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## Accepted Manuscript

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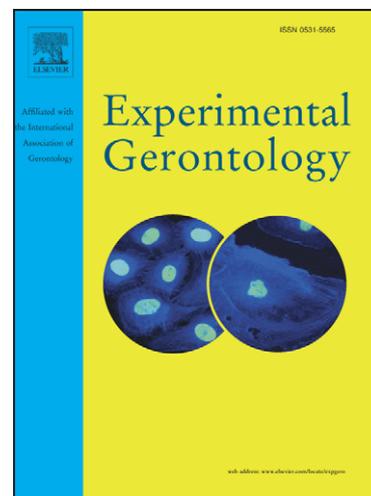
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**Age-related changes in choroid plexus and blood-cerebrospinal fluid barrier function in the sheep.**

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**Running title: Age-related changes in sheep choroid plexus**

**Key words: Choroid plexus, cerebrospinal fluid, cerebrospinal fluid secretion, cerebrospinal fluid proteins, blood-cerebrospinal fluid barrier, blood-brain barrier, sodium uptake.**

**Abstract**

2

3 Dysfunction of the choroid plexuses (CPs) and the blood-cerebrospinal fluid barrier (BCSFB)  
4 might contribute to age-related cognitive decline and neurodegenerative disease. We used the  
5 CPs from young (1-2 years), middle-aged (3-6 years) and old (7-10 years) sheep to explore  
6 effects of ageing on various aspects of CP and BCSFB functions. Total protein in the  
7 cerebrospinal fluid (CSF) was significantly higher in old compared to young sheep and CSF  
8 secretion by the CP perfused in situ was significantly lower in both old and middle-aged when  
9 compared to young sheep, which correlated with reduced  $^{22}\text{Na}^+$  uptake and efflux by the CP.  
10 Steady-state extractions of a low and medium size molecular weight extracellular space marker,  
11  $^{14}\text{C}$ -mannitol and  $^3\text{H}$ -polyethylene glycol, respectively, were significantly higher in CPs from old  
12 compared to young animals; however there was no significant difference in steady-state  
13 extraction of a high molecular weight marker,  $^{125}\text{I}$ -bovine serum albumin. This indicates  
14 increased passive BCSFB permeability for small and medium sized molecules in old sheep. CP  
15 redox activity was significantly lower in the old animals as assessed by the MTT assay, however  
16 there was no significant difference in ATP content and energy charge of the CP with age  
17 suggesting adequate baseline energy reserve capacity. These data indicate that normal ageing  
18 processes alter protein content in the CSF, CSF secretion, integrity of the BCSFB and  $\text{Na}^+$  flux in  
19 the epithelial layer, which could impact on CSF homeostasis and turnover.

20

21

## 1 1. Introduction

2  
3 The choroid plexuses (CPs) are highly vascularized branched structures with a layer of  
4 secretory epithelial cells projecting numerous villi into the four ventricles of the brain. The  
5 largest two CPs are found in the two lateral ventricles, a small extension in the third ventricle  
6 and a complex choroid plexus spreading its two “arms” across the fourth ventricle (Davson &  
7 Segal, 1996). The main function of the CP epithelium is to actively secrete cerebrospinal fluid  
8 (CSF), with the CPs of the lateral ventricles being the major site for CSF secretion. The CSF  
9 physically cushions the brain and reduces relative brain weight by almost a third, and also  
10 contributes to homeostasis of metabolites, acting as a metabolic ‘sink’ in regard to the brain  
11 extracellular fluid (Davson & Segal, 1996). The latter function is enhanced by the constant slow  
12 current of the brain interstitial fluid (ISF) bulk flow towards the CSF (Abbott, 2004). The CP  
13 epithelium constitutes a physical barrier between blood and cerebrospinal fluid (the blood-CSF  
14 barrier - BCSFB) by virtue of the complexity of the tight junctions between adjacent epithelial  
15 cells.

16 CSF has a unique composition, different from plasma due to the presence of specific ion  
17 transporters, exchangers and other transport systems in the CP epithelium for moving substances  
18 between blood and CSF (Redzic & Segal, 2004). Transporters include those for sugars, amino  
19 acids, vitamins, nucleosides as well as receptor-mediated transport of peptides and hormones  
20 which are largely directed into CSF, and a range of transporters for removing xenobiotics from  
21 CSF (Strazielle & Ghersi-Egea, 1999). The CP also synthesizes proteins, such as transthyretin,  
22 transferrin, insulin-like growth factor II and binding proteins that are secreted into CSF and play  
23 specific role in brain homeostasis (Dickson et al., 1985; Stylianopoulou et al., 1988).

24 With increasing age, morphological changes have been demonstrated in the CP and  
25 associated CSF circulatory system. The CP epithelial cell layer becomes flattened in humans,  
26 losing about 11% in height by age 88 and the epithelia basement membrane is coarser, thicker  
27 and more irregular (Serot et al., 2000). The CP epithelial cells also have cellular inclusions that  
28 increase in number with age, including the ubiquitous lipofuscin age-pigment (Wen et al., 1999)  
29 and Biondi ring tangles (Eriksson & Westermarck, 1986). It appears that normal ageing of the CP  
30 affects its metabolism, since a study on rats revealed that both total ATPase activity and activity  
31 of Na, K-ATPase was significantly higher in the CPs homogenates from young adult rats (6-8  
32 months) compared to old (26-28 months) (Kvitnitskaia-Ryzhova & Shkapenko, 1992). More  
33 recently mRNA expression of Na, K-ATPase was seen to decline in aged rat CP (Masseguin et al  
34 2005) along with water channel Aquaporin 1 protein and mRNA expression. These changes are  
35 likely to affect the rate of the CSF secretion, since it depends on ion and water translocation.  
36 However, measurement of total CSF secretion rate in humans has produced conflicting results.  
37 May et al., (1990) have shown, using the Masserman method (Masserman, 1934), that there was  
38 a significant reduction in the CSF secretion in a group of 67-84 year old healthy subjects, when  
39 compared to 21-36 year olds. On the other hand, Gideon et al., (1994), using more sophisticated

