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Complement inhibition and statins prevent fetal brain cortical abnormalities in a mouse model of preterm birth

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ABSTRACT

Premature babies are particularly vulnerable to brain injury. In this study we focus on cortical brain damage associated with long-term cognitive, behavioral, attentional or socialization deficits in children born preterm. Using a mouse model of preterm birth (PTB), we demonstrated that complement component C5a contributes to fetal cortical brain injury. Disruption of cortical dendritic and axonal cytoarchitecture was observed in PTB-mice. Fetuses deficient in C5aR (−/−) did not show cortical brain damage. Treatment with antibody anti-C5, that prevents generation of C5a, also prevented cortical fetal brain injury in PTB-mice. C5a also showed a detrimental effect on fetal cortical neuron development and survival in vitro. Increased glutamate release was observed in cortical neurons in culture exposed to C5a. Blockade of C5aR prevented glutamate increase and restored neurons dendritic and axonal growth and survival. Similarly, increased glutamate levels – measured by ^{1}HMRS – were observed in vivo in PTB-fetuses compared to age-matched controls. The blockade of glutamate receptors prevented C5a-induced abnormal growth and increased cell death in isolated fetal cortical neurons. Simvastatin and pravastatin prevented cortical fetal brain developmental and metabolic abnormalities -in vivo and in vitro. Neuroprotective effects of statins were mediated by Akt/PKB signaling pathways. This study shows that complement activation plays a crucial role in cortical fetal brain injury in PTL and suggests that complement inhibitors and statins might be good therapeutic options to improve neonatal outcomes in preterm birth.

1. Introduction

Preterm birth is an important cause of perinatal mortality and morbidity. Between 23 and 32 weeks of pregnancy, the human fetal brain is at a critical stage of development and vulnerable to injury. 25 to 50% of infants that were born prematurely experience long-term cognitive, behavioral, attentional or socialization deficits [1–3]. MRI studies in preterm infants showed decreased volume in the cerebral cortex, suggesting a role for the cortex in these long term complications [1]. Unfortunately, the mechanisms behind these long term disabilities are still unclear, preventing therefore the development of diagnostic methods and therapies.

During pregnancy, the developing brain is particularly susceptible to ischaemic and inflammatory insults, the latter often due to maternal intrauterine inflammation/infection. Despite the growing association between inflammation with fetal brain injury [4–6], the mediators and receptors involved remain unknown.

Using a mouse model of inflammation-induced PTB [7,8], we demonstrated that complement activation, in particular C5a, plays a crucial role in the cervical ripening that leads to PTB [7]. Here, we investigate if C5a also causes damage in the developing fetal brain cortex in this model. We focused on the cerebral cortex because cognitive deficits are by far the dominant neurodevelopmental sequelae in infants born preterm.

In an attempt to find a treatment to prevent fetal cortical brain damage we studied the effects of complement inhibition in this model. Furthermore, we hypothesized that statins which prevented complement-mediated tissue injury in several animal models [9–11], would also prevent fetal cortical brain developmental abnormalities.

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2. Material and methods

2.1. Animals

All housing and experimental procedures were performed in compliance with the UK Home Office Animals Scientific Procedures Act 1986 (Home Office project licence number 60/4305).

C57BL/6 from commercial vendors and C5a receptor (C5aR)-deficient mice, generated by homologous recombination technology, by Dr. Craig Gerard (Harvard Medical School) [12] were used in all the experiments. It has been described that subclinical genital tract infections are often associated with spontaneous preterm birth in women. Thus, we used a mouse model of spontaneous PTD which resembles this clinical scenario [4]. In this model, mice received LPS (*Escherichia coli* serotype 055:B5) (250 μg/mouse) intravaginally on day 15 of pregnancy [4].

This dose showed to induce cervical ripening [4] but not systemic disease in the mother. It has been documented that the vagina is a poor route for systemic delivery primarily due to the poor absorption across the vaginal epithelium [13].

To determine whether LPS administered through the vaginal canal ascends to the uterus and/or reaches systemic circulation, a group of mice were administered intravaginal LPS labeled with FITC (Sigma Chemicals, St Louis, MO). FITC-LPS content in the vagina, cervix, uterine horns, amniotic fluid and blood was measured 12 h after LPS administration with a Perkin-Elmer luminescence spectrometer (San Jose, CA, USA). A standard curve was constructed by plotting fluorescence (arbitrary units) against different concentrations of FITC-LPS.

In this model, 100% of the mice deliver preterm before gestational day 17. The pregnant mice treated with LPS were euthanized 18 to 24 h after treatment. The age-matched control mice, that received intravaginal saline, were euthanized at the same time point. Amniotic fluid and fetal brains were collected in the LPS-treated mice and age-matched controls. To inhibit C5, mice were treated on days 10 and 12 of pregnancy with anti-C5 mAb (1 mg, intraperitoneally) [14] or murine IgG as a control and on day 15 they received intravaginal LPS.

A group of mice received pravastatin (10 μg/mouse, i.p.) or simvastatin (20 μg/mouse, i.p.) [9] 24 h before and 2 h after LPS intravaginal administration. Fetal genotypes (C5aR +/− and C5aR −/−) were determined by polymerase chain reaction. The primers for C5aR were obtained from Applied Biosystems (Foster City, CA).

2.2. Simvastatin and pravastatin solutions

Simvastatin (Sigma Chemical) was prepared as a 4 mg/ml stock. Briefly 4 mg of simvastatin was dissolved in 100 μl of ethanol and 150 μl of 0.1 N NaOH and incubated at 50 °C for 2 h, then the pH was adjusted to 7, and the total volume was corrected to 1 ml. The stock solution was diluted to the appropriate concentration in sterile PBS. Pravastatin (Sigma Chemical) was directly dissolved in sterile PBS.

2.3. Measurement of C5a

C5a is rapidly cleaved to the more stable metabolite C5adesArg. Thus, we measured C5adesArg to estimate C5a levels. C5adesArg levels in amniotic fluid and fetal brain cortex was measured by sandwich ELISA as previously described [7,8] using rat anti-mouse C5a and biotin rat anti-mouse C5a (BD Biosciences Pharmingen). The fetal cortical brain tissue was isolated using a dissecting microscope.

2.4. Isolation of fetal cortical neurons

Cortical neurons from fetal brains were isolated as described by Kim and Magrane [15]. After surgical removal of the cerebellum, midbrain and hippocampus the cortical tissue was sequentially incubated with trypsin and DNAse to dissociate the neurons. More than 98% of the cells stained positive for βIII tubulin and negative for GFAP indicating that there is no glial contamination. After centrifugation the dissociated neurons were resuspended in a neuronal culture medium [15] and plated on laminin/polylysine-coated dishes (5×10^5 cells/cm^2). These cells extend their neurites and establish synapses in culture after 10 days in culture and thus represent an accessible model to study cortical brain development and evaluate synapses and neuritic networks in vitro [15]. Using these primary neuronal cultures we investigated the effects of complement component C5a on brain development by evaluating neuritic networks formation in vitro and we tested statins as a therapeutic approach to prevent brain injury. Cortical neurons from day 16 fetuses were isolated and cultured on coverslips coated with laminin/polylysine. After 7 days in culture (7 days in vitro (7DIV)) neurons were exposed to different treatments (C5a (100 nM) (C5a), glutamate (glu) (50 μM), C5aR antagonist peptide (AcPhe[L-ornithine-Pro-D-cyclohexylalanine-Trp-Arg]) (C5aR-AP) (1 μM) [16], MK-810 (1 μM), LY294002 (50 μM) or only media (control)).

