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Research report

Stress during pregnancy alters dendritic spine density and gene expression in the brain of new-born lambs

Bérengère Petit[a,*], Alain Boissy[a], Adroaldo Zanella[b], Elodie Chaillou[c], Stéphane Andanson[a], Sébastien Bes[a], Frédéric Lévy[c], Marjorie Coulon[a]

[a] INRA-VetAgro Sup, UMR 1213 Herbivores, 63122 Saint-Genès Champanelle, France
[b] Departamento de Medicina Veterinária Preventiva e Saúde Animal, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, 13635-900 Pirassununga, Brazil
[c] INRA-CNRS-Université de Tours-IFCE, UMR85, UMR7247, Physiologie de la Reproduction et des Comportements, 37380 Nouzilly, France

HIGHLIGHTS

- Prenatal stressors lead to neurodevelopmental alterations in lamb at birth.
- Prenatal stress increases dendritic spines density in corticolimbic structures.
- Prenatal stress modifies the gene expressions implied in cerebral development.
- Brains alterations could reflect changes in synaptic transmission.

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ABSTRACT

Rodent studies show how prenatal stress (PS) can alter morphology in the cortico-limbic structures that support emotional and cognitive functions. PS-induced alteration is less well described in species with a gyrencephalic brain and complex earlier fetal development, and never in sheep at birth to rule out postnatal environment effects or influences of maternal behavior. This study aimed to assess the consequences of a mild chronic stress in pregnant ewes on the neurobiological development of their lambs at birth. During the last third of gestation, 7 ewes were exposed daily to various unpredictable and negative routine management-based challenges (stressed group), while 7 other ewes were housed without any additional perturbation (control group). For each group, a newborn from each litter was sacrificed at birth to collect its brain and analyze its expression levels of genes involved in neuronal dendritic morphology (*Dlg4*, *Rac1*, *RhoA*, *Doc2b*), synaptic transmission (*Nr1*, *Grin2A*, *Grin2B*) and glucocorticoid receptor (*Nr3C1*) in hippocampus (HPC), prefrontal cortex (PFC) and amygdala (AMYG). Results revealed that lambs from stressed dam (PS lambs) showed under-expression of *Rac1* and *Nr1* in PFC and overexpression of *Dlg4* in AMYG compared to controls. To assess the morphological consequences of gene dysregulations, the dendritic morphology of pyramidal neurons was explored by Golgi–Cox staining in HPC and PFC. PS lambs had higher dendritic spine density in both structures and more stubby-type spines in the CA1 area of HPC than controls. This is the first demonstration in sheep that PS alters fetal brain, possibly reflecting functional changes in synaptic transmission to cope with adversity experienced in fetal life.

1. Introduction

Early development is a period when the brain is particularly vulnerable to the deleterious effects of stress. Various studies in laboratory species show that stressful events experienced by pregnant females have negative consequences on the offspring's neurobiological development and behavior [1]. In rodents, alterations observed consecutively to prenatal stress are associated with modifications of corticolimbic structures supporting the regulation of emotions, especially the hippocampus (HPC), prefrontal cortex (PFC) and amygdala (AMYG), including alterations in dendritic morphology and spine density [2,3]. The majority of studies investigating the neurobiological consequences of prenatal stress on offspring have been carried out in rodents, and the literature reports multiple stress paradigms such as exposure of pregnant rats, mice or guinea-pigs to repeated restraint, noise, social stress,

* Corresponding author. Tel.: +33 0 4 73 62 42 98; fax: +33 473624118.
E-mail address: berengere.petit@hotmail.fr (B. Petit).

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or chronic variable stress [4]. In addition, a substantial amount of neuroendocrine and neural development occurs in the rodent brain only after birth [5]. However, the consequences of prenatal stress on behavior and neurobiology of young are understudied in large animals like sheep that have a gyrencephalic brain and earlier cerebral development [6]. Sheep offers an alternative prenatal stress model with greater predictive value due to their similarities to humans in terms of brain organization and development. In sheep, the corticolimbic regions are well established in fetal stage at 90–92 days of gestation and the brain undergoes another important growth phase up to birth [6]. In addition, the last third of gestation in sheep is a key period for neuronal growth and a vulnerable period for brain development [6].

Neuronal structures in the brain are made of dendritic branches that extend or retract from which highly-specialized structures called dendritic spines emerge or disappear [7]. Dendritic spines can take various shapes and are classed on the basis of structure and maturity as 'filopodia' (most immature), 'stubby', 'thin' and 'mushroom' (most mature) [8]. Spine numbers and morphology are thought to reflect the amount of connectivity between neurons regulating excitatory neurotransmission in the brain network. It has been shown that dendritic structures are sensitive to stress and undergo transformations in an activity- and experience-dependent manner [9]. Thus, changes in spine density/morphology are crucial elements for synaptic function, plasticity and pattern of connectivity [9].