1 MRI techniques, measured the supratentorial CSF production in vivo and found almost identical  
2 CSF production rates (900-1000 ml per 24 h) in young and elderly healthy subjects. This is  
3 contradictory to findings by Stoquart-ElSankari et al (2007), who used sensitive phase-contrast  
4 MRI pulse sequences at the aqueductal and cervical level and revealed that CSF stroke volumes  
5 were significantly reduced in the elderly group. However, it was not clear whether this reflected  
6 impaired function of CPs, or was a consequence of age-related attenuation of cerebral blood  
7 flow (Stoquart-ElSankari et al 2007). Studies which measured CSF proteins revealed elevation of  
8 proteins originating from plasma (Reiber, 2003; Silverberg et al., 2003) in healthy elderly  
9 subjects. These data suggest that the normal ageing process might lead to alterations in a  
10 range of CP functions.

11 This study investigated the effects of aging on basic parameters of CP function  
12 and CSF composition in healthy adult sheep aged from 1 to 10 years: gross protein  
13 content in the CSF, Na<sup>+</sup> uptake and efflux by the CP, CSF secretion rate and paracellular  
14 permeability of the CP. Sheep were chosen for study, because they offer a  
15 chronologically long-lived model (average and maximum life expectancy 7.1 and 12  
16 years, respectively, Miller 1988) compared to rodents. In addition to that, the size of the  
17 lateral ventricle's CPs and blood vessels supplying them allows in situ perfusion to be  
18 performed, a technique which enables direct measurement of the CSF secretion and  
19 steady-state flux experiments.

20

## 21 **2. Materials and Methods**

### 22 **2.1. Surgery**

23 All procedures were carried out under Home Office license in accordance with the  
24 Scientific procedures Act UK 1986 (HMSO, London, UK). Clun Forest strain sheep between 14  
25 months and 10 years (60-90 Kg) were used, and categorized into 3 groups: aged 1-2 years young  
26 adult, 3-6 years middle age and 7-10 years old sheep. Sheep were anaesthetized with i.v  
27 thiopentone sodium (20 mg.Kg<sup>-1</sup>) and injected with heparin (1 000 U.Kg<sup>-1</sup>). Blood and CSF  
28 samples were collected from the carotid artery and cisterna magna, respectively, by needle  
29 puncture. After the sheep was sacrificed by exsanguination the skull and the dura mater were  
30 opened and the brain removed.

31

### 32 **2.2. CSF protein analysis**

33 Both blood and CSF samples were immediately centrifuged at 10 000 g for 10 minutes.  
34 Any CSF samples contaminated with blood indicated by erythrocyte precipitation after  
35 centrifugation were discarded. The total protein content in triplicate samples of both CSF and  
36 serum were analyzed using a protein assay kit (Bio Rad), with bovine serum albumin (BSA) as a  
37 standard.

38

### 2.3. The rate of the CSF secretion by the isolated choroid plexus

CSF secretion was measured in isolated lateral ventricle CPs, perfused in situ. The method has previously been published (Preston & Segal, 1990; Strazielle & Preston, 2003). Briefly, the circle of Willis supplying the choroidal arteries to each lateral ventricle CP in sheep were cannulated and perfusion commenced with a Ringer buffer (in mM NaCl 123, KCl 4.8, NaH<sub>2</sub>PO<sub>4</sub> 1.22, CaCl<sub>2</sub> 2.4, MgSO<sub>4</sub> 1.22, NaHCO<sub>3</sub> 25, glucose 5) containing 4% bovine serum albumin to maintain colloid osmotic pressure. The buffer was warmed to 37°C and gassed with 95% O<sub>2</sub> / 5% CO<sub>2</sub>. The perfusate outflow was collected from the Great vein of Galen, into which the veins from each CP flow. The cerebral hemispheres were opened to gain access to the CSF side of the plexuses, and the ventricles were flooded with artificial CSF (aCSF) (in mM NaCl 148, KCl 2.9, NaH<sub>2</sub>PO<sub>4</sub> 0.25, CaCl<sub>2</sub> 2.5, MgCl<sub>2</sub> 1.8, NaHCO<sub>3</sub> 26, glucose 5) which was also pre-warmed to 37°C and gassed with 95% O<sub>2</sub>, 5% CO<sub>2</sub>.

CSF secretion rate was determined within the first hour of perfusion, measured using the erythrocyte concentration technique (Deane & Segal, 1985). Washed sheep erythrocytes were added to Ringer with final haematocrit of 25% (Deane & Segal, 1985). Concentration of erythrocytes in the venous outflow due to water extraction by CSF secretion, produces an arterio-venous difference in haematocrit proportional to the rate of the secretion. CSF secretion rate was calculated as:

$$K_f = F_v (V/A - 1) \text{ ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1} \quad (1)$$

where  $F_v$  is venous perfusion flow rate (ml.g<sup>-1</sup> wet weight), V and A are venous and arterial haematocrit readings respectively (Deane & Segal, 1985).

