The last third of gestation is a vulnerable period for brain development in sheep (McIntosh et al., 1979). After a first major growth phase of the brain, the hippocampus and the prefrontal cortex are well established regions in fetal sheep at 90–92 days of gestation. The feotal brain then undergoes a second ''growth spurt'' from 90 days to birth, as reflected in changes in brain weight and DNA content. In addition, increases in protein:DNA ratios and cholesterol content in the forebrain from gestational day 81 to birth suggest that the third trimester is an important period for neuronal growth and myelinization (McIntosh et al., 1979). Some mechanisms, such as high levels of 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2) in the placenta catalyze the rapid inactivation of cortisol to cortisone, which will not activate glucocorticoid receptors and may offer protection to the developing fetus from the mother's high glucocorticoid levels (Meaney et al., 2007). Prenatal exposure to excess glucocorticoids has been shown to alter the mRNA level of glucocorticoid receptor (GR) in rodents (Charil et al., 2010). The hippocampus and prefrontal cortex are rich in glucocorticoid and mineralocorticoid receptors and are particularly vulnerable to glucocorticoid excess, especially in early life (Charil et al., 2010). Therefore, we next measured cortisol and cortisone concentration and the expression of the GR gene (Nr3C1- nuclear receptor subfamily 3, group C, member 1) in the hippocampus and prefrontal cortex of the AVS and GEN lambs. Compromised GR function in the nervous system impairs HPA axis regulation, resulting in increased glucocorticoid levels that may contribute to behavioral and cognitive deficits (Brunton and Russell, 2011) by altering neuronal morphology and function (Cook and Wellman, 2004; Mizoguchi et al., 2004). So then we examined the effects of aversive and gentle handling in late pregnant ewes on apical dendritic spine density and morphology in pyramidal neurons in hippocampus and prefrontal cortex tissue. Finally, we also examined alterations in the expression of genes regulating spine morphology and brain development: Ras homolog gene family, member A (RhoA), Ras-related C3 botulinum toxin substrate 1 (Rac1), postsynaptic density protein 95 (PSD-95), purine-rich element binding protein A (PURα), and carboxypeptidase E (CPE). The Rho family of small GTPases, including RhoA and Rac1, regulates the dynamics of the actin cytoskeleton and is an important contributor to spine formation and elimination. Overexpression or suppression of these molecules as well as their interacting proteins results in changes in the density of dendritic spines (Bhatt et al., 2009). PSD-95, a postsynaptic scaffolding protein that acts to promote maturation of dendritic spines, has also been reported to play an important role in synaptic remodeling (Charych et al., 2006). PURα is an essential protein for postnatal brain development which localizes specifically to dendrites and can regulate Rho at multiple levels, including basal protein levels, subcellular compartmentalization, and turnover of active RhoA in order to promote dendritic maturation (Mishra et al., 2012). CPE is a multifunctional protein that plays many nonenzymatic roles in the endocrine and nervous systems. CPE has a role in modeling the cytoarchitecture of neurons with respect to dendritic growth and the dendritic spine formation that can impact synaptogenesis and neural function (Cawley et al., 2012).

Changes in spine density and morphology could have direct and profound effects on corticolimbic function. Indeed, dendritic spines are the primary sites of excitatory synapses on pyramidal neurons, and their morphological plasticity may play a pivotal role in the remodeling of neural circuitry that underlies higher brain functions such as learning and memory (Alvarez and Sabatini, 2007). According to previous study of prenatal stress in rodents (Weinstock, 2008), we hypothesized that we would observe a difference in spine density and morphology on pyramidal neurons in the

hippocampus and in prefrontal cortex between AVS lambs and GEN lambs explained by a difference in their glucocorticoids concentration and expression of genes involved in brain development and spine morphogenesis. These differences could explain the increased fearfulness observed in AVS lambs (Coulon et al., 2011).

2. Methods

2.1. Animals and treatments

All experimental procedures were performed in a working farm setting (Sandnes Research Station, Norway). Animal care and all procedures were completed in accordance with the authorization of the Norwegian Ministry demands for FELASA category C, researcher, and approved by the Norwegian Animal Research Authority.

