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Effects of *in utero* endotoxemia on the ovine fetal brain: A model for schizophrenia?

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1. ABSTRACT

Infections during pregnancy can adversely affect the development of the fetal brain. This may contribute to disease processes such as schizophrenia in later life. Changes in the (cyto-) architecture of the anterior cingulate cortex (ACC), particularly in GABA-ergic interneurons, play a role in the pathogenesis of schizophrenia. We hypothesized that exposure to infection during pregnancy could result in cyto-architectural changes in the fetal ACC, similar to the pathogenesis seen in schizophrenia. Fetal sheep of 110 days GA (term=150 days GA) received an intravenous injection of 100 ng or 500 ng lipopolysaccharide (LPS) or saline as control. After delivery at 113 days GA, the cyto-architecture of the cingulate cortex (CC) was examined by immunohistochemistry. High dose LPS exposure resulted in a decreased density of GFAP-, calbindin D-28K- and parvalbumin-immunoreactive cells in the CC. In addition, these cells and calretinin-immunoreactive cells showed a changed morphology with reduced cell processes. This study provides further evidence that intra-uterine endotoxemia can induce changes in the fetal brain which correspond with changes seen in schizophrenia.

2. INTRODUCTION

Infections during pregnancy are a widespread phenomenon and may have serious consequences on the development of the fetus *in utero*. Chorioamnionitis is a bacterial infection of the placental membranes and amniotic fluid which is highly associated with preterm birth. Often chorioamnionitis presents as a clinically silent infection and is only diagnosed postnatally by histo-pathological analysis of the placenta (1, 2). Chorioamnionitis may not only provoke a maternal immune response, but can also induce an inflammatory response of the fetus. The latter can adversely affect the development of the fetal brain leading to pathologies such as periventricular leukomalacia (PVL) and cerebral palsy (3, 4). The neurological outcome after preterm birth is also linked to the development of several disorders in later life, one of which is schizophrenia (5-7). In addition, infections during the second trimester of pregnancy were identified to play a role in the etiopathogenesis of schizophrenia (8, 9).

Schizophrenic psychoses are a group of diseases that are characterized by the breakup of the personality (10). Emotional stability is disturbed and thinking is highly

limited. The disease may show a chronic progress or progress in episodes, and often results in persisting changes of personality. A multitude of post mortem studies showed various neuropathological findings in the anterior cingulate cortex (ACC) of the brains from patients with schizophrenia (11-13). The ACC is part of the limbic system and is the central location for visceral regulation processes and higher cognitive processes like memory, attentiveness, affect regulation, concentration, and pain (14). Anatomically, the ACC is located on the cingulate gyrus in the medial cortex area above the corpus callosum. Magnet resonance tomography studies showed that the largest loss of cortical volume in the brains of patients suffering from schizophrenia can be found, amongst others, in the anterior cingulate cortex (15).

On a molecular and structural level, the GABA-ergic interneurons play a major role in the pathogenesis of schizophrenia. GABA-ergic interneurons, which can be differentiated immunohistochemically by the calcium-binding proteins parvalbumin, calbindin D-28K and calretinin, have an inhibitory function on neuronal circuits and a decisive influence on the cortico-limbic circulation. They contribute to maintenance of synchronized oscillations in neuronal networks, process information from the cortex and the limbic system, and have an influence on gating of sensory information (16). Benes *et al.* showed a reduced interneuron count and changes in both pyramidal cells and GABA-ergic interneurons in the ACC in schizophrenia (17). Also the prefrontal cortex of schizophrenia patients seems affected with a decreased density of inhibitory neurons which was not seen in patients who suffered from bipolar disease (18, 19).

As infections during pregnancy seem to play a role in development of schizophrenia (8, 9), we hypothesized that fetal exposure to infection *in utero* could result in cyto-architectural changes in the fetal ACC, similar to the pathogenesis seen in patients with schizophrenia. To test this hypothesis, we used a sheep model in which lipopolysaccharide (LPS) was injected intravenously in instrumented fetal lambs. Our group has developed several well established sheep models to study both the effects of chorioamnionitis and endotoxemia/sepsis on the fetal brain (20-23). In this study, the (cyto-) architecture of the cingulate cortex (CC) was examined using immunohistochemistry with focus on the different subsets of GABA-ergic interneurons which were shown to play a major role in the pathogenesis of schizophrenia.