### 2.4. Functional integrity of blood-CSF barrier

Molecules of varying molecular mass (180 Da – 68 KDa) with radioactive-labels were added to the Ringer perfusate in tracer concentrations for up to 2 hours and their extraction at the blood side of the CPs calculated in order to assess the tightness of the BCSFB. Labeled markers used were <sup>125</sup>I-bovine serum albumin (BSA, MW 68 KDa; specific activity 0.59 KBq.ml<sup>-1</sup>, final concentration in perfusate 11 mg/L), <sup>3</sup>H-polyethylene glycol (PEG) (MW 4 KDa; specific activity 0.59 KBq.ml<sup>-1</sup>, final concentration in perfusate 100 pM) and <sup>14</sup>C-mannitol (MW 180 Da; specific activity 1.48 KBq.ml<sup>-1</sup>, final concentration in perfusate 0.68 μM). Arterial and venous Ringer samples were taken every 5 minutes and tracer activities (disintegrations per minute, DPM) in 100 μl of each sample determined by liquid scintillation counting (LKB Wallac Rackbeta Spectral 1219 counter) after the addition of 3.5 ml liquid scintillation fluid (Ecoscint, National Diagnostics). Extraction was calculated as:

$$\text{Extraction (\%)} = [(F_a A^* - F_v V^*) / F_a A^*] \times 100 \quad (2)$$

1 where  $F_a$  and  $F_v$  were the arterial and venous perfusate flow rates, respectively ( $\text{ml min}^{-1}\text{g}^{-1}$  wet  
2 weight) and  $A^*$  and  $V^*$  were the activity of tracer ( $\text{DPM ml}^{-1}$ ) in arterial and venous effluent,  
3 respectively (Strazielle & Preston, 2003).

#### 5 **2.5. $^{22}\text{Na}$ uptake and efflux in isolated CPs, incubated in vitro**

6 In separate studies, lateral ventricle CPs (approximately 80mg each) were freshly  
7 harvested from brain immediately after decapitation, divided into 10 mg sections and pre-  
8 incubated for 10 min in aCSF at  $37^\circ\text{C}$  gassed with  $95\%\text{O}_2/5\%\text{CO}_2$ . They were then transferred to  
9 1 ml warmed ( $37^\circ\text{C}$ ), gassed aCSF, which contained  $^{22}\text{Na}$  ( $37 \text{ KBq.ml}^{-1}$ ) plus an extracellular  
10 marker ( $^3\text{H}$ -mannitol,  $37 \text{ KBq.ml}^{-1}$ ) for a total of 60 min incubation. Uptake was calculated as an  
11 accumulation ratio:

$$13 \text{ Uptake} = \text{CP dpm.g}^{-1} / \text{CSF dpm.ml}^{-1} \quad (3)$$

14  
15 The net  $^{22}\text{Na}$  uptake was calculated from the difference between  $^{22}\text{Na}$  and  $^3\text{H}$ -mannitol uptake.  
16 The effect of the  $\text{Na}^+$ ,  $\text{K}^+$  ATPase inhibitor ouabain on  $^{22}\text{Na}$  uptake was studied by including  
17 1mM ouabain in pre-incubation and incubation aCSF of one CP, with the contralateral CP acting  
18 as control.

19 Studies of efflux transport from CPs were done after initial loading of CP samples  
20 with  $^{22}\text{Na}$  for 1 hour. Then, CPs were rinsed briefly in saline and placed in 2 ml fresh aCSF  
21 which did not contain radioactivity. Samples of aCSF ( $50 \mu\text{l}$ ) were taken every 10 seconds  
22 thereafter for 2 minutes, as described earlier (Johanson et al., 1990; Preston et al., 1993). The  
23 total radioactivity in CP at time zero was calculated, and a plot made of CP radioactivity on a log  
24 scale versus time. The slope of the line gives the efflux rate constant  $k$  ( $\text{sec}^{-1}$ ). In some  
25 experiments ouabain (1 mM) was added to the contralateral CP in the last 20 minutes of  
26 incubation and to the efflux bath.

#### 28 **2.6. Choroid plexus ATP content and redox activity (MTT) assay**

29 In separate studies, after anesthesia the skull of sheep was reflected, an incision made  
30 into each cerebral hemisphere to the level of the roof of the lateral ventricles and the CPs  
31 harvested. The tissues were immediately frozen in liquid nitrogen and stored for up to 24 hrs.

32 For in vitro analysis of ATP content, CPs were homogenized with 0.5 ml buffer  
33 containing 10% Tween-X and 25% DMSO, and ATP content measured using a commercially  
34 available Luciferin-Luciferase assay kit, Sigma (Preston et al., 1995) .

35 In order to estimate the energy charge of the CPs we measured the ATP, ADP and AMP  
36 content in the CP homogenates by HPLC. Tissues were defrosted in 20% methanol in phosphate  
37 buffered saline (PBS) (v/v), pH 7.3, at  $-5^\circ\text{C}$ , and homogenized with 10% methanol in PBS. Then  
38 0.1ml of 2M 2-hydroxy-5-benzoic acid added to each sample, and samples centrifuged at 10 000

1 g for 10min. Clear supernatant was collected and stored at -20°C. HPLC analysis was performed  
2 on a Hewlett Packard 1100 system with UV detector and binary pump using the conditions  
3 explained previously (Isakovic et al., 2004). Briefly, molecular separation was by gradient  
4 elution on a thermostatted Zorbax SB-C18 reverse phase column (15cm x 4.6 µm, 25°C, flow  
5 rate 1ml/min, injected volume 20µl). The mobile phase consisted of two elution buffers: 0.1mM  
6 KH<sub>2</sub>PO<sub>4</sub>, pH 6.00 containing 8 mM tetrabutylammonium hydrogensulfate (buffer A) against  
7 buffer B (70% (v/v) buffer A, 30% methanol). The UV absorbance was measured at 254 nm with  
8 diode array detector and peaks detected on Hewlett Packard LC Control Software using external  
9 standards consisting of nucleotides AMP, ADP and ATP dissolved in PBS. The concentrations of  
10 these nucleotides were then estimated in the samples, and the energy charge (EC) estimated for  
11 each sample as:

$$12 \quad EC = \frac{[ATP] + 0.5 [ADP]}{[ATP] + [ADP] + [AMP]} \quad (4)$$

13

14 The cellular redox activity was determined using the MTT (3-[4,5-dimethylthiazol-2-yl]-  
15 2,5-diphenyltetrazolium bromide) assay for tissue viability (Preston et al., 1998; Porciuncula et  
16 al., 2003). MTT salt is reduced to form an insoluble formazan dye during both mitochondrial and  
17 cytosolic redox reactions and is indicative of cellular metabolic activity. CPs were incubated in  
18 warmed gassed aCSF containing 0.5 mg.ml<sup>-1</sup> MTT for 20 minutes then transferred into 2 ml  
19 isopropanol for solubilization. Appearance of the resulting dye was measured with a Unicam  
20 spectrophotometer with absorbance set at 490 and 630 nm. The final results were expressed as  
21 absorbance (arbitrary units) U. mg<sup>-1</sup> tissue wet weight.