The dose of C5a was selected from dose-response studies (Supplemental material). A group of neurons was preincubated with pravastatin (20 μg/ml) or simvastatin (40 μg/ml) 6DIV and on 7DIV C5a was added to the media (P + C5a; S + C5a)). A group of control neurons and a group of neurons preincubated with LY294002 for 12 h were exposed to pravastatin or simvastatin on 6DIV and on 7DIV C5a was added to the media. Formation of neuritic networks in each experimental group was evaluated on 10DIV by immunohistochemistry using BIII tubulin antibodies after fixation with paraformaldehyde 4%. Glutamate release to the media and viability of neurons were measured on 7DIV, 6 h after the respective treatment. Glutamate was measured using a commercial kit (Biovision Incorporated, Milpitas, CA, USA and lactate dehydrogenase (LDH) activity was determined by measuring the linear rate of consumption of NADH absorption (340 nm) during the reduction of pyruvate to lactate using a spectrophotometer. Caspase-3 activity in isolated cortical neurons was measured by a fluorometric assay based on the hydrolysis of acetyl Asp-Glu-Val-Asp 7-amido-4-methylcoumarin (Ac-DEVD-AMC) by caspase 3, resulting in the release of the fluorescent 7-amino-4-methylcoumarin (AMC) (Sigma Chemicals, St Louis, MO, USA). After each treatment, the cortical neurons were lysed with Tris buffer (50 mol/L Tris–HCL, 1 mmol/L EDTA, and 10 mmol/L EGTA at pH = 7.4) containing 10 μmol/L digitonin for 20 min at 37 °C. The lysates were subjected to a quick centrifugation at 20,000 ×*g* and cell-free supernatants were incubated with Ac-DEVD-AFC, 50 μmol/L) for 1 h at 37 °C. The caspase-3 activity was measured by florescence using a microplate reader with excitation at 360 nm and emission at 460 nm. The caspase-3 activity was expressed as fluorescent units (FUs/mg protein).

2.5. Proton magnetic resonance spectroscopy studies (^{1}HMRS)

This non-invasive in vivo imaging modality was used to study the biochemical and metabolic profile in fetal brain during preterm. All MRI experiments were performed using a 7-T horizontal bore NMR spectrometer (Agilent, Yarnton, UK), equipped with a high-performance gradient insert (12-cm inner diameter), maximum gradient strength 400 mT/m. 18 to 24 h after their respective treatments, the mice were anesthetized with 1.8% isofluorane in oxygen/air (50/50,1 L/min) and placed in a cradle (Rapid Biomedical GmbH, Rimpar, Germany). The rectal temperature and respiration rate were monitored throughout the experiments, and body temperature was maintained at 37 °C with a heat fan. A birdcage coil (33-mm diameter) was used for radio frequency transmission and signal reception.

In order to place the spectroscopy voxel (Fig. 2H) in the brain, good anatomical details are required. To this end, respiration-gated T2-weighted fast spin echo images (echo train length of 4 or 8) of 1 mm slice thickness in 3 orthogonal directions were collected with the following parameters: repetition time (TR) ≈ 2500 ms depending on the respiration rate; effective echo time = 50 ms; field of view = 35 mm × 35 mm; matrix = 192 × 192, 2 signal

averages. For localized spectroscopy a PRESS (PointRESolved Spectroscopy) sequence was used with VAPOR water suppression, outer volume suppression and the following parameters: TR = 3000 ms, TE = 23 ms, number of signal averages = 240 or 480, voxel size = 2.8 × 2.8 × 2.8 mm. The voxel was positioned in the center of the fetal brain. Magnetic field homogeneity within the voxel was optimized manually. At the end of the PRESS sequence, low resolution fast spin echo images were acquired to verify that the voxel location hadn't changed due to movement of the fetuses. Spectra were analyzed using LCModel (http://s-provencher.com/pages/lcmodel.shtml).

2.6. Immunohistochemistry (IHC)

Fetal brains were harvested from LPS-treated mice and age-matched controls (18 to 24 h after induction of preterm labor) and incubated overnight in PFA 4% with 10% sucrose for cytoprotection. Fetal brain tissue was then frozen in the O.C.T. compound, and cut into 10 μm sections. A list of the antibodies and their respective dilutions used for IHC is shown in Table 1. The FITC staining was quantified using NIS-Elements Research Imaging software (Nikon Inc. Tech Co., Ltd., Tokyo, Japan). Exposure time was fixed and the camera settings were kept constant. The cortical brain tissue was segmented based on red/green/blue intensity, and the area was restricted to eliminate debris. Measurements data per area were calculated using the following parameters: sum density (the sum of individual optical densities of each pixel in the area being measured) and mean density (statistical mean of density values of pixels). NIS-Elements BR (Nikon Inc., Tokyo, Japan) uses density calibration curves for evaluation of these parameters. 10 views per slide were analyzed in each experimental condition.

2.7. Apoptosis and neurodegeneration in fetal brain tissue

Apoptosis in the fetal brain cortex frozen sections was evaluated using caspase-3–protease – involved in cell death in immature neurons – immunohistochemistry (Table 1).

Neurodegeneration was measured using Fluoro-Jade B (Millipore, Millerica, MA, USA), a polyanionic fluorescein derivative which sensitively and specifically binds to degenerating neurons [17]. FluoroJade B staining was performed on fetal brain frozen sections following the protocols suggested by the manufacturer.

2.8. Immunoblotting

To investigate the effects of statins on the expression of PKB and phosphorilated PKB, isolated fetal cortical neurons were incubated on day 6 (6DIV) with pravastatin (20 μg/ml), simvastatin (40 μg/ml) or just media (control group). 24 h later (7DIV), expression of PKB and phosphorylated PKB was assessed by Western blotting. Cortical cells were homogenized in ice-cold lysis buffer (50 mmol/L Tris, pH 7.4, 0.27 mol/L sucrose, 1 mmol/L sodium orthovanadate, pH 10, 1 mmol/L EDTA, 1 mmol/L EGTA, 10 mmol/L sodium β-glycerophosphate, 50 mmol/L NaF, 5 mmol/L sodium pyrophosphate, 1% [w/v] Triton X-100, 0.1% [v/v] 2-mercaptoethanol, one tablet of complete TM protease inhibitor [Roche, Burgess Hill, U.K.]), and 50 μg of protein were run on 4–12% Bis–Tris gels for Western blotting. Protein signals were visualized using enhanced chemiluminescence (Pierce Biotechnology, Rockford, IL) by exposure to an Amersham HyperfilmTH ECL film (Amersham). The antibodies used are described in Table 1.

2.9. Statistical analysis

Data are expressed as mean ± SD. Statistical differences between groups were determined using one-way ANOVA with subsequent two-tailed Student *t* test.

3. Results

3.1. Intravaginal administration of LPS does not reach systemic circulation

Our results show that 85 ± 2% of the LPS dose administered intravaginally remained in the cervix 12 h after LPS administration. Only 13 ± 4% of the administered LPS-FITC dose was found in the amniotic fluid. The levels of LPS-FITC in systemic circulation were undetectable, suggesting that intravaginal LPS ascends to the cervix but it is not absorbed to systemic circulation. This is in agreement with the absence of maternal systemic disease (fever, piloerection and lethargy) observed in pregnant females that received intravaginal LPS. In addition, after giving birth prematurely the females survive without any health complications. The offspring – born around day 16 – die because of the effects of immaturity. Mice born at day 16 are equivalent to extremely preterm human babies (<28 weeks) according to the World Health Organization classification. Children born very preterm are reported to have an increased frequency of social, emotional, and behavioral problems at school age compared with their peers born at term [18].