3. MATERIALS AND METHODS

3.1. Experimental setup of the animal study

The animal studies were performed at the University of Maastricht, the Netherlands. The protocol was approved by the Animal Ethics Committee of the University of Maastricht and was in accordance with the guidelines of the responsible governmental agencies. The experimental setup of the animal studies was published previously (24). Briefly, twenty-one fetal sheep with a gestational age (GA) of 106-108 days (term=150 days) were instrumented *in utero* of which 14 fetuses were included in further analysis for brain structure.

Before the surgical instrumentation, ewes were anaesthetized using sodium thiopental (1 g / 70 kg body weight intravenously for initiation of anesthesia) and 0.5 - 1.0% halothane (with 1:1 nitrogen oxide and oxygen for maintenance) (25). A midline abdominal incision was performed and the fetal hind legs were identified and exposed by means of incision in the uterus. Polyvinyl catheters (Maxxim Medical BV, Den Bosch, the Netherlands) with an outer diameter of 1.25 mm and an inner diameter of 0.75 mm were inserted through a tibial vein or artery in the fetal hind legs up to vena cava inferior or aorta abdominalis. The skin of the fetuses was closed with cyanoacrylate glue (Cyanolit; Toagosei, Tokyo, Japan). Furthermore, an intra-uterine pressure catheter was placed. After closure of the uterus, the lost amniotic fluid was replaced with 0.9% saline solution (temperature: 39° C). The protruding catheters were filled with heparin (100 IU/ml; Heparin-Natrium; Braun, Melsungen, Germany) and directed outwards via a small incision in the flank of the mother where they were collected in a bag sewn to the skin of the ewe. Antibiotics (1g ampicillin, Pentrexyl; Bristol–Myers, Woerden, the Netherlands) and post-operative pain relief (buprenorphine 10µg/kg bodyweight, Temgesic; Schering, Netherlands) was provided to all ewes. After a recovery period of three days, the fetuses (110 ±1 day GA) received an intravenous bolus injection of lipopolysaccharide (LPS; E.coli O127:B8; Sigma-Aldrich, Saint Louis, MO, USA) of either 100 ng (n=4) or 500 ng (n=5) LPS in 5 ml saline solution (0.9%). Control animals (n=5) received an intravenous bolus injection of saline. Fetal heart rate and fetal mean arterial pressure were monitored throughout the experiment. Three days after the injection, the ewes were anaesthetized using sodium thiopental (1 g / 70 kg body weight intravenously for initiation of anesthesia) and 0.5 - 1.0% halothane (with 1:1 nitrogen oxide and oxygen for maintenance). The fetus was exposed by an incision in the centre line of the abdomen and uterus. The thorax of the fetus was opened and a catheter was inserted into the left ventricle for perfusion-fixation with 10% paraformaldehyde.

3.2. Tissue processing

After the fetuses were sacrificed, the fetal brains were removed from the skull. The cerebral hemispheres were separated and the left hemisphere was divided into different brain regions after which they were fixated in 10% paraformaldehyde. Each region was cryo-protected (10%, 20% and 30% sucrose in 0.1% PBS, 24h per solution at 4°C), embedded in Tissue-Tek O.C.T Compound (Sakura; Alphen aan den Rijn, the Netherlands) and deep-frozen. Frontal 50µm thick sections were cut on a cryostate (CM3050; Leica, Wetzlar, Germany) for immunohistochemical and stereological analysis.