In the HPC, chronic stress exposure causes morphological changes, notably a reduction in neuronal processes with less extensive dendritic trees and decreased dendritic length in the apical part [10–13]. The AMYG, in addition to playing a pivotal role in processing emotional information, is an important component of the neural circuitry mediating stress responses [14], and it also undergoes plastic changes when exposed to stress [15]. The PFC is associated with cognitive operations and emotional processes through extensive connections with other cortical and subcortical regions [16]. Stress *in utero* interferes considerably with the development of apical pyramidal neurons of layer II/III (external), leading to a marked drop in dendritic complexity [2,17] and spine numbers [18,19].

A recent study in sheep has shown effects of a moderate prenatal stress in one-month-old lambs characterized by increased dendritic spine density and modified expressions of genes involved in brain development and spine morphogenesis in both HPC and PFC [20]. However, maternal stress hormones could affect a developing fetus; change in maternal behavior could also drive anatomical changes. Indeed, prenatal stress is shown to affect mother–infant interaction [21]. Therefore, the goal of this study on newborn sheep was to explore cellular and molecular correlates of prenatal stress-induced changes that could link structural plasticity in the corticolimbic structures to symptoms of stress disorders. It is essential to understand some of the putative mechanisms that have been implicated in the influence of prenatal stress on synaptic morphology. We thus explored how patterns of expression of key molecular markers of spine morphology and cerebral development change with dendritic morphology in response to daily long-term prenatal exposure to various stressful events in newborn male lambs immediately after birth so as to rule out other environmental factors.

In the three key structures HPC, PFC and AMYG, we quantified the expression of the glucocorticoid receptor gene (*Nr3c1*) and genes regulating dendritic spine morphogenesis (*RhoA*, *Rac1*, *Dlg4*) and synaptic plasticity (*Nr1*, *Grin2A*, *Grin2B*, *Doc2b*). Corticolimbic structures are rich in glucocorticoid receptors and particularly vulnerable to glucocorticoid variations, especially in early life [22]. Prenatal exposure to excess glucocorticoids has been shown to decrease mRNA levels of glucocorticoid receptor [22]. *RhoA* and *Rac1* are involved in different processes of spine morphogenesis, motility and stability. *Rac1* is essential for spine head morphing, promoting late spine head growth and stabilization [23]. *Dlg4* regulates microtubule dynamics and the motor proteins that support the cytoskeletal machinery modulating dendritic spine morphology [24]. The *Dlg4* gene encodes the cytoskeletal-associated protein postsynaptic density-95 (DLG4, also named PSD-95) that allows NMDA receptor activation by the binding of its NR2 subunits (encoded by *Grin2A* and *Grin2B* genes). NMDA receptors count among the major classes of ionotropic glutamatergic receptors that play a central role in both emotion and cognition [25]. Finally, DOC2, encoded by the *Doc2b* gene, is a vesicular protein localized to presynaptic terminals that may be involved in the early stages of preparing vesicles for exocytosis [26]. We then analyzed the neuromorphological expression of these gene dysregulations to highlight the concomitant changes in density and morphology of dendritic spines in corticolimbic areas of prenatally stressed lambs (PS lambs) at birth.

We hypothesized that corticolimbic structures of PS lambs at birth would show changes in the expression of genes involved in synaptic transmission, underlying modifications in spine morphogenesis and density. The results could help sketch out hypotheses on the functional consequences of stress-induced structural plasticity and how these structural changes may contribute to adaptive behaviors as well as maladaptive responses associated with stress.

2. Methods

2.1. Animals and treatments

At 21-month-old, 40 primiparous Romane ewes were mated by natural service using Romane rams after estrus synchronization with progesterone. Over the last third of pregnancy (7 weeks, between day 94 and day 143 of gestation), 20 ewes were daily exposed to repeated unpredictable and uncontrollable aversive events whereas the other 20 ewes were housed without additional stressful events. The experimental design to induce stress in ewes during gestation was built according to a validated protocol of aversive events [27]. The aversive events, including social isolation, mixing, dog handling, transport, simulation of shearing, delay in feeding times, occurred at different times of day and night with no forewarning (stressors are described in more details in Table 1) This 7-week exposure to unpredictable and uncontrollable aversive events has been shown to induce a chronic mild stress in sheep, as described elsewhere [27] and in our companion paper [28]. At lambing, we focused our analysis on 7 male lambs from ewes reared in standard conditions (Control lambs) and 7 male lambs from ewes stressed during gestation (PS lambs).

The experiment was carried out in accordance with EC Council Directive of 24 November 1986 (86/609/EEC). The experimental protocol was reviewed and approved by an institutional review board (Authorization number CE38-1) entitled the (Comité Régional d'Ethique en Matière d'Expérimentation Animale Région Auvergne) (CEMEA Auvergne).

2.2. Tissue collection

Immediately after birth, the 7 PS lambs and 7 Control lambs were anesthetized (Rompun® 2%, Bayer, 1 mL) and euthanized (Dolethal®, Vétoquinol, 5 mL injected directly into the heart) to collect their brains. Within 10 min, the brains were removed, the right hemisphere was processed for Golgi histology [29,30] as described in Coulon et al. [20], and the left hemisphere was processed for molecular biology analysis.