3.2. Fetal brain cortical abnormalities in LPS-treated mice

Fetal brains were harvested 18 to 24 h after the induction of PTB with intravaginal LPS. The mice that were euthanized showed signs of preterm labor (vaginal bleeding and cervical dilatation). Immunohistochemical results were compared to age matched-control fetuses. Microtubule associated protein-2 (MAP-2) was used as a marker of intact neuronal cell bodies and neurofilament 200 (NF200) was used to evaluate dendritic and axons structure. Decreased staining for NF200 and MAP-2 was observed in the cortex of fetuses born preterm compared to age-matched controls (Fig. 1A) (staining intensity (arbitrary units):

Table 1
Antibodies used for immunohistochemical studies and western blotting (WB).

Clone	Specificity	Manufactured by	Dilution	Used in	Developed with
mAb 10/92	Mouse C5aR	Hycult biotech	1/50	Fetal brain	Anti-rat-HRP/DAB
Anti-MAP2	MAP2	Sigma-Aldrich	1/100	Fetal brain	Anti-rabbit FITC
Anti-NF200	NF-200	Sigma-Aldrich	1/400	Fetal brain	Anti-rabbit FITC
Ab 18207	BIII tubulin	Abcam	1/800	ICN	Anti-rabbit FITC
Ab 44976	Caspase-3	Abcam	1/500	Fetal brain	Anti-rabbit HRP/DAB
11E7	Akt (pan)	Cell Signaling	1:2000	WB	Anti-rabbit-HRP/CHEM
(D9E) XP	Phospho-Akt (Ser473)	Cell Signaling	1:4000	WB	Anti-rabbit-HRP/CHEM

ICN = isolated cortical neurons; CHEM = chemiluminescence; WB = Western Blot.

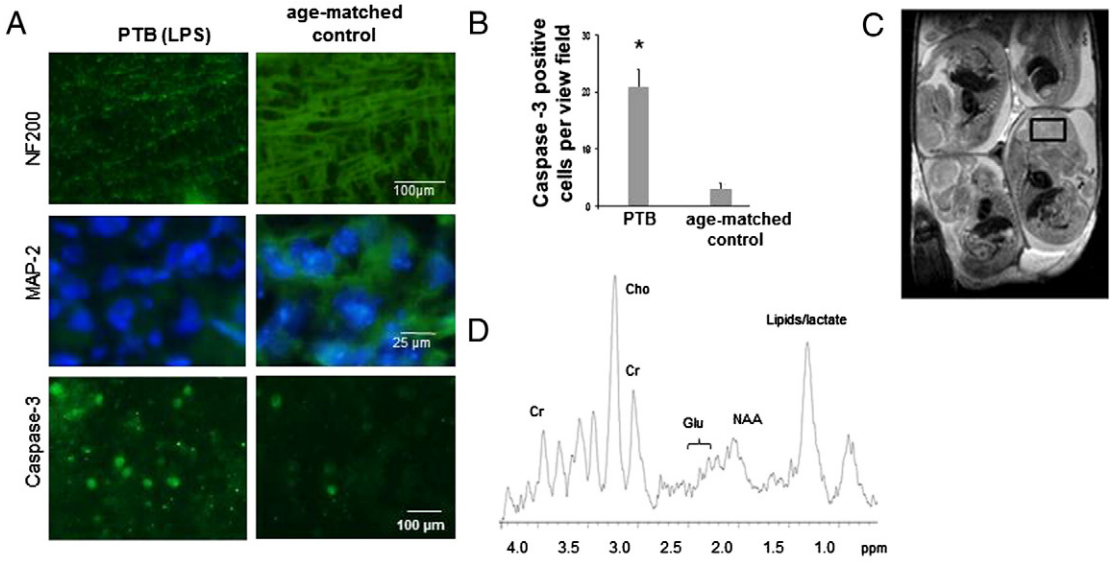
NF200: age-matched control = 267 ± 35; PTB (LPS) = 61 ± 29*; MAP-2: age-matched control = 287 ± 34, PTB (LPS) = 73 ± 22*, *different from age-matched control, $p < 0.01$), suggesting a disruption of cortical dendritic and axonal cytoarchitecture at this stage of fetal cortical brain development. Increased cell death was observed in the fetal brain cortex in the PTB-model. The number of neurons expressing active caspase-3 – involved in cell death in immature neurons – was higher in the fetal brain cortex from fetuses in the PTB group compared to age-matched controls (Fig. 1A, B).

3.3. Metabolic abnormalities in fetal brains in PTB mice — proton magnetic resonance spectroscopy (^{1}HMRS) studies

^{1}HMRS was used to assess the metabolic profile of fetal brains in the uterus. Fetal brain ^{1}HMRS studies were performed in the LPS-treated mice that showed signs of preterm labor (vaginal bleeding and cervical dilatation) and in age matched controls. Fig. 1C shows the voxel placement in the fetal brains. A ^{1}HMRS spectrum of a control mouse fetal brain is represented in Fig. 1D. Four metabolic peaks can be easily identified in the spectrum: creatine plus phosphocreatine (Cr), choline-containing compounds (Cho), N-acetylaspartate (NAA) and lipids. In addition more complex peaks from glutamate (Glu) can be identified (Fig. 1D). The ^{1}HMRS signal from creatine (measure of the global brain function or integrity) was used to normalize the signals from other metabolites measured within the same voxel. A significant increase in Glutamate/total Creatine (Glu/tCr) but not in other metabolites was observed in the fetal brains from pregnancies complicated by preterm birth compared to those of age-matched controls (Fig. 1E). It has been suggested that excessive signaling by excitatory glutamate can cause excitotoxicity leading to cell injury and death [19]. Given the claimed relation between glutamate overexcitatory effects and cell death we investigated neurodegeneration using FluoroJade B, a marker for neurons that undergo cell death presumably by excitoxicity [20]. A Significant increase of FluoroJade B positive cells was observed in the cortex of fetuses in the PTB-model (Fig. 1F). The combined MRI and histochemical results suggest that fetal cortical brain injury could be caused by glutamate excitotoxicity.