3.3. Immunohistochemistry

Different subsets of GABA-ergic interneurons were identified by immunoprocessing for calretinin (CR: antibody #7699/3H, Swant, Bellinzona, Switzerland), calbindin D-28K (CB: antibody #300, Swant) and

parvalbumin (PV: antibody #235, Swant). Anti-GFAP (glial fibrillary acidic protein) antibody (#G3893, Sigma-Aldrich) was used to visualize astrocytes. All procedures were performed using the free floating method. Unspecific binding was blocked by incubating the sections in 5% bovine serum albumin (BSA) for 60 min. Subsequently, the endogenous peroxidase activity was blocked with 7% H202. After rinsing with Tris-buffered saline (TBS, pH 7,6), sections were incubated for 48 h at 4°C with the diluted primary antibody (calretinin 1:5000, calbindin D-28K 1:2000, parvalbumin 1:2000, GFAP 1:5000 in TBS). After rinsing with TBS, the sections were incubated with a secondary biotin-labeled antibody for 2 h at room temperature. The immunostaining was enhanced with Vectastain ABC peroxidase Elite kit (PK-6200, Vector Laboratories, Burlingame, USA) followed by a 3,3`-diaminobenzidine (DAB) staining. Subsequently, the brain sections were rinsed, mounted on glass slides coated with APES (3-aminopropyltriethoxysilane; Sigma-Aldrich) and coverslipped with 80% glycerol in TBS (26).

3.4. Nissl staining

Sections for Nissl staining were mounted on APES-coated glass slides and were allowed to dry. The sections were immersed in 0.01% cresyl violet for 25 minutes and post-fixed in 100% ethanol. Finally, slides were coverslipped using DePeX (Serva; Heidelberg, Germany).

3.5. Qualitative and quantitative histological analysis

The Nissl-stained slides were used to select the brain sections that contained the CC. Stereological analysis of the density of CR-, CB-, PV- or GFAP-immunoreactive cells in the CC were performed on a stereology workstation consisting of a modified light microscope (Olympus BX50; Olympus, Tokyo, Japan) with Olympus UPlanApo objectives, motorized specimen stage (Ludl Electronics; Hawthorne, NY, USA), digital measuring sensor for determination of the depth of focus (Ludl Electronics), CCD color video camera (HV-C20AMP; Hitachi, Tokyo, Japan) and stereology software (Stereo Investigator Version 7.00.03; MBF Bioscience, Williston, VT). Virtual three-dimensional regions (UVCS) within the CC were determined within the brain sections in accordance with the criteria of systematic-and-random (SRS) sampling (27, 28). Then, CR-, CB, PV- or GFAP-immunoreactive cells were counted within the UVCS according to the principles of design-based stereology. The dimensions of the UVCS were as following: base area: 50 µm x 50 µm, distance between the UVCS in XY directions: 100 µm for PV-immunoreactive cells, 200 µm for CR-, CB- and GFAP-immunoreactive cells (29). For determining cell densities, the total number of counted cells was divided by the total number of analyzed UVCS multiplied by the volume of the UVCS. Analysis of the morphology of the cells was only performed descriptively.

3.6. Photography

Photomicrographs were taken using an Olympus DP70 digital camera assembled onto an Olympus AX-70 microscope with CellP software (version 2.3; Soft Imaging System, Münster, Germany).

3.7. Statistical analysis

Results are given as means ± standard error of mean (SEM). The groups were compared using one-way ANOVA with Bonferroni tests for post-hoc analysis. Statistical analysis was performed using GraphPad Prism v4.0 (GraphPad Software, San Diego, CA, USA). Significance was accepted at $p<0.05$.

4. RESULTS

LPS exposure did not induce major changes in the architecture of the CC (Figure 1). In those animals that received a low dose of LPS (100 ng), no changes in the brain (cyto-) architecture were observed. In contrast, intra-uterine exposure to high dose of LPS (500 ng) reduced the density of several GABA-ergic interneuron subsets and GFAP-immunoreactive cells, which are mainly astrocytes. Exposure to 500 ng of LPS resulted in a decreased mean density of GFAP-, CB D-28K- and PV-immunoreactive cells (Figure 2). The mean density of CR-immunoreactive cells did not change after LPS exposure.

