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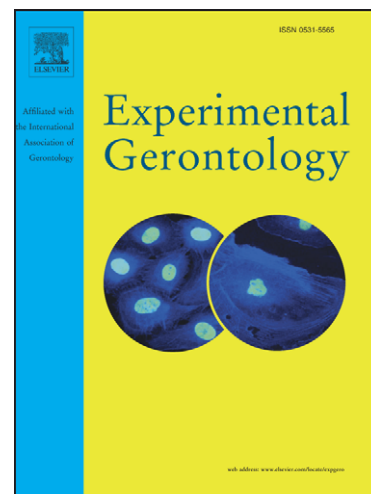
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Age-related changes in choroid plexus and blood-cerebrospinal fluid barrier function in the sheep.

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Abstract

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

1. Introduction

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

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

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

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

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

2. Materials and Methods

2.1. Surgery

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

2.2. CSF protein analysis

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

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, NaH₂PO₄ 1.22, CaCl₂ 2.4, MgSO₄ 1.22, NaHCO₃ 25, glucose 5) containing 4% bovine serum albumin to maintain colloid osmotic pressure. The buffer was warmed to 37°C and gassed with 95% O₂ / 5% CO₂. 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, NaH₂PO₄ 0.25, CaCl₂ 2.5, MgCl₂ 1.8, NaHCO₃ 26, glucose 5) which was also pre-warmed to 37°C and gassed with 95% O₂, 5% CO₂.

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:

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

where F_v is venous perfusion flow rate (ml.g⁻¹ 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 ¹²⁵I-bovine serum albumin (BSA, MW 68 KDa; specific activity 0.59 KBq.ml⁻¹, final concentration in perfusate 11 mg/L), ³H-polyethylene glycol (PEG) (MW 4 KDa; specific activity 0.59 KBq.ml⁻¹, final concentration in perfusate 100 pM) and ¹⁴C-mannitol (MW 180 Da; specific activity 1.48 KBq.ml⁻¹, 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:

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

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

2.5. ^{22}Na uptake and efflux in isolated CPs, incubated in vitro

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

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

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

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

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

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

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

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

g for 10min. Clear supernatant was collected and stored at -20°C. HPLC analysis was performed on a Hewlett Packard 1100 system with UV detector and binary pump using the conditions explained previously (Isakovic et al., 2004). Briefly, molecular separation was by gradient elution on a thermostatted Zorbax SB-C18 reverse phase column (15cm x 4.6 µm, 25°C, flow rate 1ml/min, injected volume 20µl). The mobile phase consisted of two elution buffers: 0.1mM KH₂PO₄, pH 6.00 containing 8 mM tetrabutylammonium hydrogensulfate (buffer A) against buffer B (70% (v/v) buffer A, 30% methanol). The UV absorbance was measured at 254 nm with diode array detector and peaks detected on Hewlett Packard LC Control Software using external standards consisting of nucleotides AMP, ADP and ATP dissolved in PBS. The concentrations of these nucleotides were then estimated in the samples, and the energy charge (EC) estimated for each sample as:

$$EC = ([ATP] + 0.5 [ADP]) / ([ATP] + [ADP] + [AMP]) \quad (4)$$

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

2.7. Materials

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

2.8. Data analysis

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

3. Results

3.1. CSF protein content

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

unpaired t-test), Table 1. However, total plasma protein content did not differ between the two groups (77.12 ± 6.13 and 82.65 ± 8.13 g.l⁻¹ in young and old sheep, respectively, $p > 0.05$ by unpaired t-test). The total protein CSF/plasma ratio was estimated for each animal and was 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).

3.2. CSF secretion rate

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

3.3. Functional integrity of blood-CSF barrier

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

3.4. ^{22}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 Na^+ , Cl^- and 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 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}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.

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}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}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}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 ± 0.10 in young and 0.721 ± 0.04 in old sheep, $p > 0.05$).