Pregnant *Norwegian-dala* ewes predicted to give birth to twins within 22 days of each other were identified via ultrasound examination and selected from a flock of animals living in the same barn. They were moved to three experimental tents six weeks before the earliest expected parturition. Each tent contained two groups of four ewes housed in 10 m^2 straw-bedded group pens, with acoustic contact within and between tents. Visual and direct physical contacts were not possible between tents and were partially blocked within tents by a 1.5 m corridor with opaque canvas fences. Ewes were fed by a familiar shepherd with hay ad libitum and appropriate quantities of standard concentrate feed twice daily (0830 and 1400 hours). Access to water was ad libitum.

One group in each tent received gentle treatment (GEN), while the other group received aversive treatment (AVS), 10 min, twice daily, during the last 5 weeks before the first expected parturition. These two treatment groups were chosen for two reasons. First, in our previous study (Zanella et al., 2009), we showed that ewes receiving positive human interaction had the same behavior toward humans as the ewes receiving minimal handling. Second, we used AVS and GEN groups to ensure that the quantity of human interaction was comparable across treatments. Thus, our two treatments differed only by the type (gentle or aversive) rather than quantity of human interaction, and were chosen to maximize potential group differences. The four animals housed together were moved together by one handler to a test pen (7.5 m $\times$ 3 m) in another tent 10—16 m away from their home pen for a treatment session. This tent was closed to minimize the likelihood that the voice of the handler would carry to other tents. As the ewes were treated in groups, they rarely vocalized during or after treatment sessions. In addition, we did not observe stressful behavior of the other ewes in their home pen (no vocalization or vigilance behavior) when a group was treated. Two handlers (unfamiliar females) performed the treatment sessions (one handler per treatment session) and rotated every second day throughout the treatment period; thus, all groups received handling from all handlers. Treatments were stopped when the first ewe in the tent lambed, and averaged 32 $\pm$ 0.25 days. After lambing, contact with humans (familiar shepherds) was limited to that necessary to perform common farm routines (feeding, maintenance, visual inspection of animals).

GEN occurred at predictable times, approximately midway between morning and afternoon feedings. When animals arrived in the experimental pen, the human handler sat on a chair, talking and behaving calmly. The handler made slow movements, and avoided looking directly into the eyes of the animals, as direct gaze has been shown to stress sheep, as reflected in increased locomotor activity and urination (Beausoleil et al., 2005). When the ewe approached, the handler opened her hand to allow the ewe to sniff it voluntarily. The ewe was stroked if she was within reach and showed no avoidance reaction to physical contact. AVS was unpredictable, occurring at random times across days. The type of aversive treatment also varied randomly across weeks, and consisted of different combinations of suddenly walking with swift and erratic movements, speaking in a loud voice, shouting intermittently, and staring directly into the ewes' eyes. This manipulation has been shown to produce alterations in emotional behavior in 1-month-old offspring (Coulon et al., 2011).

Salivary samples from each ewe were collected once a week before and after the treatment session. Sampling was carried out 15 min before the treatment session and 15 min after the ewes were returned to their home pen by the handler who applied the treatments. A small enclosure was built within the pen with portable metal bars where the four ewes were in close physical contact with each other. This was meant to curb their movements so that restraining by the human during the saliva collection was moderate and stress for animal was minimal. Saliva was collected on cotton buds and took 30—60 s per ewe and this would not allow time for the stress of contact with the stockperson collecting saliva samples to influence the cortisol content of saliva. Ewes were sampled in a randomized order every time (Hild et al., 2011). Cortisol concentrations were marginally higher in AVS ewes compared to GEN ewes after the treatment sessions (Hild et al., 2011), suggesting that AVS was mildly stressful.

At the first signs of imminent lambing (e.g., restlessness, appearance of water bag, pawing of the ground), ewes were temporarily placed in individual pens, a standard husbandry practice. Videos were recorded during the first 2 h postpartum to assess maternal behavior for another experiment (Hild et al., 2011). After 24—48 h, ewes and their lambs were returned to their home pens. Average pregnancy duration for AVS and GEN ewes was comparable (144.9 $\pm$ 1 days and 146.1 $\pm$ 0.6 days, respectively). Forty-three of forty-eight lambs survived, a typical survival rate for twin lambs. One lamb per ewe was randomly selected for behavioral testing at one month after parturition (Coulon et al., 2011); the non-tested sibling was used for the current study. Lambs stayed in their home pen with the dam and sibling throughout the experimental period.