22

### 23 **2.7. Materials**

24 <sup>14</sup>C-mannitol (2.18 GBq / mmol), <sup>3</sup>H-PEG (74.7 MBq / g) and <sup>22</sup>Na (37 MBq / ml), were  
25 obtained from Perkin Elmer UK. <sup>125</sup>I-BSA (37MBq / mg) was purchased from MP Biomedicals.  
26 All other materials were from Sigma unless stated.

27

### 28 **2.8. Data analysis**

29 All values were expressed as mean ± SEM. ANOVA with Tukey post-hoc analysis, paired or  
30 unpaired t-test were used as appropriate to compare means from different ages of sheep. Values  
31 of p < 0.05 were considered statistically significant.

32

33

34

## 3. Results

35

### 36 **3.1. CSF protein content**

37 The total protein in 24 samples of old sheep CSF was 0.79±0.12 g.l<sup>-1</sup>, which was  
38 significantly higher than in 36 samples of the young sheep CSF, 0.54±0.03 g.l<sup>-1</sup>, (p<0.05 by

1 unpaired t-test), Table 1. However, total plasma protein content did not differ between the two  
2 groups ( $77.12 \pm 6.13$  and  $82.65 \pm 8.13$  g.l<sup>-1</sup> in young and old sheep, respectively,  $p > 0.05$  by  
3 unpaired t-test). The total protein CSF/plasma ratio was estimated for each animal and was  
4 significantly higher in old sheep ( $9.6 \pm 4 \times 10^{-3}$ ), than in young ( $7 \pm 3 \times 10^{-3}$ ,  $p < 0.05$  unpaired t-test).

### 5 6 **3.2. CSF secretion rate**

7 The CSF secretion rate, venous outflow rate, perfusion pressure and preparation  
8 temperature were monitored continuously for the first 30 minutes to ensure stability of the  
9 preparation. CSF secretion rate was then assessed at 10 minute intervals for the next 30 minutes  
10 in order to determine mean secretion rate in 28 preparations. The mean secretion rate for each  
11 preparation was plotted against age and this is presented in Figure 1. The averages for each age  
12 group were  $0.148 \pm 0.013$  ml.min<sup>-1</sup>.g<sup>-1</sup> for young sheep (1-2 years, n=11),  $0.092 \pm 0.02$  ml.min<sup>-1</sup>.g<sup>-1</sup>  
13 for sheep aged 3-6 years (n=7) and  $0.070 \pm 0.013$  ml.min<sup>-1</sup>.g<sup>-1</sup> for sheep aged 7+ years (n=10)  
14 showing a significant age-related decline in middle and old age groups compared to young (slope  
15 of line  $-0.012 \pm 0.003$  and y-intercept  $0.169 \pm 0.03$  ml.min<sup>-1</sup>.g<sup>-1</sup>  $p < 0.05$ ). There was a trend for CP  
16 weights to increase with age, but CSF secretion expressed per CP also showed a significant age  
17 associated decline also from  $0.0109 \pm 0.0012$  ml.min<sup>-1</sup> per CP in young  $0.0079 \pm 0.0010$  ml.min<sup>-1</sup>  
18 per CP in old ( $p < 0.05$ ).

### 19 20 **3.3. Functional integrity of blood-CSF barrier**

21 Choroid plexus epithelial cells are joined together by encircling tight junctions (Redzic and  
22 Segal. 2004), which are concentrated in short lengths of several complex strands close to the CSF  
23 (apical) side. This normally prevents free passage of molecules between the blood and CSF via  
24 the paracellular route between cells, thus forming the BCSFB. To study the functional integrity  
25 of the BCSFB, we measured the extraction (%) of three radiolabelled markers from perfusate and  
26 plotted this against age (Figure 2a), or against log relative molecular mass (Figure 2b). These  
27 molecules of increasing molecular weight were chosen as markers of paracellular permeability  
28 since no specific receptor- or carrier-mediated transport of these molecules exist in mammalian  
29 cells with the exception of albumin which may undergo slower transcytosis. The smallest  
30 compound, mannitol (180 Da) had a steady-state extraction of around 9% in young adult animals  
31 illustrating the small passive leakiness of the blood-CSF barrier. In old sheep extraction was  
32 significantly higher and almost double than in young (17%). Extraction of PEG (4 000 Da) was  
33 smaller than mannitol in young sheep, but again old sheep showed a significant extraction of  
34 more than 15%. Extraction of the largest molecule, BSA (68 000 Da) was close to zero in young  
35 sheep. A very small measurable extraction was seen in old sheep, but a statistically significant  
36 difference could not be resolved compared to young sheep after 2hr steady-state ( $p > 0.05$ ). The  
37 results for middle-aged sheep were not different to those for young sheep for any of the  
38 compounds.