In addition, descriptive analysis of the sections revealed several morphological changes in the examined cell types. GFAP-immunoreactive cells were present in abundance throughout the cortex of control animals (Figure 3). Exposure to high dose of LPS resulted in a reduction of the processes of GFAP-immunoreactive cells, particularly in the perivascular area. CR-immunoreactive multipolar cells were present in all layers of the cortex in the control animals (Figure 4). Animals that were exposed to LPS did not show a reduced density of CR-immunoreactive cells. Instead, descriptive analysis showed a change in the morphology of these cells with more unipolar cells (Figure 4). CB-immunoreactive cells were mainly found in layer II and III of the cortex in control animals (Figure 5). The density of CB-immunoreactive cells was significantly decreased after LPS exposure (Figure 2). Furthermore, the cells seemed to be arranged in clusters what was not seen in the control animals (Figure 5). Exposure to LPS resulted in a decreased density of PV-immunoreactive cells which are normally found in layers III and IV of the cortex (Figure 6).

5. DISCUSSION

In the present study, we examined the effects of intravenous LPS exposure on the fetal cingulate cortex. Exposure to a high LPS dose (500 ng) resulted in a decreased density of GFAP-, calbindin D-28K- and parvalbumin-positive cells in the CC of the fetus. Although several studies already underlined the profound effects of *in utero* LPS exposure on fetal brain development (21, 23), this study is the first to report cyto-architectural changes in GABA-ergic interneuron subsets in the CC. The changes observed in the present study in the ovine fetal brain were detected in a brain region that has been associated with the pathogenesis of schizophrenia. The post- mortem pathologic changes in the brain of schizophrenia patients include, amongst others, (cyto)-architectural changes in the ACC, particularly for GABA-ergic interneurons (16, 30). It is of note that the altered pattern of different subsets of GABA-ergic interneurons in the ACC of patients with

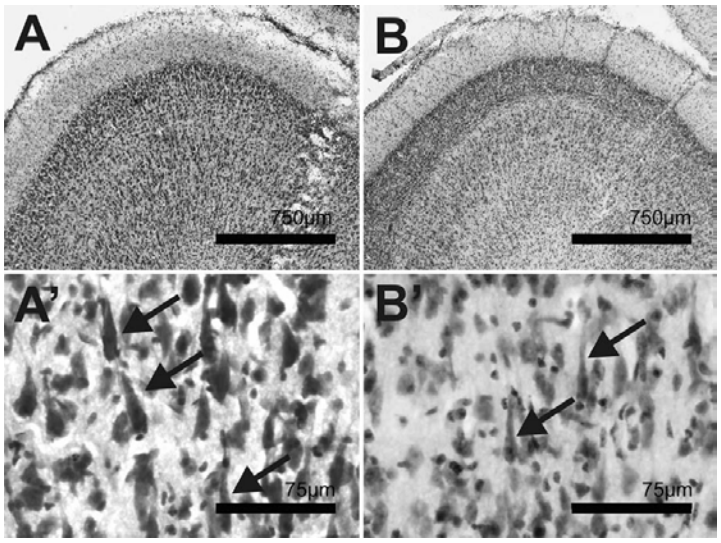

Figure 1. Architecture of the cingulate cortex (CC) after LPS exposure. Representative images of the Nissl-staining on the cingulate cortex (CC) are shown for controls (A) and 500 ng LPS exposed animals (B). LPS exposure did not induce major changes in the architecture of the CC. Arrow: Oblong neurons (similar to von Economo neurons in the human anterior cingulate gyrus).