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 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-ElSankari 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 (Wadhwani 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 Na^+ , K^+ ATPase. This enzyme is located at the apical face and uses the energy of
 37 ATP hydrolysis to pump 2K^+ into and 3Na^+ out of the cell against their concentration gradients.
 38 In addition, intracellular accumulation of Cl^- (in exchange for HCO_3^- at the basolateral face)
 39 generates a concentration gradient, which drives movement of Cl^- , Na^+ and K^+ across the apical

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

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

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

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

Age	n	CSF Total protein (mg.ml ⁻¹)	Plasma Total protein (mg.ml ⁻¹)	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*

Data are mean ± SEM. * p<0.05, unpaired t-test.

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

Age	MTT assay (absorbance U.mg ⁻¹)	n	ATP (μmol.g ⁻¹)	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

Data are expressed as mean ± SEM. * p<0.05 ANOVA.

Figure Legends

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

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

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

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

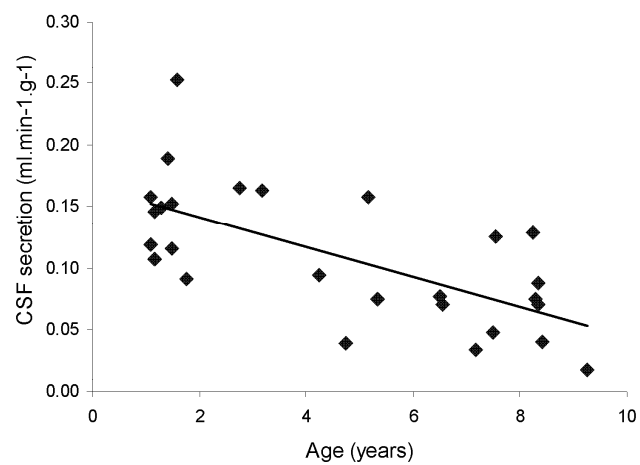
Figure 1

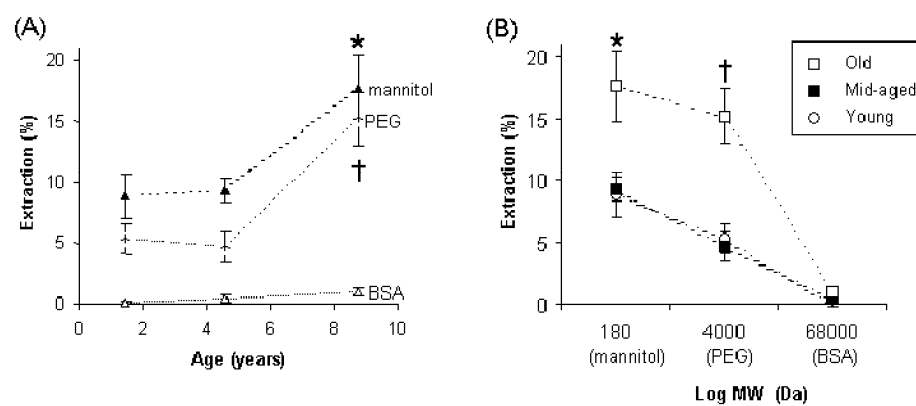
Figure 2

Figure 3

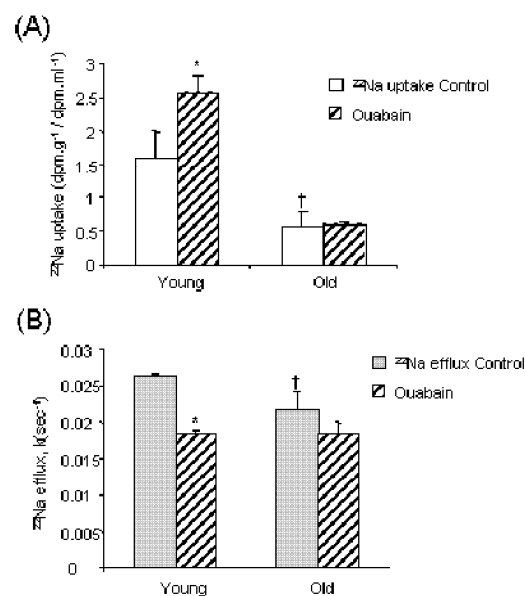


Figure 4