39

### 3.4. $^{22}\text{Na}$ accumulation and efflux

The driving force for the CSF secretion by the CP is activity of  $\text{Na}^+, \text{K}^+, \text{ATPase}$  which uses energy of ATP hydrolysis to generate a unidirectional flux of  $\text{Na}^+$ ,  $\text{Cl}^-$  and  $\text{HCO}_3^-$  across the epithelial layer (Brown et al., 2004). The activity of this enzyme, located at the apical side of the epithelium, generates a gradient for  $\text{Na}^+$  entry into the epithelial cells via Na ion transporters located at mainly at the basolateral side, facing the CP interstitial fluid. In our study, pieces of CP were incubated in the aCSF and initially the apical side of the epithelium was exposed to  $^{22}\text{Na}$ . However, since the experiment lasted for 1h, the tracers would be expected to diffuse and reach CP interstitial fluid at basolateral side; therefore, uptake data also reflects processes which take place at the basolateral side of the CP epithelium.

$\text{Na}^+$  efflux from CP epithelium takes place across the apical CSF side mediated by the  $\text{Na}^+ - \text{K}^+ - 2\text{Cl}^-$  co-transporter and by the action of the  $\text{Na}^+, \text{K}^+, \text{-ATPase}$ , which unlike other epithelia is also located at the apical side (Redzic and Segal, 2004). The  $^{22}\text{Na}$  accumulation into CP was significantly lower after 1 hour in old compared to young sheep (Figure 3a). Addition of 1 mM ouabain to inhibit  $\text{Na}^+, \text{K}^+, \text{-ATPase}$ , increased  $^{22}\text{Na}$  accumulation in young tissue ( $p < 0.05$  vs. control) as expected if cellular efflux was inhibited, but there was no significant increase in old CPs ( $p > 0.05$  vs. control). The control rate of  $^{22}\text{Na}$  efflux from old CPs was significantly lower than in young (Figure 3b). Ouabain did not affect this efflux from old CPs but did reduce efflux rate from young tissue by almost 1/3. compared to control.

### 3.5. ATP content and redox activity (MTT) assay

Our data from the MTT assay indicated that redox activity in the CPs from old animals was significantly lower than in young and middle aged animals (Table 2) indicating attenuated cytoplasmic glycolysis and/or mitochondrial function. However, the in vitro ATP content using the Luciferin-Luciferase assay did not show any difference between young and old tissues (Table 2) indicating sufficient baseline energy production. ATP content and energy charge of the tissue was also estimated using HPLC A typical chromatogram of the standard (Figure 4A) and aqueous extracts from young (Figure 4B) and old (Figure 4C) CPs is shown in Figure. 4. All CP samples revealed clear peaks of AMP, ADP and ATP, but peaks for nucleosides could not be detected, meaning that concentrations of free nucleosides were below the threshold for detection ( $\bullet 400 \text{ nM}$ ). The AMP retention time was 9.5-9.8 min and retention times for ADP and ATP were very close, 14.4 and 14.8 min, respectively. The ATP content did not change with age and was  $6.81 \pm 0.61 \mu\text{g} \cdot \text{mg}^{-1}$  protein in young ( $n=4$ ) and  $7.67 \pm 0.20 \mu\text{g} \cdot \text{mg}^{-1}$  protein in old sheep, ( $n=4$ ),  $p > 0.05$ , confirming the findings from the in vitro assay. The estimated energy charge was also not different between groups ( $0.711 \pm 0.10$  in young and  $0.721 \pm 0.04$  in old sheep,  $p > 0.05$ ).

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#### 4. Discussion

This study focused on age-related changes in choroid plexus function and integrity of the isolated BCSFB, which has received less attention than the blood brain barrier (BBB). Four significant alterations in the old sheep's CP-CSF system were found in this study: a) increased protein content in CSF; b) increased paracellular permeability of BCSFB to extracellular space markers, mannitol and PEG; c) decreased CSF secretion rate; d) reduced  $\text{Na}^+$  accumulation and efflux by the CP..

The observation of increase in protein concentration in old ovine CSF in this study is consistent with human studies, where elevation of protein in CSF was found in healthy old populations (Garton et al., 1991) starting at age 40 (Muller et al., 1954), and in patients with neurological diseases (Reiber, 2003). The increase in total protein content could reflect either addition of proteins from adjacent nervous tissue, or addition from the circulation across a 'leaky' BCSFB. However the increase in total protein content of CSF also correlates with decreased CSF secretion (Reiber, 2001, 2003) and CSF flow rate influences the CSF protein concentration (May et al., 1990).

It is widely agreed that 60-90% of the total CSF volume is formed by the CPs. The rest most likely originates from the brain interstitial fluid bulk flow towards the CSF, which has an estimated flow rate of 100–300 and 100-150  $\text{nL min}^{-1} \text{g}^{-1}$  in rat and rabbit, respectively (Cserr et al., 1981; Redzic & Segal, 2004). As mentioned above, several previous measurements of total CSF production have produced conflicting results. However, it should be noted that Masserman's technique could be affected by a number of factors other than the rate of the CSF production, such as compliance of the ventricular system (Davson et al., 1963), which might be affected by aging. More sophisticated studies in humans, which used magnetic resonance imaging phase mapping, also produced conflicting results (Gideon et al., 1994, Stoquart-EISankari et al 2007); the results obtained in our study are the first ones in aged animals which report the rate of the CSF secretion by the CP in isolation, without any interference by other factors. Since an alteration in CP blood flow rate due to age may affect CSF secretion rate (Zheng et al., 2003), we held the perfusion flow rate constant as far as possible, averaging  $3.9 \pm 0.6 \text{ ml.min}^{-1} \text{.g}^{-1}$  in young ( $n=7$ ) and  $4.2 \pm 0.6 \text{ ml.min}^{-1} \text{.g}^{-1}$  in old sheep ( $n=5$ ). Under these conditions, we detected a consistent and significant reduction in CSF secretion in old animals of approximately 50% compared to young adult sheep, and similar to the situation seen previously in old rats (Preston, 2001).