3.4. C5a is required for fetal brain injury in preterm fetuses

Knowing that complement activation plays a crucial role in this model of PTB [7,8] we went on to study complement activation in brains from the fetuses born prematurely. Increased levels of complement split product C5a was observed in the amniotic fluid and fetal brains in PTB-mice compared to those of appropriate controls (Fig. 2A). C5a receptors were identified in fetal cortical neurons by immunohistochemistry (Fig. 2B). Prompted by these findings, we investigated if C5a could be involved in the cortical fetal brain injury observed in fetuses that are born preterm. To answer this question we mated C5aR+/− mothers with C5aR−/− mice to generate C5aR−/− fetuses. Next, we induced preterm delivery in C5aR+/− pregnant females and cortical fetal brain damage was assessed in the resulting C5aR+/− and C5aR−/− fetuses in the litter. Interestingly C5aR+/− females deliver preterm as wild type mice and C5a levels in fetal brains after LPS treatment were



E

	Tau/tCR	tCh/tCR	tNAA/tCR	Glu/tCR
control (n=6)	2.89±0.29	0.76±0.17	0.32±0.14	1.00±0.16
PTB (n=6)	2.94±0.51	0.80±0.17	0.33±0.09	1.49±0.32*

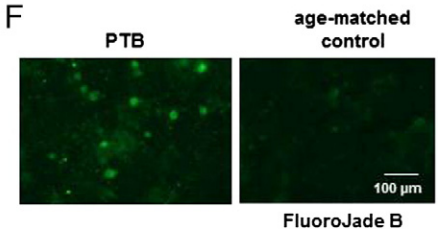


Fig. 1. Fetal cortical brain injury in preterm birth. A — Fetal brains sections from LPS-treated mice (n = 6) and age-matched controls (n = 7) were stained with anti-filament 200 (NF200) (marker for axons), anti-microtubule associated protein 2 (MAP-2) (marker for mature neurons) and anticaspase-3 (marker of apoptotic neurons). Decreased staining for NF200 and MAP-2 in the fetal brains in LPS-treated mice suggests a disruption of cortical dendritic and axonal cytoarchitecture. Increased number of caspase-3 positive cells is also observed in the fetal cortex in LPS-treated mice compared to controls. B — Active caspase-3-positive cells per view-field in the fetal cortical brain in the LPS and control group (means.d.). Original magnification = ×400. C — The spectroscopy voxel (2.8 × 2.8 × 2.8 mm) was positioned in the center of the fetal brain in respiration-gated T2-weighted fast spin echo images. D — ^{1}HMRS spectrum obtained from a fetal brain from a control mouse showing the different metabolic peaks Cr (creatine), Cho (choline), Glu (glutamate), N-acetyl-aspartate (NAA), lipids and lactate. Resonant frequencies of the chemical species are expressed in parts per million (ppm). E — Table showing the different metabolic peaks. Increased glutamate/Cr was observed in fetal brains from LPS-treated mice compared to controls. F — FluoroJade B, high affinity fluorescent marker for the localization of neuronal degeneration, staining in the cortex of fetal brains in LPS-treated mice and age-matched controls. Increased number of Fluorojade B positive cells in the fetal cortical brain in LPS-treated mice.

A

C5a			
Amniotic fluid (ng/ml)		Cortical fetal brain (ng/mg)	
LPS (PTL) (n=5)	Control (n=6)	LPS (PTL) (n=8)	Control (n=8)
3304.7±865.7*	29.0±6.2	1765±346*	69±21

* different from control, p<0.01

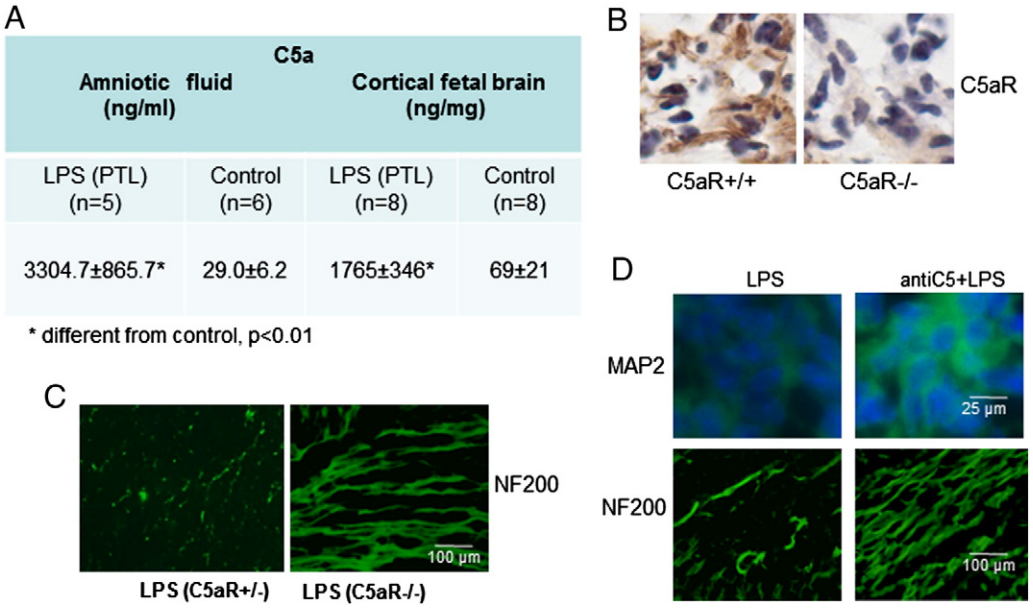


Fig. 2. Role of C5a in fetal cortical brain injury in preterm birth. A — Complement split product C5a in amniotic fluid and fetal cortical brain in LPS-treated mice and age-matched controls. B — Immunohistochemical detection of C5aR on fetal cortical brain in mice. C5aR−/− mice were used as negative control. C — NF200 staining in the brain cortex of C5aR+/− and C5aR−/− fetuses in LPS-treated mice. NF200 and MAP-2 staining in the brain cortex in fetuses from mice treated with LPS + antiC5 antibody. D — NF200 and MAP-2 staining in the fetal brain cortex of LPS-treated mice treated with antibody antiC5.

comparable to wild type mice (1622 ± 513 ng/ml vs 1765 ±346 ng/ml). C5aR−/− fetuses, but not C5aR+/− fetuses in LPS-treated mice showed conserved axonal and dendritic architecture at this stage of development, judged by NF200 staining, comparable to age-matched controls staining (intensity (arbitrary units): NF200: LPS (C5aR+/−) = 45 ± 13*, LPS (C5aR-/−) = 198 ± 26*; *different from C5aR+/−, $p < 0.01$) (Fig. 2C). In support of the role of C5a in cortical fetal brain injury, treatment with antibody anti-C5 – that blocked generation of C5a – prevented cortical fetal brain abnormalities in LPS-treated mice. Staining for NF200 and MAP-2 in fetal brains from antiC5 + LPS treated mice was not different from that of the age-matched controls (staining intensity (arbitrary units): NF200: age-