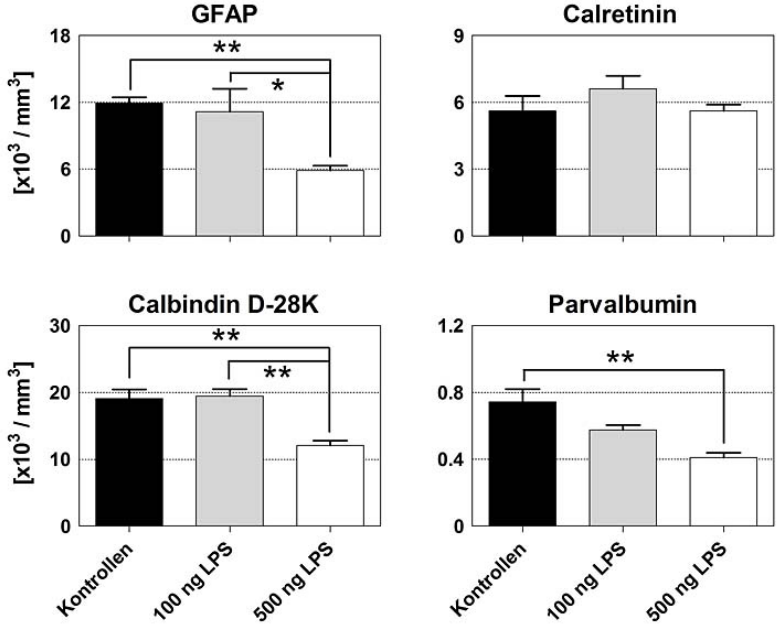

Figure 2. Mean density of GFAP, calretinin, calbinin D-28K and parvalbumin-positive cells in the cingulate cortex (CC) of fetal sheep. At day 110+/-1 of gestation, the fetuses received either an injection of saline (black bar; controls) or 100 ng lipopolysaccharides (LPS) (grey bar) or 500 ng LPS (white bar). Results of the post-hoc Bonferroni tests on pairwise comparison between the groups with p values < 0.05 are shown (*: $p<0.05$; **: $p < 0.01$).

schizophrenia was confirmed in subsequent studies (31-33), and shows correspondence to the pattern of changes in cell density in the CC of the fetal sheep after exposure to 500 ng LPS as demonstrated in the present study. As such, this pilot study demonstrates that antenatal factors can play a role in the development of postnatal diseases.

GABA-ergic interneurons play an inhibitory role in neuronal circuits and have a decisive influence on the cortico-limbic circulation (34). Due to the increasing evidence of an impairment of the GABA-ergic interneuron subpopulations in the pathology of schizophrenia, understanding the disturbances of prenatal development and disturbed formation of neuronal circuits during early adult life – when the disease mainly becomes clinically manifest – may provide more insight into the pathophysiology of this disorder (35). There are several underlying pathological mechanisms by which exposure to

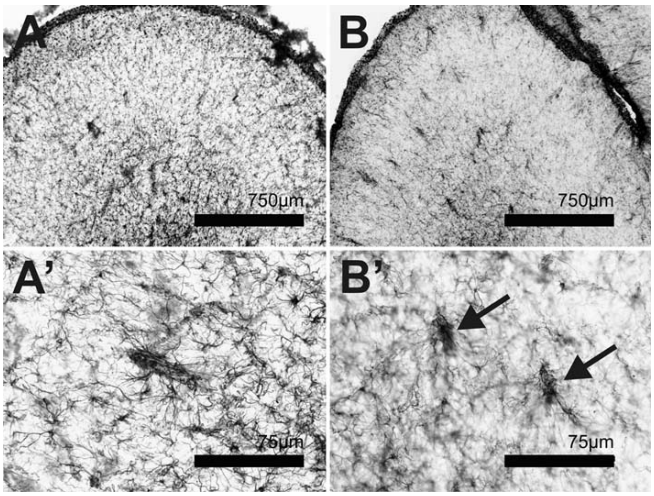

Figure 3. LPS exposure decreased the density of GFAP-positive cells in the cingulate cortex (CC). The reduced density of GFAP-positive cells as well as the reduced GFAP signal within the cell processes is mainly seen in the area of the perivascular astrocytes after exposure to high dose LPS. Representative images of the GFAP-staining on the CC are shown for controls (A) and 500 ng LPS exposed animals (B). Arrow: GFAP-positive cells which show reduced processes.