The implications of reduced CSF secretion are that turnover of the bulk ventricular CSF would be slowed. Additional age-related changes in the CSF dynamics are increased CSF volume in aged humans and rats (Foundas et al., 1998; Preston, 2001; Silverberg et al., 2001; Silverberg et al., 2003) and increased resistance to CSF drainage in aged humans (Albeck et al., 1998). A reduction of CSF turnover may affect protein concentration in the CSF and reduce the CSF /

1 brain ISF concentration gradient (which is normally  $<0.1$  for a number of compounds (Davson et  
2 al., 1963; Abbott, 2004) which impairs the “sink” action of the CSF for drainage of brain ISF.  
3 Silverberg and colleagues (Silverberg et al., 2001; Silverberg et al., 2003) have investigated the  
4 use of ventricular shunts to increase CSF turnover in patients with mild to moderate Alzheimer’s  
5 disease, and have found stabilization of cognitive decline in one trial.

6 The clinical interpretation of increased CSF protein from blood origin, is not however  
7 confined to reduced CSF flow, but has been interpreted as increased barrier permeability to  
8 plasma proteins as a result of increased ‘leakiness’ of the barrier (Kleine et al., 1993; Tumani et  
9 al., 1998; Farrall and Wardlaw 2007). In our study, this was assessed by the steady-state CP  
10 extraction of three radiolabeled lipid insoluble compounds with molecular weight ranging from  
11 180 Da to 68 KDa. The steady-state method was chosen since the only factors affecting  
12 extraction in these conditions are the rate at which the compound is perfused (which was  
13 constant for all ages) and the rate at which it leaves the CP, but is unaffected by any differences  
14 in extracellular volume between CPs. There are contradictory findings surrounding changes in  
15 CP interstitial fluid volume with aging but in rat and humans no volume change is seen, although  
16 accumulations of lymphoid cells and collagen deposits are noted (Serot et al 2001, Jovanovic et  
17 al., 2007, Shuangshoti and Netsky 1970). The compounds chosen are essentially neither  
18 metabolized by the CP epithelium nor do specific transport sites / receptors exist at this  
19 epithelium although plasma proteins such as albumin can be transcytosed by the developing CP  
20 (Johansson et al 2008). Therefore, their extraction from the basolateral (vascular) space should  
21 be by simple diffusion via the paracellular spaces. Simple diffusion across the cellular layer  
22 could be limited by both paracellular diffusion rates (in which case the rate of diffusion across  
23 that layer is inversely related to the molecular weight) and by size of cellular pores or gaps (in  
24 which case the rate of diffusion shows a typical “cut-off” effect). Our results revealed that the  
25 BCSFB in aged sheep is leakier than in young adult sheep only in regard to the low and medium  
26 molecular weight compounds. There was a linear correlation of extraction versus log molecular  
27 weight in young animals (Figure 2), indicating a diffusion-coefficient related restriction.  
28 However, in old animals the lack of linearity also suggested a cut-off effect for albumin.  
29 Permeability studies on the aging BBB, suggest similar findings. No change (Wadhvani et al.,  
30 1991) or small region specific increases in sucrose permeability were seen with aging in rats  
31 (Rapoport et al., 1979; Goldman et al., 1992) and no change in permeability to horseradish  
32 peroxidase or albumin in senescent mice (Rudick & Buell, 1983) or to fluorescein  
33 isothiocyanate-labelled dextran in senescent rats (Buchweitz-Milton & Weiss, 1987).

34 The generally accepted view of CSF secretion mechanisms by the CP is that ion transport  
35 is fundamental to secretion of fluid (Brown et al 2004). The driving force of these processes is  
36 the activity of  $\text{Na}^+$ ,  $\text{K}^+$  ATPase. This enzyme is located at the apical face and uses the energy of  
37 ATP hydrolysis to pump  $2\text{K}^+$  into and  $3\text{Na}^+$  out of the cell against their concentration gradients.  
38 In addition, intracellular accumulation of  $\text{Cl}^-$  (in exchange for  $\text{HCO}_3^-$  at the basolateral face)  
39 generates a concentration gradient, which drives movement of  $\text{Cl}^-$ ,  $\text{Na}^+$  and  $\text{K}^+$  across the apical

1 side into CSF by the  $\text{Na}^+-\text{K}^+-2\text{Cl}^-$  co-transporter (Wu et al., 1998). Ultimately, the unidirectional  
2 flux of  $\text{Na}^+$  and  $\text{Cl}^-$  from basolateral (blood) into apical (CSF) space generates an osmotic  
3 gradient accompanied by the movement of water across the epithelial layer (Speake et al., 2001,  
4 Redzic and Segal, 2004). In our study, in addition to the reduction in CSF secretion in old sheep,  
5 we found that the accumulation of  $^{22}\text{Na}^+$  was significantly lower in old sheep when compared to  
6 young (Figure 3a). That might reflect either altered distribution of  $\text{Na}^+/\text{H}^+$  exchangers or different  
7 activities / properties of with age., Masseguin et al (2005) have shown reduced  $\text{Na}^+$ ,  $\text{K}^+$  ATPase  
8 protein and mRNA expression in the lateral and fourth ventricle CPs from aged rats along with  
9 reduced water channel Aquaporin 1 and carbonic anhydrase II, all of which could contribute to  
10 reduced fluid secretion. To explore Na transport functionally, we inhibited  $\text{Na}^+$ ,  $\text{K}^+$  ATPase by  
11 ouabain, and the accumulation of  $^{22}\text{Na}^+$  significantly increased in young sheep, which was to be  
12 expected. In contrast, ouabain did not increase accumulation of  $^{22}\text{Na}^+$  in old sheep CP, further  
13 implicating a functional deficiency of  $\text{Na}^+$ ,  $\text{K}^+$  ATPase activity with age (Figure 3a).