2.2. Dendritic spine analysis

Five AVS lambs (three females, two males) from AVS ewes and five GEN lambs (three males, two females) from GEN ewes were randomly selected from the three tents for brain morphology and gene expression studies. One month old lambs were used so that we could observe the behavior of their siblings at 3 weeks (Coulon et al., 2011) without

altering the social group by removing the lambs used in the present study. Lambs (33–36 postnatal days of age) were taken individually from their home pens and immediately euthanized (30 mg/kg pentobarbital *iv)*. Brains were removed within 10 min and the right hemisphere was processed for Golgi histology (Gibb and Kolb, 1998; Cook and Wellman, 2004). Brains were immersed 38 days in Golgi-Cox solution, followed by immersion in 30% sucrose in saline. The rostral pole of PFC and all of the hippocampus were dissected and coronal sections (200 μm) were cut on a vibratome. Sections were mounted on gelatin-subbed slides, alkanized, developed, fixed, dehydrated, cleared, and coverslipped.

Dendritic spine type and density were assessed on terminal segments of apical dendrites of pyramidal neurons in hippocampal area CA1 (10 neurons per lamb) and the superficial, middle, and deep layers of the medial wall of the rostral pole of prefrontal cortex (6 neurons per lamb). Pyramidal neurons were identified by their characteristic soma shape, prominent apical dendrite, and dendritic spines. Each terminal segment (≥30 μm long) was traced at 1000×. Dendritic spines were counted and expressed as spines/μm and average for each animal for each brain area. Spines were labeled as thin, stubby or mushroom type (Peters and Kaiserman-Abramof, 1970) based on morphology described by Michelsen et al. (2007) as follows: thin spines, long narrow necks and small to medium-sized heads; mushroom spines, short necks and large heads; stubby spines, short protrusion with no clear necks. These three spine types are readily identifiable using light microscopy in Golgi-stained tissue, and many previous studies have used similar techniques to quantify differences in spine density and morphology in Golgi-Cox stained material (e.g., Auffret et al., 2009; Irwin et al., 2000; Konur et al., 2003; Magariños et al., 2011). Data were collected by an experimenter blind to the experimental treatments.

2.3. Quantitative real-time PCR and glucocorticoid analyses

At birth, placenta samples were snap frozen in liquid nitrogen, and stored at −80 °C. Following euthanasia, PFC (from anterior to the primary motor cortex to the rostral pole) and all of the hippocampus were dissected from the left hemisphere, snap frozen in liquid nitrogen, and stored at −80 °C. Samples were powdered and homogenized in liquid nitrogen and aliquoted for glucocorticoid extraction and quantification and RNA extraction for RT-qPCR analyses.

Glucocorticoid analyses. Aliquots (150 mg) from PFC, hippocampus and placenta were homogenized with an Ultraturrax T25 homogenizer, extracted with 10 ml ethyl acetate, and evaporated under nitrogen stream on a heating block. Total protein concentration in the samples was determined using the Bio-Rad Bradford Total Protein Assay. Cortisol levels were assayed via ELISA (Correlate-EIA™ Kit) with the following modifications for sheep: incubation time was 3 h and sample volume was 100 μl. Samples were randomized across one plate to minimize intra-assay variability. Cortisone levels were assayed using a cortisone enzyme immunoassay (EIA). For a detailed description of both assays' performance, see Rettenbacher et al. (2004) and Hild et al. (2011).