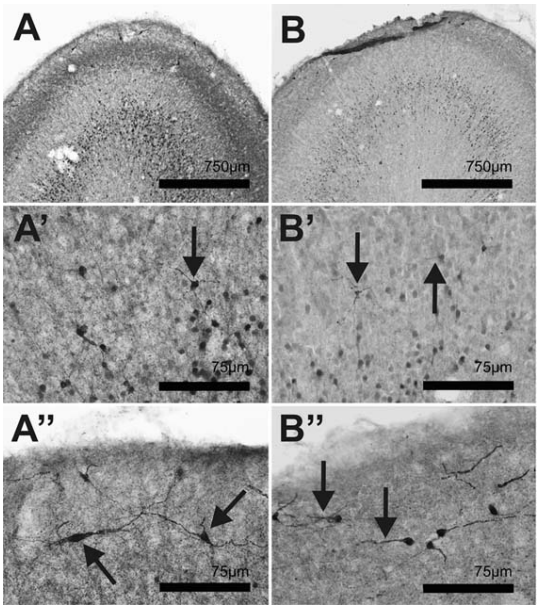

Figure 4. LPS exposure induced changes in the morphology of calretinin-positive cells in the cingulate cortex (CC). The morphology of the calretinin-immunoreactive cells changed from multipolar cells to unipolar cells in the cingulate cortex after exposure to 500 ng LPS. Representative images of calretinin-positive cells are shown for controls (A) and 500 ng LPS exposed animals (B). Arrow: Multipolar (A) vs. unipolar (B) calretinin-positive cells in layer I of the cortex.

infection during fetal development can lead to a similar neuropathology as seen in schizophrenia. The risk to develop a neuropsychiatric disease like schizophrenia could be promoted by a prenatal maternal infection with consecutive cytokine-controlled inflammatory response that may result in a persistent damage to the developing brain (36, 37). Here, glia cell reactions represent a possible immune response to inflammatory stimuli and play a decisive role in the developing brain. Glia cells in turn may produce immune modulators (TNF-α, IL-1b, IL-6, IL-12, IL-10, TGF-β) and supply nutrients to ripening neurons (38). Glial dysfunction may therefore also result in neuronal dysfunction (39, 40).

Rodent models of pre- or neonatal LPS exposure have shown changes in some GABA-ergic interneurons in the hippocampus which were linked to behavioral changes consistent with schizophrenia (41-43). However, no studies address the changes in the CC which seems to be a key region in the pathology of schizophrenia. This is in part due to the differences in neurospecific stages of development between rodents and humans. As such, rodents are only