14 Since efflux of  $\text{Na}^+$  from the CP mainly depends on  $\text{Na}^+$ ,  $\text{K}^+$  ATPase activity we then  
15 measured  $^{22}\text{Na}^+$  efflux and found that the baseline rate was significantly lower in old compared to  
16 young sheep CPs (Fig 3b). Ouabain did not significantly inhibit  $^{22}\text{Na}^+$  efflux in old CPs, but did  
17 reduce efflux from young, confirming an age-related decline in  $\text{Na}^+$ ,  $\text{K}^+$  ATPase activity At the  
18 present time it not straight forward to investigate whether this change in activity can be attributed  
19 to a change in  $\text{Na}^+$ ,  $\text{K}^+$  ATPase content in our model since current antibodies for identifying the  
20 protein are not sufficiently specific for the sheep. However correlations between  $\text{Na}^+$ ,  $\text{K}^+$  ATPase  
21 function and expression (transcript and protein) would be useful to further our understanding.

22 Activity of  $\text{Na}^+$ ,  $\text{K}^+$  ATPase is highly energy dependent, adequate energy availability in  
23 the form of ATP is essential to maintain CSF secretion. The chronological accumulation of  
24 mitochondrial dysfunction has been proposed as a potential mechanism in the physiological  
25 processes of aging. Cottrell et al., (2001) have shown an age-related increase in cytochrome c  
26 oxidase-deficient cells in both hippocampal pyramidal neurons and choroid plexus epithelial  
27 cells in rat indicating mitochondrial deficiency. The MTT assay in this study demonstrated a  
28 decline in CP cell redox potential between mid and old aged groups (Table 2); this assay is  
29 considered to reflect indirectly activity of mitochondrial dehydrogenases, so it is consistent with  
30 the findings in the previous study (Cottrell et al., 2001). However the MTT method is not  
31 specific for mitochondrial activity and will reflect cytosolic MTT reduction, for example during  
32 glycolysis (Berridge and Tan 1993, Takahashi et al 2002). Initial MTT uptake by cells also  
33 requires adequate endocytosis of the compound, and any age related changes here may contribute  
34 to the age-related differences. By comparison, two methods for ATP assessment showed no  
35 change in total ATP content or energy charge, and the values obtained were close to those for the  
36 rat brain (Delaney & Geiger, 1996). This suggests there is sufficient reserve capacity in the CP to  
37 maintain ATP production under basal conditions and it is therefore unlikely that lack of ATP  
38 could explain the reduction in CSF secretion rate in this model.

1            In summary, this study has described age-related changes in the CP function and CSF  
2 composition which are likely to be interlinked: impaired Na<sup>+</sup> accumulation and efflux would  
3 impact on CSF secretion, which was reduced with age. The resulting stagnation of CSF flow  
4 would contribute to elevation of CSF proteins as seen by the 37% increase in CSF/plasma ratio  
5 for total protein. Increasing permeability of the barrier to small/mid sized molecules would  
6 further elevate molecular accumulation in CSF of the oldest age group. These alterations  
7 underpin a decline in key functions of the CP which would impact on turnover and homeostasis  
8 of the CSF in later life.

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2

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2 **Table 1.** CSF and plasma total protein concentration in sheep.

Age	n	CSF Total protein (mg.ml <sup>-1</sup> )	Plasma Total protein (mg.ml <sup>-1</sup> )	CSF/plasma ratio
Young group	36	0.54 ± 0.03	77.12 ± 6.13	0.0070± 0.003
Old group	24	0.79 ± 0.12*	82.65 ± 8.13	0.0096± 0.004*

3

4 Data are mean ± SEM. \* p&lt;0.05, unpaired t-test.

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2 **Table 2.** ATP content and MTT assay, sheep choroid plexus.

3

Age	MTT assay (absorbance U.mg <sup>-1</sup> )	n	ATP (μmol.g <sup>-1</sup> )	n
Young group	0.090 ± 0.009	12	0.34 ± 0.04	8
Middle age group	0.110 ± 0.020	3	0.44 ± 0.04	3
Old group	0.046 ± 0.005*	11	0.41 ± 0.06	6

4

5 Data are expressed as mean ± SEM. \* p&lt;0.05 ANOVA.

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2 **Figure Legends**

3

4 **Figure 1.** CSF secretion rates in different ages of sheep. Each point represents data from one  
5 sheep. The slope of the line is  $-0.122 \pm 0.0026$ , ( $p < 0.001$ , linear regression).

6

7 **Figure 2.** The extraction of radiolabelled mannitol, PEG (polyethylene glycol 4000), or BSA  
8 (bovine serum albumin) from the perfusate in different ages of sheep. **(A).** The extraction (%) of  
9 plotted against mean age for each group. **(B).** Extraction (%) in each age group, plotted against  
10 log molecular weight (MW). Values are mean  $\pm$  SEM. \* $p < 0.05$  difference in mannitol extraction  
11 by age (old compared to young and middle aged); † $p < 0.05$  difference in PEG extraction by age  
12 (old compared to young and middle aged).

13

14 **Figure 3.** **(A).** Net  $^{22}\text{Na}$  uptake (after  $^3\text{H}$ -mannitol correction) in the absence (control) or  
15 presence of 1mM ouabain in young and old sheep CP. **(B).**  $^{22}\text{Na}$  efflux rate constants ( $k$ ,  $\text{sec}^{-1}$ ) in  
16 the absence (control) or presence of 1mM ouabain in young and old sheep. Values are mean  $\pm$   
17 SEM of 4-5 measurements. \*  $p < 0.05$  difference from control using paired t-test; † $p < 0.05$   
18 difference by age using unpaired t-test.