Quantitative real-time PCR. We used RT-qPCR to examine expression of the GR gene (Nr3C1) as well as several genes related to dendritic spine morphology and brain development: RhoA (Ras homolog gene family, member A), Rac1 (Ras-related C3 botulinum toxin substrate 1), PSD-95 (postsynaptic density protein 95), Purine-rich element binding protein A (PURα) and carboxypeptidase E (CPE). Total RNA from 100 mg aliquots from PFC and hippocampus was isolated using Trizol extraction and dissolved in RNase-free water. Concentration of the samples was measured using a NanoDrop spectrophotometer, and quality assessed using Agilents Bioanalyzer 2100 expert. 1 μg of total RNA was used for cDNA synthesis using oligo(dT) primers and SuperscriptIII (Marjara et al., 2010). cDNA was diluted 1:5 and 2 μl of this was used as template in each RT PCR reaction. All samples were run in duplicates for all genes tested. RT-qPCR was performed as described by Marjara et al. (2010), on a LightCycler® 480 Real-Time PCR System (Roche) using the LightCycler® 480 SYBR Green I Master mix (LightCycler® 480 Software; Roche). Crossing point values (Cp) were calculated using the maximum-second-derivative method and relative expression of each specific candidate gene for individual sample was calculated as $2^{\text{Cp-target}}/2^{\text{Cp-reference}}$ ratio of a target gene versus reference gene/housekeeping gene. All primers were tested on a cDNA that was serially diluted in 1:5 and efficiency of each primer was calculated (see Table 1) above 1.9. As in previous studies (Marjara et al., 2010, 2011), we used 7 points of 1:5 dilutions starting from 1:50 and ending at 1:781,250 to ensure that we obtained at least 4 valid points for our efficiency calculation. See Table 1 for sequence and annealing temperature used for each primer. To find a suitable housekeeping gene for our study, a validation of three of the most commonly used reference genes in *Ovis aries*—Actin B (Act B), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and succinate dehydrogenase complex subunit A flavoprotein (SDHA)—was performed (Lampo et al., 2009). SDHA was the most suitable reference gene for the treatment and tissues tested, and was thus used for normalization of the qPCR data.

2.4. Statistics

Given the non-normal distribution of data and relatively small *N*'s, non-parametric statistics were used. Cortisol and cortisone concentrations, spine density, and gene expression were compared between AVS and GEN lambs using Mann–Whitney *U* tests. Spearman correlations were calculated between PFC and CA1 spine density and grooming behavior of the ewes and between PFC and CA1 spine density and cortisol and cortisone concentrations. All statistical analyses were two-tailed with a significance level set at 0.05. The results are expressed as means ± standard error of the mean (SE). No significant sex-related difference was observed for any measure (Mann–Whitney *U* tests).

3. Results

3.1. Effect of prenatal treatment on glucocorticoids

Cortisone concentration was higher in placenta of GEN ewes compared to AVS ewes ($U = 3$, $p < 0.05$, Fig. 1a). No other

Table 1 Selected candidate genes and reference genes used in *Ovis aries* and their primers: sequence, annealing temperature, amplicon size in base pair, efficiency and accession number.

Gene name	Primer name	Sequence	AT/Amp[a]	Eff[b]	Accession number
Glucocorticoid receptor	GR-NR3C1-Fwd GR-NR3C1-Rev	GAAGTCATTGAACCCGAGGTG CCATTTCACTGCTGCAATCAC	60/125	2.1	EU371026
Ras-related C3 botulinum toxin substrate 1	RAC1-Fwd RAC1-Rev	CACCACTGTCCCAACACAC GCGTCAGCTTCTTCTCCTTC	59/100	2.0	FJ943986
Ras homolog gene family	RHOA-Fwd RHOA-Rev	CCGGAAGTCAAGCATTTCTGTC ACTGGCTCCTGCTTCATCTTG	59/116	2.0	FJ943984
Postsynaptic density protein	PSD95-Fwd PSD95-Rev	GTCTCAGGGTCAACGATAGCA GTAGAGGCGGACGATAGAGC	59/160	1.94	M96853
Carboxypeptidase E	CPE-Fwd CPE-Rev	CGTGCCTGGAGGAATGCAAG CCTCGGTGTATCTGATGAATGT	60/162	1.9	AF063109
Purine-rich element binding protein A	PURA-Fwd PURA-Rev	GACGTGAAGCAGAACGCCAAG ATGAAGTCGCCCAGGTAGTC	60/125	2.0	NM_001009447
Actin beta	Actb-Fwd Actb-Rev	CGCAGACAGGATGCAGAAAGA GCTGATCCACATCTGCTGGAA	60/148	1.90	DQ386889
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH-Fwd GAPDH-Rev	GATCCTGCCAACATCAAGTG TCACAAACATGGGAGCGTCA	62/160	1.97	AF035421
Succinate dehydrogenase complex subunit A flavoprotein	SDHA-Fwd SDHA-Rev	CAGCAGAAGAAGCCGTTTGAG CACAGTCGGTCTCGTTCAAAG	60/127	1.96	DQ386895

[a] Annealing temperature/amplicon size in base pair
[b] Efficiency.

significant differences in stress hormones in placenta were observed between GEN and AVS ewes (Fig. 1a).