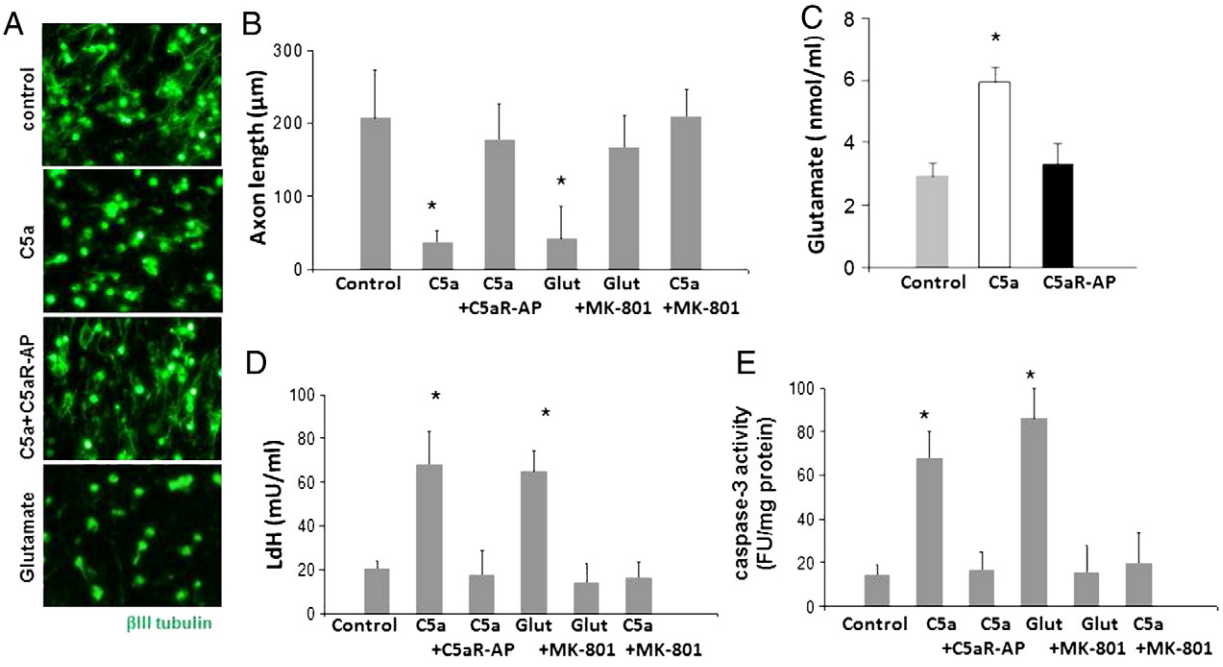


Fig. 3. Role of glutamate in C5a-induced neurotoxicity in vitro. A — Neuritic network formation in isolated fetal cortical neurons incubated with C5a, glutamate and C5a + C5aR-AP. Cells were identified as neurons by positive BIII tubulin staining. B — Graph bar shows length of axons in isolated cortical neurons incubated under different conditions (C5a, glutamate, C5a + C5aR-AP, glutamate + MK-801, C5a + MK-801). *Different from untreated, $p < 0.01$. Five to six cortical neurons preparations were used for each experimental condition. C — Glutamate levels in the supernatants of fetal cortical neurons incubated under different conditions (A). D — Cell death in isolated cortical neurons subjected to different conditions was measured by the release of lactate dehydrogenase (LdH) – marker of membrane integrity – to the media. *Different from untreated, $p < 0.05$ (n = 5/experimental condition). E — Fluorometric caspase 3 activity in cortical neurons subjected to different treatments.

matched control = 267 ± 35, LPS = 61 ± 29*, antiC5 + LPS = 238 ± 23; MAP-2: age-matched control = 287 ± 34, LPS = 73 ± 22*, antiC5 + LPS = 268 ± 32; *different from age-matched control, $p < 0.01$), suggesting appropriate axonal and dendritic structure (Fig. 2D).

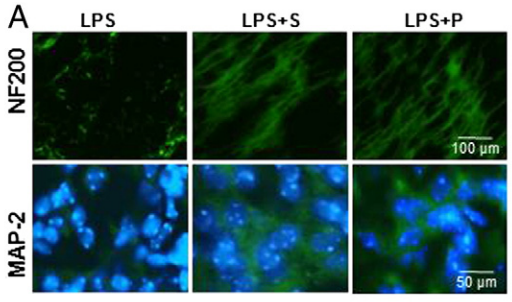
3.5. Neurotoxic effects of C5a

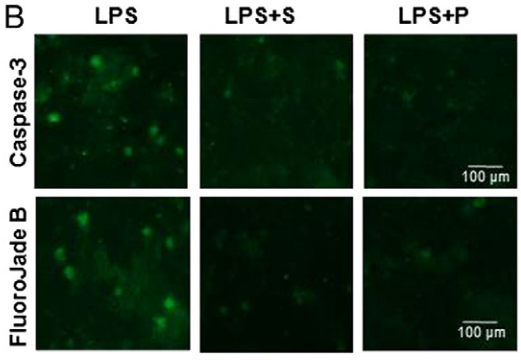
To confirm the detrimental effects of C5a on the developing fetal brain we studied isolated cortical neurons from day 16 fetuses. Immunohistochemical procedures determined that most cultured cells (>95%) express βIII tubulin (Fig. 3A), protein associated with cortical neurons and not found in glial cells. These cells extend their neurites and establish synapses becoming mature neurons after 10 days in culture (10DIV). To study the effects of C5a on developing neurons in vitro we exposed the cells on 7DIV to C5a. As a quantitative measure of neuronal injury during neuronal development, we analyzed the growth of projections that emanated from the neuronal cell bodies. At day 10, long axons were observed in cultured control cortical neurons while the length of axons in C5a-exposed neurons was considerably reduced after the addition of C5a to the media on 7DIV (Fig. 3A, B). The blockade of C5a increased the numbers of dendrites and the length of axons compared to neurons incubated with only C5a (Fig. 3A, B). Axonal and dendritic architecture in C5aR-AP treated cells was comparable to control cells (Fig. 3A). Increased levels of glutamate were measured in the supernatant of cortical neurons incubated with C5a (Fig. 3C). In addition, isolated cortical neurons incubated with glutamate showed disrupted neuronal morphology (shorter axons and dendrites) similar to cells exposed to C5a (Fig. 3A and B). Addition of the N-methyl-D-asparate antagonist, MK-801 (dizocilpine maleate) prevented C5a and glutamate-induced diminished axonal length (Fig. 3B). This raises the possibility that C5a neurotoxicity might be mediated by glutamate excitotoxicity. Excitation resulting from stimulation of the ionotropic glutamate receptors is known to cause neuronal apoptosis [19]. Thus, we looked for signs of neuronal death/apoptosis. Increased release of lactate dehydrogenase (LdH), marker of cell injury and death, was observed in cortical neurons cultures exposed to C5a or glutamate (Fig. 3D). The addition of C5aR-AP and MK-801 prevented C5a-induced increased cell injury and death (Fig. 3D).

The fluorometric assay of caspase 3 activity showed that there was a significant difference in activity between the control cells and the cells incubated with C5a and glutamate ($p < 0.001$) (Fig. 3E). Caspase activity in control neurons averaged 14 ± 5 FU/mg protein (Fig. 3E). In contrast, cortical neurons incubated with C5a or glutamate had a significantly higher caspase activity, a 4.5–5.0 times increase over controls. Caspase activity was reduced in cortical neurons incubated with C5a plus C5aR-AP or MK-801 when compared to cells incubates with C5a or glutamate alone (Fig. 3E).

3.6. Pravastatin and simvastatin prevent brain injury in PTL

Statins showed beneficial effects in several neurological disorders [16–18]. Thus, we hypothesized that statins could prevent fetal cortical brain injury in our mouse model of PTB. In accordance with our hypothesis, the disruption in cortical neuron cytoarchitecture observed in the fetal brains from the PTB mice was not observed when the mice were pretreated with pravastatin or simvastatin (Fig. 4A). Robust staining for NF200 and MAP-2 was observed in the cortex of the fetal brains from the simvastatin + LPS (S + LPS) and the pravastatin + LPS (P + LPS)-treated mice, comparable to age-matched untreated controls (Fig. 4A) (staining intensity (arbitrary units: NF200: age-matched control = 238 ± 45;=S + LPS = 251 ± 32, P + LPS = 228 ± 61; MAP-2: age-matched control = 247 ± 49, S + LPS = 271 ± 43, P + LPS = 228 ± 29)). The mice treated with LPS and pravastatin or simvastatin also showed a decreased number of caspase-3 positive cells in the fetal cortical brain compared to the LPS-treated mice (number positive cells/view field: LPS = 21 ± 3, S + LPS = 4 ± 1, P + LPS = 5 ± 2, age-matched untreated control = 3 ± 1) (Fig. 4B). A decreased number of FluoroJadeB positive cells was observed in the fetal brains in mice treated with LPS + statins, suggesting a protective effect of statins against cell death and neurodegeneration (Fig. 4B). Reduced levels of