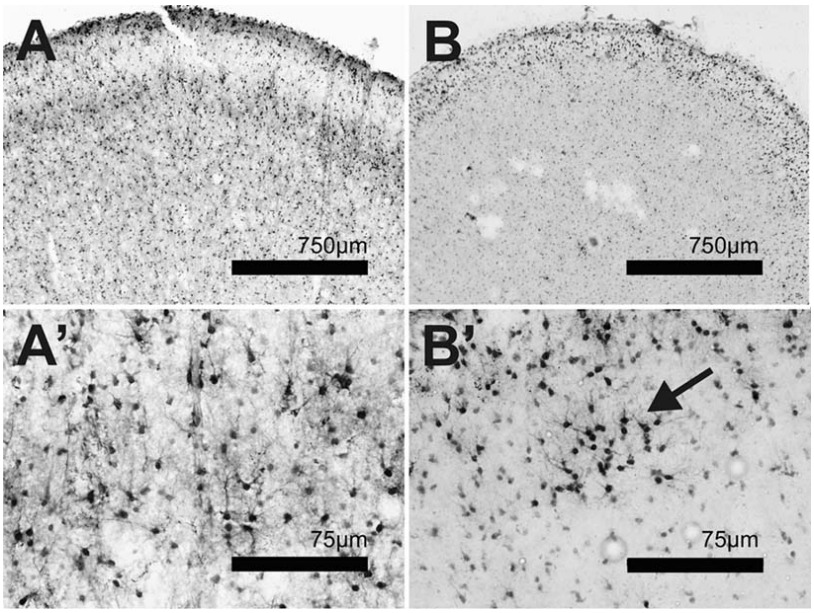

Figure 5. The density of calbindin D-28K-positive cells decreased after LPS exposure in the cingulate cortex (CC). Exposure to high dose LPS (500 ng) decreased the density of calbindin D-28K-immunoreactive cells in the cingulate cortex as well as the allocation of such cells in "clusters" compared to controls. Representative images of calbindin D-28K-positive cells are shown for controls (A) and 500 ng LPS exposed animals (B). Arrow: Clusters of calbindin D-28K-positive cells in LPS exposed animals.

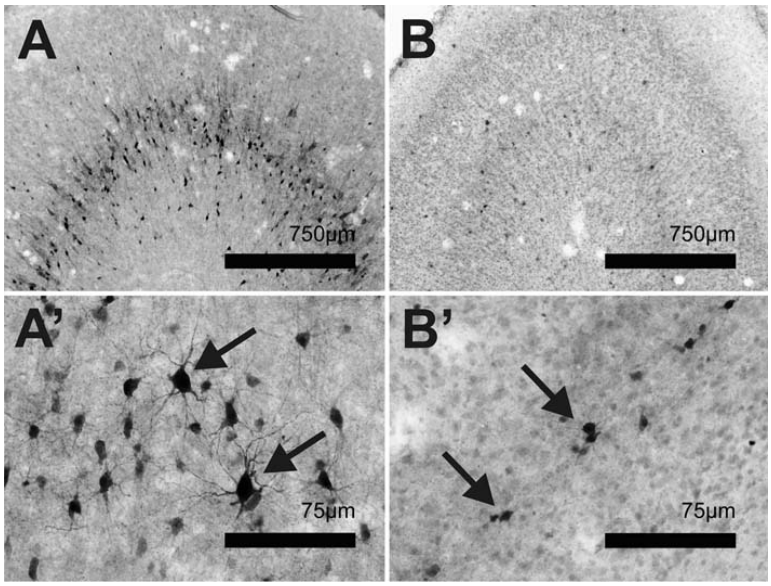

Figure 6. LPS exposure induced changes in the density and morphology of parvalbumin-positive cells in the cingulate cortex (CC). Exposure to high dose LPS (500 ng) decreased the density of parvalbumin-immuoreactive cells as well as loss of immunoreactivity within the cell processes. Representative images of parvalbumin-positive cells are shown for controls (A) and 500 ng LPS exposed animals (B). Arrow: Loss of immunopositivity in the cell processes in LPS exposed animals.

limitedly suitable for creation of an animal model with regard to possible intra-uterine disturbances of brain development during the second trimester of human pregnancy. With regard to prenatal development processes in the brain, sheep show a significantly higher similarity to humans as rodents do.

Accordingly, exposure of fetal sheep to a high dose of LPS on day 110 of gestation could serve as a new animal model for key aspects of the neuropathology of schizophrenia with the adverse event occurring around the end of the second trimester of pregnancy which has been shown be the critical point in time in relation to

disturbances in the fetal brain development (8, 9). However, this pilot study does impose some limitations. The study was conducted with only 14 animals and provides some quantitative measurements and descriptive data. To clarify the role of intra-venous LPS exposure on changes in the ovine fetal GABA-ergic interneuron subsets, further studies will be necessary. At the given moment, only limited information is available concerning behavioral examination of this animal model. However, behavioral similarities between experimentally manipulated animals and specific symptoms of human suffering from a certain neuropsychiatric disease often represent only intuitive criteria for evident application of an animal model. Most importantly, it seems impossible to find an adequate correlate with an animal model for the positive symptoms of schizophrenia (e.g. hallucinations and delusions).

In summary, the results of the present paper suggest that exposure of fetal sheep to intravenous LPS at day 110+/-1 of pregnancy is a possible new animal model for key aspects of the neuropathology of schizophrenia, with significant benefits as compared to rodent models of the disease.

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