In hippocampus and PFC, cortisol and cortisone levels and GR gene expression did not differ between AVS and GEN lambs (Fig. 1b and c).

3.2. Effect of prenatal treatment on dendritic spine density and morphology

Completely stained neurons in PFC and hippocampal area CA1 were plentiful, and thin, mushroom, and stubby spines were

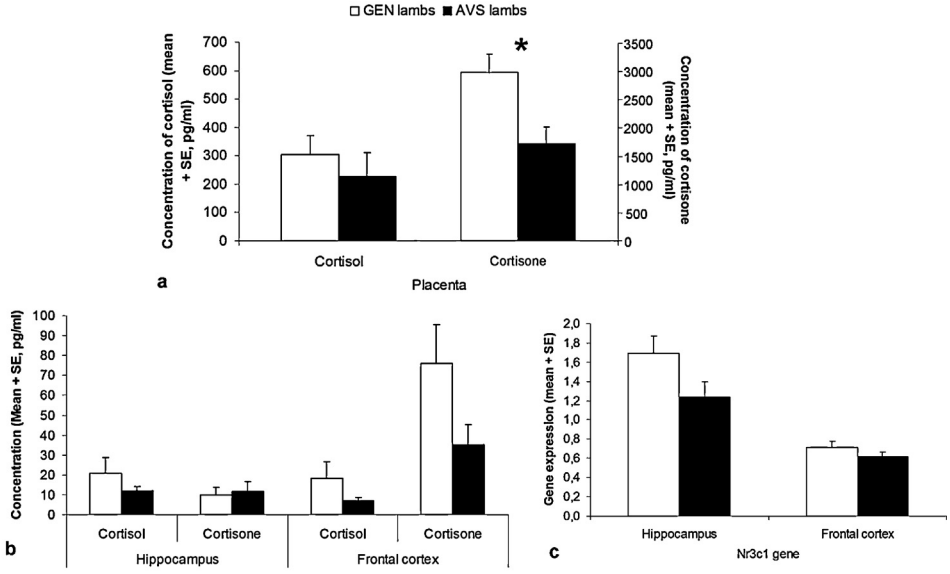

Figure 1 Aversive prenatal handling decreases placental cortisone in lambs. (a) Placental cortisone, but not cortisol, is lower in AVS relative to GEN lambs ($*p < 0.05$). (b) Cortisol and cortisone in hippocampus and PFC of GEN versus AVS lambs are not significantly different. (c) Glucocorticoid receptor (Nr3C1) mRNA levels in hippocampus and PFC of AVS lambs and GEN lambs.