19

20 **Figure 4.** Typical HPLC analysis of ATP content in choroid plexuses from old and young sheep.  
21 **(A).** AMP, ADP and ATP standards. **(B).** Representative analysis of a young CP. **(C).**  
22 Representative analysis of an old CP.

Figure 1

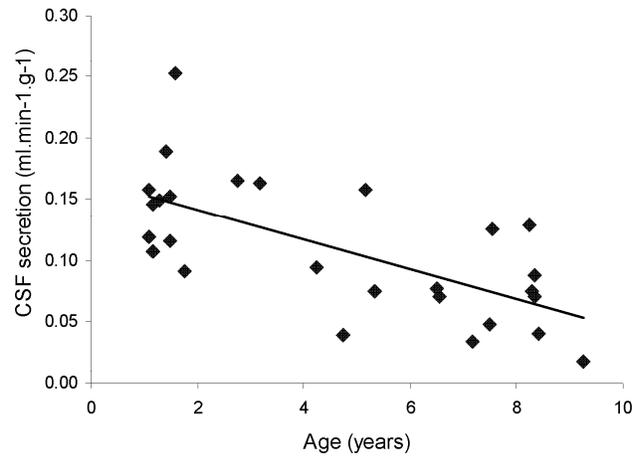
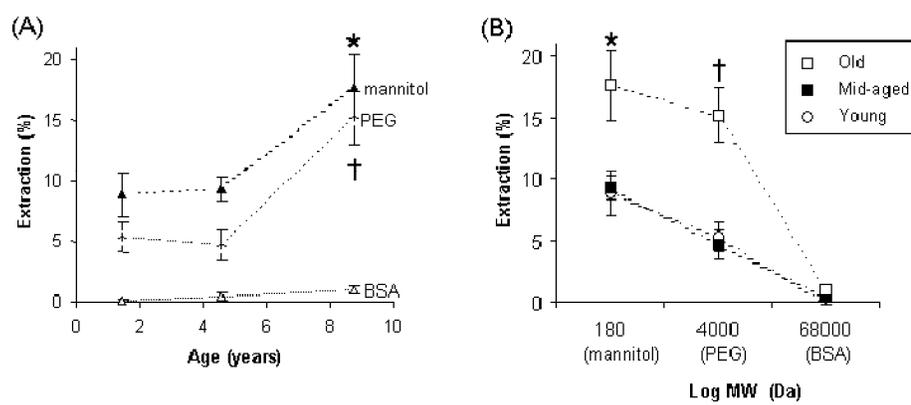


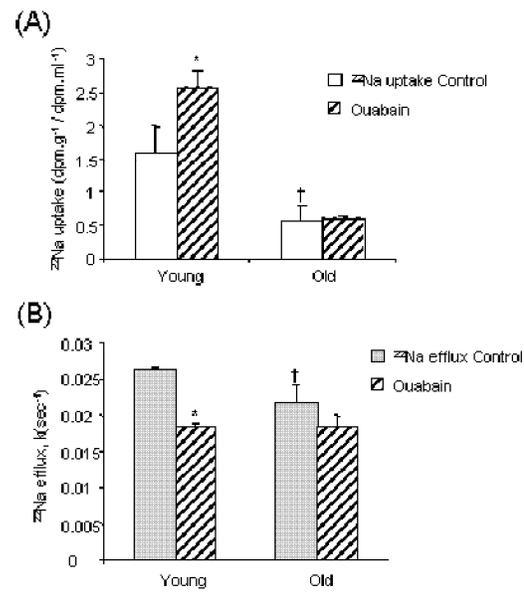
Figure 2



1

A

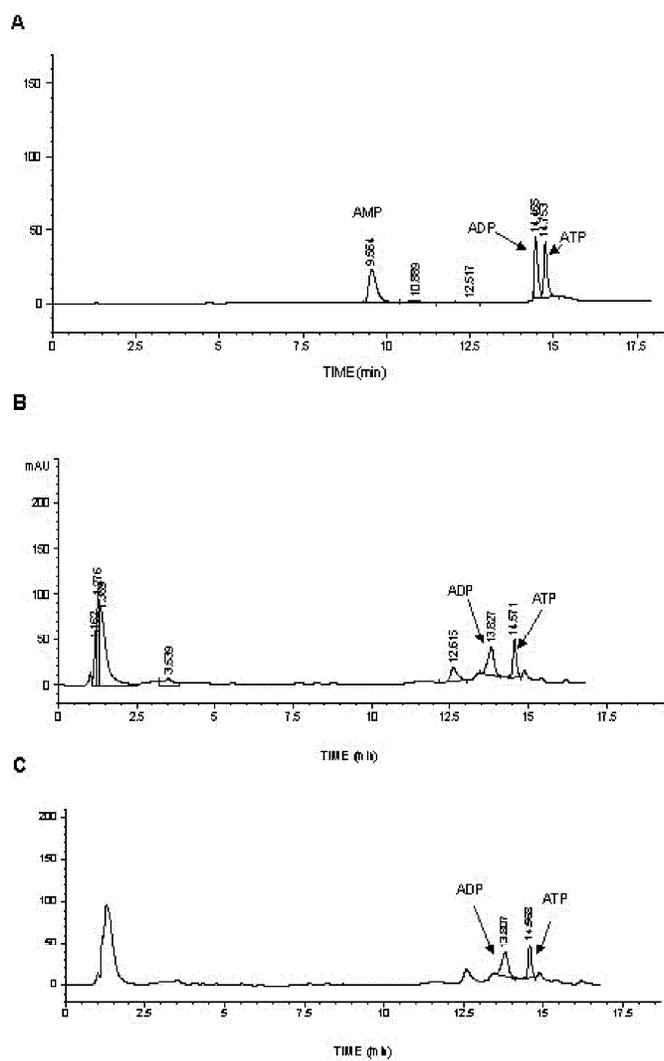
Figure 3



1

A

Figure 4



1

A