C

C5a (ng/mg) in fetal brain			
LPS (PTL) (n=8)	Control (n=8)	LPS+P (n=8)	LPS+S (n=8)
1765±346*	69±21	128±38	149±29

* different from control, p<0.01

D

	Tau/tCR	tCh/tCR	tNAA/tCR	Glu/tCR
Age-matched control (n=5)	2.89±0.29	0.76±0.17	0.32±0.14	1.00±0.16
LPS (n=5)	2.94±0.51	0.80±0.17	0.33±0.09	**1.49±0.32***
LPS+S (n=6)	2.97±0.50	0.63±0.11	0.34±0.12	1.00±0.12

Fig. 4. Statins prevent fetal cortical brain injury in vivo. A — NF200 and MAP-2 staining in the cortex of fetal brains in LPS + P- and LPS + S-treated mice. n = 5–7 mice/group. B — Caspase and fluorojade staining in the fetal brain cortex of control, LPS-, LPS + S and LPS + P-treated mice. C — C5a levels (ng/mg) in fetal brains from control, LPS-, LPS + S and LPS + P-treated mice. D — Table showing the different metabolic peaks values measured by ^{1}HMRS of the fetal brains. *Different from age-matched control, $p < 0.01$.

C5a were found in fetal brains from mice treated with LPS + statins (Fig. 4C).

The protective effects of statins were also observed in the ^{1}HMRS studies. Spectroscopy metabolic studies revealed a significant reduction in glutamate signal in mice treated with simvastatin plus LPS when compared to mice that only received LPS (Fig. 4D).

We then investigated if statins could prevent C5a-induced toxicity in developing neurons. Isolated cortical neurons incubated with simvastatin or pravastatin + C5a did not show abnormal dendrite and axon architecture (Fig. 5A). Axons in cortical neurons incubated with statins and C5a were longer than in cells incubated with only C5a and the axon length was comparable to control neurons (Fig. 5B). In addition, statins also prevented C5a-induced increased release of glutamate and Ldh (Glutamate (nmol/ml): control (n = 5): 2.9 ± 0.2; C5a (n = 5):5.9 ± 0.5*; S + C5a (n = 6):2.8 ± 0.2; P + C5a (n = 5):3.3 ± 0.4; LdH (mU/ml): control (n = 5):17 ± 6; C5a (n = 5): 53 ± 13*, S + C5a (n = 5):20 ± 8, P + C5a (n = 5):23 ± 6; *different from control, $p < 0.01$). Furthermore, statins diminished caspase-3 activity in isolated cortical neurons exposed to C5a (Fig. 5C).

Akt/PKB signaling pathway was reported to be a crucial mechanism in the beneficial effects of statins in neuronal injury models [21,22]. Thus, we looked into this mechanism in our in vitro model. In agreement with these studies, we observed increased PKB phosphorylation in cortical neurons incubated with simvastatin or pravastatin (Fig. 5D, E) compared to untreated cells. Treatment with the PKB/Akt inhibitor LY294002 blocked the neuroprotective effects of simvastatin and pravastatin in C5a-induced neurotoxicity on cortical cells (Fig. 5B), confirming that the beneficial effects of statins on cortical neuron development are dependent on protein kinase B (PKB) signaling. Cortical neurons incubated with LY294002 plus statins showed abnormal axonal growth in response to C5a comparable to cells treated with C5a alone (Fig. 5C).

4. Discussion

Prematurity is an important cause of neonatal brain injury and long-term neurodevelopmental disabilities. Unfortunately, the mechanisms behind fetal brain injury are still unclear, preventing therefore the development of diagnostic methods and therapies. In this study we discovered the complement system as a crucial mediator in cerebral cortex injury in fetuses born preterm.

Using a mouse model of preterm birth triggered by a subclinical vaginal inflammation/infection, we found that C5a is neurotoxic on fetal cortical neurons.

The presence of fetal brain abnormalities in our mouse model emphasizes the validity of our approach to use a mouse model that resembles the clinical scenario in extremely preterm (<28 weeks) human babies and allowed us to identify the mediators/effectors of brain damage and test potential therapies. Cortical brain injury, characterized by abnormal cytoarchitecture of axons and dendrites in neurons and increased cell death, was observed in preterm fetuses but not in age-matched controls. The presence of cortical brain damage/neuronal death in LPS-treated mice that deliver preterm and not in age-matched controls suggests that prenatal exposure to inflammation may have detrimental effects on neurological development in addition to the termination of the intrauterine environment. The absence of