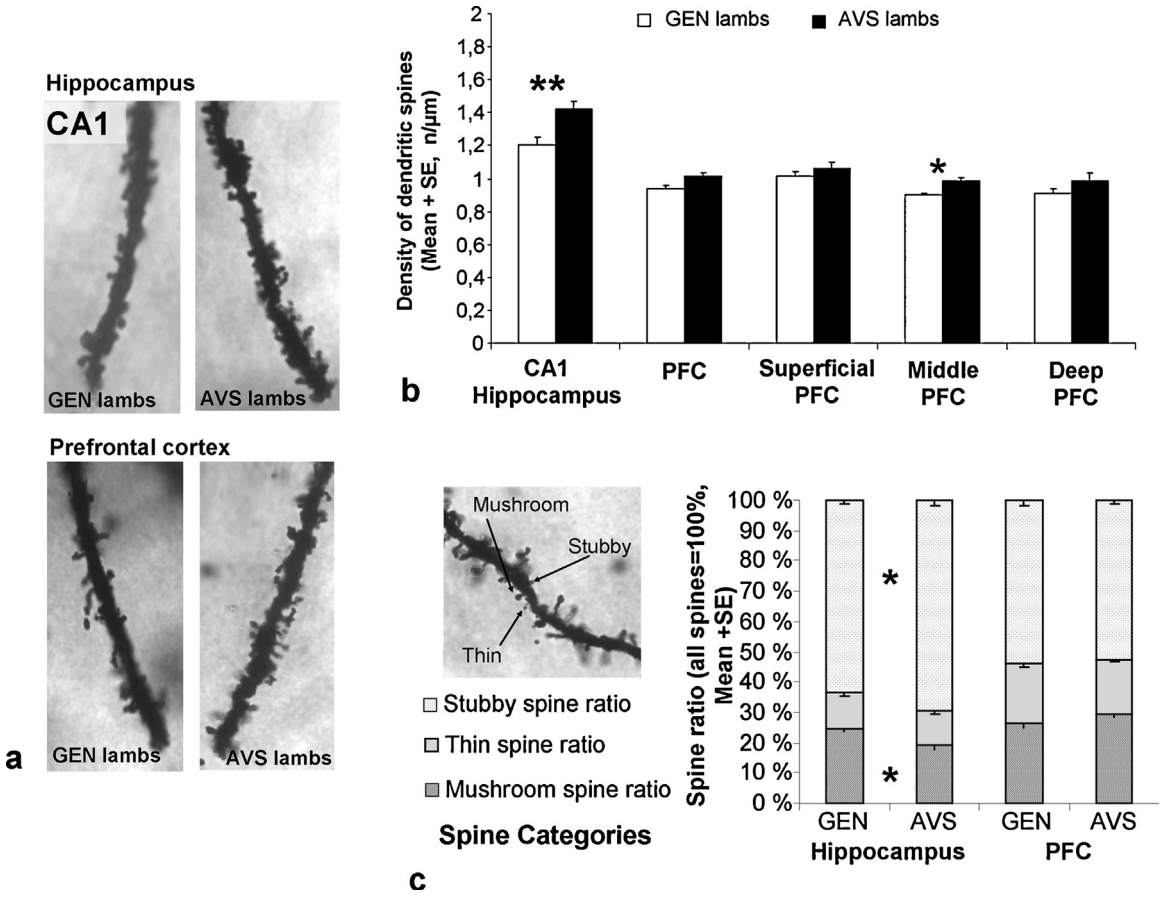

Figure 2 Aversive prenatal handling increases spine density in hippocampus and PFC and alters spine morphology in hippocampus (a) terminal apical branches of pyramidal neurons in CA1 and PFC of GEN and AVS lambs. (b) Spine density was increased in CA1 (**$p < 0.01$) and in the middle (*$p < 0.05$), but not superficial or deep, layers of PFC. (c) *Left*. Spines were classified as stubby, thin, or mushroom. *Right*. In CA1, the proportion of mushroom spines was decreased in AVS lambs compared to GEN lambs (*$p < 0.05$), while the proportion of stubby spines was increased (*$p < 0.05$).

readily identifiable (Fig. 2a and b). The within-group variability, as reflected in the SE, was small and comparable to that seen in other studies using similar methods (e.g., Magariños et al., 2011; Martínez-Téllez et al., 2009; Silva-Gómez et al., 2003). Prenatal aversive handling altered spine density in both hippocampal area CA1 and PFC (Fig. 2a and b). AVS lambs ($N = 5$) had significantly higher spine density in CA1 (1.42 ± 0.04 versus 1.02 ± 0.04 spine/µm, $U = 0$, $p < 0.01$, Fig. 2c) and in the middle layers of the prefrontal cortex (0.98 ± 0.03 versus 0.89 ± 0.02 spine/µm, $U = 3$, $p < 0.05$, Fig. 2c) than GEN lambs ($N = 5$). Maternal behavior (time spent grooming) was not correlated with spine density in either hippocampus or PFC ($R = 0.02$ and $R = -0.2$, respectively; *NS*). We found overall no significant correlation of spine density with stress hormones excepted for a significant negative correlation ($R = -0.66$, $p < 0.05$) between spine density in PFC and placental cortisone concentration.

In PFC, the proportions of different spine types were not significantly different between AVS and GEN lambs. However, in CA1, the proportion of mushroom spines was lower in AVS lambs compared to GEN lambs (19% versus 25%, $U = 3$, $p < 0.05$), while the proportion of stubby spines was higher (69% versus 64%, $U = 2$, $p < 0.05$).

3.3. Effect of prenatal treatment on gene expression

AVS lambs had less expression of RhoA in PFC compared to GEN lambs ($U = 3$, $p < 0.05$, Fig. 3), and less expression of PSD-95 in hippocampus compared to GEN lambs ($U = 3$, $p < 0.05$, Fig. 3). Expression of PURα and CPE was significantly reduced in PFC of AVS lambs ($U = 3$, $p < 0.05$, Fig. 3). However, there were no significant differences between AVS and GEN lambs in the expression of these genes in the hippocampus (Fig. 3). The small variability for gene expression is similar to that seen in other studies (e.g., Marjara et al., 2011).

4. Discussion

This study provides the first demonstration of changes in corticolimbic morphology and gene expression associated with an ecologically valid prenatal aversive treatment in a field study. Prenatal aversive handling of ewes resulted in significant changes in apical dendritic spine density and morphology in the hippocampus and PFC of one-month-old offspring, with concomitant decreases in expression of spine

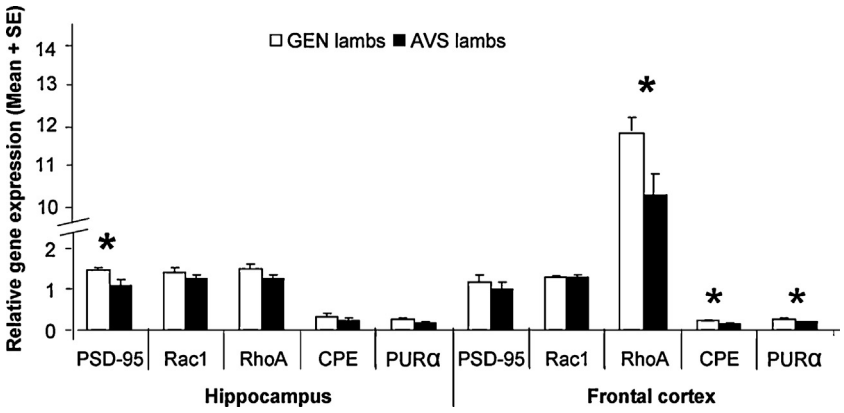

Figure 3 The expression of genes involved in brain development and spine morphogenesis was lower in hippocampus and prefrontal cortex in AVS compared to GEN lambs. RhoA, and Rac1, PSD-95, CPE and PURα mRNA levels in hippocampus and PFC of GEN and AVS lambs. AVS lambs had less expression of PSD95 in hippocampus, and RhoA, CPE and PURα in PFC (*$p < 0.05$).

morphology-related genes in AVS relative to GEN lambs. Our data are consistent with previous laboratory studies showing similar changes in spine density (Murmu et al., 2006; Martınez-Téllez et al., 2009; Mychasiuk et al., 2012) and morphology (Michelsen et al., 2007) in CA1 of prenatally stressed rats, and demonstrate that even for animals domesticated over 12,000 years ago, an apparently mild stressor, resulting from human to animal interactions, can have similarly profound impacts on corticolimbic morphology.

Cortisone concentration was higher in placenta of gently handled ewes compared to aversively handled ewes. Placental 11β-HSD2 normally catalyzes the inactivation of cortisol to cortisone and may offer protection to the developing fetus from the mother's relatively high glucocorticoid levels (Meaney et al., 2007). Thus, the reduced cortisone in AVS placenta may reflect reduced activity of placental 11β-hydroxysteroid dehydrogenase 2 and consequently higher levels of cortisol reaching the fetus of AVS ewes. Thus, the amount of cortisol exposure in utero may have been increased despite lack of a significant increase in placental cortisol. This could potentially impair HPA axis negative feedback (Brunton and Russell, 2011), increasing glucocorticoid exposure in response to stress and possibly contributing to the changes in emotional behavior (e.g., Lay et al., 1997; Roussel et al., 2005, 2006; Jarvis et al., 2006; Coulon et al., 2011) and dendritic spine density and morphology in the AVS lambs. This is consistent with our finding of a strong negative correlation between cortisone level in placenta and spine density in PFC in lambs.