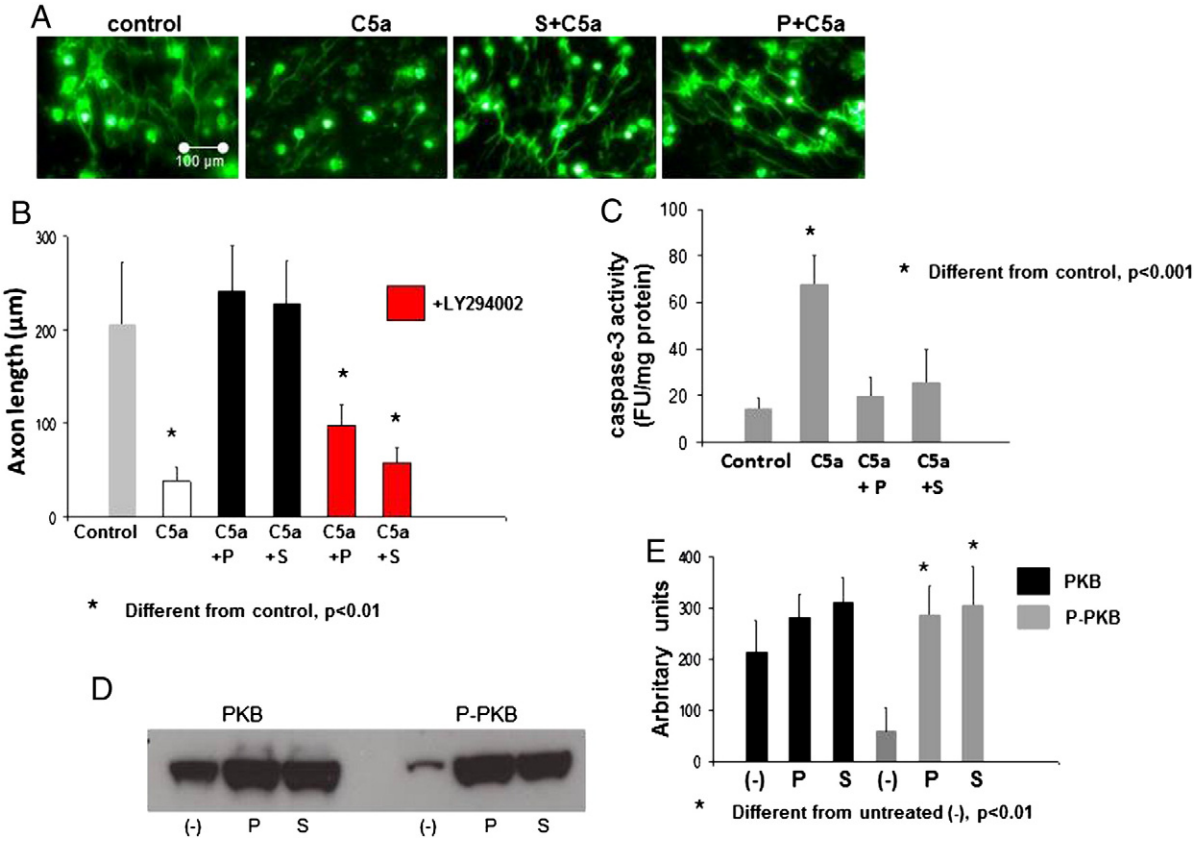


Fig. 5. Statins prevent fetal cortical brain injury in vitro. A — Pravastatin (P) and simvastatin (S) restore axonal development of isolated cortical neurons incubated with C5a. The microphotographs represent one of five similar experiments. Cortical neurons were identified by BIII tubulin (positive staining condition). B — Axon length in isolated cortical neurons incubated with C5a, P + C5a and S + C5a, LY294002 + P + C5a, LY294002 + S + C5a and just media. *Different from untreated, $p < 0.05$ (n = 5 cortical neurons preparations/experimental). C — Fluorometric caspase 3 activity in cortical neurons incubated with C5a, and C5a plus statins. D — Expression of PKB and phosphorylated PKB was assessed by Western blotting in isolated cortical neurons incubated with simvastatin (S) (lanes 3 and 7), pravastatin (P) (lanes 2 and 6) and only media (–) (lanes 1 and 5). E — Western blots (n = 3) were quantified by densitometry (arbitrary units, mean ± SD).

brain injury in LPS-treated mice that received anti-C5 antibody and in fetuses deficient in C5aR and the toxic effects of C5a in vitro suggest that C5a may be responsible for the fetal cortical brain damage observed in fetuses born prematurely. Moreover, increased C5a levels were found in amniotic fluid and fetal cortical brains in mice that delivered preterm [7]. Interestingly, Soto et al demonstrated increased C5a levels in amniotic fluid in women with spontaneous preterm labor [23]. Isolated fetal cortical neurons showed abnormal architecture and increased death when incubated with C5a and this effect was blocked by C5aR antagonists, confirming the direct neurotoxic effect of C5a. Other authors also demonstrated the neurotoxic effects of C5a in adults [24,25]. However, this is the first study, to our knowledge, reporting the involvement of C5a in fetal brain injury. Brain cells can synthesize a full complement system and also express specific complement inhibitors [26]. However, the amount of complement inhibitors in premature newborns is low relative to term newborns making the fetuses born preterm more susceptible to complement-mediated brain injury [27].

Although C5a is traditionally considered a proinflammatory molecule, several studies have demonstrated its role in the development and survival of neurons [28,29]. However, C5a inhibits apoptosis in isolated cortical neurons at lower concentrations than those used in experiments showing its neurotoxic pro-death effects [28,30]. The physiological relevance of high and low doses remains to be established.

Excessive signaling by excitatory neurotransmitter glutamate can be deleterious to neurons and cause disease [22]. Spectroscopy MRI studies showed increased glutamate levels in total brain from premature fetuses. In addition, increased reactivity with FluoroJade B – a marker for neurons that undergo cell death by excitotoxicity – was observed in fetal cortical brain in mice with LPS-induced preterm birth. That C5a induces glutamate release from isolated cortical neurons suggests that C5a-induced cortical brain injury in premature fetuses may be mediated by glutamate excitotoxicity. The blockade of glutamate receptors prevented C5a-induced abnormal cortical neurons development and increased cell death in vitro, confirming the role of glutamate in C5a-induced neurotoxic effects.

In addition to complement inhibition with antibody anti-C5, statins prevented cortical brain injury in fetuses from the LPS-treated mothers. Statins also prevented C5a-induced cortical neuron toxicity in vitro. Pravastatin and simvastatin restored the cortical brain morphology and prevented glutamate release and cell death in cortical neurons incubated with C5a. Diminished glutamate levels were also measured in fetal brains of LPS + simvastatin-treated mice by MRI spectroscopy. It has been described that a decrease in the level of membrane cholesterol can result in attenuated transporter-mediated glutamate release from nerve terminals. Therefore, lowering cholesterol may be used in neuroprotection in brain injury associated with an increase in glutamate uptake [31]. Lower levels of C5a were measured in fetal brains from statins + LPS treated mice compared to mice treated with LPS alone. It has been described that statins increase the expression of complement inhibitor DAF and thus block C5a generation [32].

It was suggested that the neuroprotective effects of statins are mediated by activation of the Akt/PKB signaling pathway [21,22] and that this pathway increases neuronal resistance to glutamate-induced apoptosis [33]. Our studies show increased phosphorylation of PKB and diminished fetal neuronal death in statin-treated mice. It is likely that the protective effects of statins can be caused by an increased resistance to glutamate through PKB signaling. The role of PKB in the protective effects of statin was confirmed by the observation that statins did not protect cortical fetal neurons from C5a-induced injury when neurons were previously treated with PKB/Akt inhibitor LY294002. Our studies were focused on the cortical neurons. However, we also need to consider the potential effects of inflammation/complement activation on other cells in the central nervous system like glial cells and astrocytes that can also contribute to fetal brain damage in prematurity.

In conclusion, we demonstrated a previously unknown role for complement anaphylotoxin C5a in cortical brain abnormal development and increased cell death in preterm fetuses. These data together with our previous observations that C5a induces cervical ripening and that complement activation does not play a role in term parturition [8] suggest that the complement system might be a good target for therapy to prevent fetal cortical brain damage and preterm birth. We also found that statins prevented C5a-induced disruption of cortical dendritic and axonal cytoarchitecture. Because statins are already approved for therapeutic use and proved not to be harmful to the fetus [34], our studies if translatable to women in clinical trials may provide a safe and fast way to prevent fetal cortical brain injury and the resulting long-term neurodevelopmental disabilities.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2013.10.011.