AVS lambs showed increased spine density in both hippocampal CA1 and PFC relative to GEN lambs, as well as a lower ratio of mushroom spines in hippocampal CA1 region. Given that mushroom spines are considered to be more 'mature' and form stronger and more stable synapses than other spine types, perhaps the dendrites may express more spines to compensate for the loss of the more stable synapses (e.g., Kirov and Harris, 1999). Dendritic spine morphology is a critical regulator of excitatory synaptic physiology (Nimchinsky et al., 2002), and alterations in dendritic spine density and morphology are associated with neurodevelopmental disorders characterized by cognitive impairment (Fiala et al., 2002) as well as cognitive impairment induced by adult stress (Conrad et al., 2012). Therefore, the changes in spine density and morphology that we observed may reflect important functional changes in synaptic transmission in prenatally stressed lambs and may contribute to the increased fear and cognitive impairment seen in these offspring (Lay et al., 1997; Roussel et al., 2005, 2006; Jarvis et al., 2006). In this study we focused on the apical dendrite as a first step, as previous studies have found effects localized to the apical dendrites (e.g., Michelsen et al., 2007; Mychasiuk et al., 2012). Examining potential changes in the basilar arbor is an important next step.

Importantly, studies in rats clearly indicate that some of the structural and functional alterations induced by prenatal stress depend on gender, time during gestation that the stress occurred, as well as its duration, severity, and type. For example, in hippocampal CA1 pyramidal neurons, spine density was increased by prenatal chronic variable stress only in males (Bock et al., 2011). On the other hand, prenatal bystander stress produces similar increases in CA1 spine density in both males and females (Mychasiuk et al., 2012). Unfortunately, in the present study, the number of lambs of each sex was not enough to allow a detailed analysis of a potential differential effect of the AVS treatment between the sexes. Future studies should address this important issue.

Altered spine density and morphology in the AVS lambs was accompanied by changes in the expression of genes associated with development and regulation of spine morphology, which again is consistent with laboratory studies in rodents (Bogoch et al., 2007; Weinstock, 2008). Specifically, we found that expression of PSD95 mRNA was lower in the hippocampus and of RhoA, CPE, and PURα mRNA was lower in PFC of AVS lambs. Downregulation of either RhoA, which plays an important role in spine morphogenesis — particularly head enlargement and spine stabilization (e.g., Tashiro and Yuste, 2008; Auer et al., 2011) — or PSD-95 in primary hippocampal neuron cultures leads to massive loss of mature dendritic spines (Surakul et al., 2011). Likewise, PURα plays a role in establishing the postsynaptic compartment (White et al., 2009), and CPE may also play a role in neural development (Cawley et al., 2004). Thus, changes in spine density and morphology in the AVS lambs could be mediated in part by the altered expression of these genes.

Finally, this study provides the first evidence that a husbandry-relevant aversive human handling of pregnant ewes

triggers changes that can alter the development of PFC and hippocampal dendritic morphology. Increased glucocorticoid exposure during gestation likely contributes to this effect. However, we do not know if the difference between AVS and GEN lambs observed would persist into adulthood or normalize. Postnatal factors could also play a role. For example, in rodents, high levels of maternal care increase glucocorticoid receptors in hippocampus and prefrontal cortex (Liu et al., 1997) and hippocampal spine density (Champagne et al., 2008). Ewes exposed to aversive handling spent more time grooming their lambs compared to ewes exposed to gentle handling (Hild et al., 2011); however, we found no correlation between maternal behavior and spine density, suggesting that differences in maternal behavior did not contribute to the changes in brain and behavior in AVS lambs. Nonetheless, further studies using cross-fostering could resolve this issue. Regardless of whether pre- or postnatal mechanisms contribute to the effect, this study demonstrates that aversive handling of pregnant ewes has consequences for the developmental outcomes of their offspring, and is the first field study demonstrating that ecologically valid aversive experiences in a real-world setting produce alterations in neuronal structure that have previously only been demonstrated in a laboratory setting.

Role of the funding source

This work was funded by the Norwegian Research Council (Grant 185163 awarded to Pr Adroaldo J. Zanella). Their author agreement is compatible with archiving requirements specified by the funding body that supports an author's research.

Conflict of interest

Each of the authors declares that he has no conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the work submitted that could inappropriately influence, or be perceived to influence his work.

Acknowledgements

This study was supported by the Norwegian Research Council (Grant 185163 awarded to Pr Adroaldo J. Zanella). The Fyssen foundation is acknowledged for financial support to Dr Marjorie Coulon. The authors wish to thank Martha Ulvund, the technicians and veterinarians from Sandnes for their care of the animals and their help in collecting the brains. The authors are grateful to Sophie Hild and Anne Schroeer for their participation in the experiment and to Charles Press for providing access to imaging facilities and Rupert Palme for the processing of cortisone EIA.

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