References

[1] J.J. Volpe, Brain injury in premature infants: a complex amalgam of destructive and developmental disturbances, Lancet Neurol. 8 (1) (2009) 110–124.
[2] K. Trewaud, A. Ure, L.W. Doyle, K.J. Lee, C.E. Rogers, K. Kidokoro, T.E. Inder, P.J. Anderson, Psychiatric outcomes at age seven for very preterm children: rates and predictors, J. Child Psychol. Psychiatry (2013), http://dx.doi.org/10.1111/jcpp.12040.
[3] R. Rathbone, S.J. Counsell, O. Kapellou, L. Dyet, N. Kennea, J. Hajnal, J.M. Allsop, F. Cowan, A.D. Edwards, Perinatal cortical growth and childhood neurocognitive abilities, Neurology 77 (16) (2011) 1510–1517.
[4] M.J. Bell, J.M. Hallenbeck, Effects of intrauterine inflammation on developing rat brain, J. Neurosci. Res. 70 (2002) 570–579.
[5] J.R. Duncan, M.L. Cock, J.-P.Y. Scheerlinck, et al., White matter injury after repeated endotoxin exposure in the preterm ovine fetus, Pediatr. Res. 52 (2002) 941–949.
[6] Ch. Härtel, D. Finas, P. Ahrens, E. Kattner, T. Schaible, et al., Polymorphisms of genes involved in innate immunity: association with preterm labor, Mol. Hum. Reprod. 10 (2004) 911–915.
[7] J.M. Gonzalez, C.W. Franzke, F. Yang, R. Romero, G. Girardi, Complement activation triggers metalloproteinases release inducing cervical remodeling and preterm birth in mice, Am. J. Pathol. 179 (2011) 838–849.
[8] J.M. Gonzalez, Z. Dong, R. Romero, G. Girardi, Cervical remodeling/ripening at term and preterm labor: the same mechanism initiated by different mediators and different effector cells, PLoS One 6 (11) (2011) e26877.
[9] P. Redecha, C. Franzke, W. Ruf, N. Mackman, G. Girardi, Activation of neutrophils by the tissue factor – factor VIIa – PAR-2 axis mediates fetal death in antiphospholipid syndrome, J. Clin. Invest. 118 (2008) 3453–3461.
[10] J. Singh, A. Ahmed, G. Girardi, Role of complement component C1q in the onset of preeclampsia in mice, Hypertension 58 (2011) 716–724.
[11] S.V. Seshan, C.W. Franzke, P. Redecha, M. Monestier, N. Mackman, G. Girardi, Role of tissue factor in a mouse model of thrombotic microangiopathy induced by antiphospholipid antibodies, Blood 114 (8) (2009) 1675–1683(20).
[12] U.E. Höpken, B. Lu, N.P. Gerard, C. Gerard, Impaired inflammatory responses in the reverse Arthus reaction through genetic deletion of the C5a receptor, J. Exp. Med. 186 (1997) 749–756.
[13] A. Hussain, F. Ahsan, The vagina as a route for systemic drug delivery, J. Control. Release 103 (2) (2005) 301–313.
[14] Y. Frei, J.D. Lambris, B. Stockinger, Generation of a monoclonal antibody to mouse C5 application in an ELISA assay for detection of anti-C5 antibodies, Mol. Cell. Probes 1 (1987) 141–149.
[15] H.J. Kim, J. Magrané, Isolation and culture of neurons and astrocytes from the mouse brain cortex, Methods Mol. Biol. 793 (2011) 63–75.
[16] A.M. Finch, A.K. Wong, N.J. Paczkowski, S.K. Wadi, D.J. Craik, et al., Low-molecular-weight peptidic and cyclic antagonists of the receptor for the complement factor C5a, J. Med. Chem. 42 (1999) 1965–1974.
[17] L.C. Schmuel, C. Alberston, W. Slikker Jr., Fluoro-Jade: a novel fluorochrome for the sensitive and reliable histochemical localization of neuronal degeneration, Brain Res. 751 (1) (1997) 37–47.
[18] A.J. Spittle, K. Treyvaud, L.W. Doyle, G. Roberts, K.J. Lee, T.E. Inder, J.L. Cheong, R.W. Hunt, C.A. Newnham, P.J. Anderson, Early emergence of behavior and social-emotional problems in very preterm infants, J. Am. Acad. Child Adolesc. Psychiatry 48 (9) (2009) 909–918.
[19] C. Matute, Glutamate and ATP signalling in white matter pathology, J. Anat. 219 (1) (2011) 53–64.
[20] P.J. van der Most, A.M. Dolga, I.M. Nijholt, P.G. Luiten, U.L. Eisel, Statins: mechanisms of neuroprotection, Prog. Neurobiol. 88 (1) (2009) 64–75.

[21] S. Carloni, S. Girelli, G. Buonocore, M. Longini, W. Balduini, Simvastatin acutely reduces ischemic brain damage in the immature rat via Akt and CREB activation, J. Exp. Neurol. 220 (1) (2009) 82–89.
[22] A.M. Dolga, I. Granic, I.M. Nijholt, C. Nyakas, E.A. van der Zee, P.G. Luiten, U.L.F. Eisel, Treatment with lovastatin prevents N-methyl-D-aspartate-induced neurodegeneration in the magnocellular nucleus basalis and behavioral dysfunction, J. Alzheimers Dis. 17 (2) (2009) 327–336.
[23] E. Soto, R. Romero, K. Richani, B.H. Yoon, T. Chaiworapongsa, E. Vaisbuch, P. Mittal, O. Erez, F. Gotsch, M. Mazor, J.P. Kusanovic, Evidence for complement activation in the amniotic fluid of women with spontaneous preterm labor and intra-amniotic infection, J. Matern. Fetal Neonatal Med. 22 (11) (2009) 983–992.
[24] T.M. Woodruff, R.R. Ager, A.J. Tenner, P.G. Noakes, S.M. Taylor, The role of the complement system and the activation fragment C5a in the central nervous system, Neuromolecular Med. 12 (2010) 179–192.
[25] M.C. Garrett, M.L. Otten, R.M. Starke, R.J. Komotar, P. Magotti, J.D. Lambris, M.A. Rynkowski, E.S. Connolly, Synergistic neuroprotective effects of C3a and C5a receptor blockade following intracerebral hemorrhage, Brain Res. 1298 (2009) 171–177.
[26] P. Gasque, Y.D. Dean, E.P. McGreal, J. VanBeek, B.P. Morgan, Complement components of the innate immune system in health and disease in the CNS, Immunopharmacology 49 (2000) 171–186.
[27] E.P. McGreal, K. Hearne, O.B. Spiller, Off to a slow start: under-development of the complement system in term newborns is more substantial following premature birth, Immunobiology 217 (2012) 176–186.
[28] H. Osaka, P. Mukherjee, P.S. Aisen, G.M. Pasinetti, Complement-derived anaphylatoxin C5a protects against glutamate-mediated neurotoxicity, J. Cell. Biochem. 73 (3) (1999) 303–311.
[29] M. Bénard, B.J. Gonzalez, M.T. Schouft, A. Falluel-Morel, D. Vaudry, P. Chan, H. Vaudry, M. Fontaine, Characterization of C3a and C5a receptors in rat cerebellar granule neurons during maturation. Neuroprotective effect of C5a against apoptotic cell death, J. Biol. Chem. 279 (42) (2004) 43487–43496.
[30] D. Pavlovski, J. Thundyil, P.N. Monk, R.A. Wetsel, S.M. Taylor, T.N. Woodruff, Generation of complement component C5a by ischemic neurons promotes neuronal apoptosis, FASEB J. 26 (9) (2012) 3680–3690.
[31] N. Krisanova, R. Sivko, L. Kasatkina, T. Borisova, Neuroprotection by lowering cholesterol: a decrease in membrane cholesterol content reduces transporter-mediated glutamate release from brain nerve terminals, Biochim. Biophys. Acta 1822 (10) (2012) 1553–1561.
[32] J.C. Mason, Z. Ahmed, R. Mankoff, E.A. Lidington, S. Ahmad, V. Bhatia, A. Kinderlerer, A.M. Randi, D.O. Haskard, Statin-induced expression of decay-accelerating factor protects vascular endothelium against complement-mediated injury, Circ. Res. 91 (2002) 696–703.
[33] H. Wu, D. Lu, H. Jiang, Y. Xiong, C. Qu, B. Li, A. Mahmood, D. Zhou, M. Chopp, Increase in phosphorylation of Akt and its downstream signaling targets and suppression of apoptosis by simvastatin after traumatic brain injury, J. Neurosurg. 109 (4) (2008) 691–698.
[34] D.M. Kusters, H.H. Lahsinoui, J.A. van de Post, A. Wiegman, F.A. Wijburg, J.J. Kastelein, B.A. Hutten, Statin use during pregnancy: a systematic review and meta-analysis, Expert. Rev. Cardiovasc. Ther. 10 (2012) 363–378.