Delayed astrocytic contact with cerebral blood vessels in FGF-2 deficient mice does not compromise permeability properties at the developing blood-brain barrier

Norman R. Saunders¹, Katarzyna M. Dziegielewska¹, Klaus Unsicker^{2#}, C. Joakim Ek^{3#*}

1. Department of Pharmacology and Therapeutics, University of Melbourne, Australia

2. Institute of Anatomy and Cell Biology, Department of Molecular Embryology, University of Freiburg, Germany

3. Institute for Neuroscience and Physiology, Department of Physiology, Sahlgrenska Academy, University of Gothenburg, 40530 Gothenburg, Sweden

#Shared senior authorship

*Corresponding author:

Dr Joakim Ek

Department of Neuroscience and Physiology, Sahlgrenska Academy

University of Gothenburg, Sweden

Phone: +46 31 7863875

Internet mail: joakim.ek@neuro.gu.se

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ABSTRACT

The brain functions within a specialised environment tightly controlled by brain barrier mechanisms. Understanding the regulation of barrier formation is important for understanding brain development and may also lead to finding new ways to deliver pharmacotherapies to the brain; access of many potentially promising drugs is severely hindered by these barrier mechanisms. The cellular composition of the neurovascular unit of the blood-brain barrier proper and their effects on regulation of its function are beginning to be understood. One hallmark of the neurovascular unit in the adult is the astroglial foot processes that tightly surround cerebral blood vessels. However their role in barrier formation is still unclear. In this study we examined barrier function in newborn, juvenile and adult mice lacking fibroblast growth factor-2 (FGF-2), which has been shown to result in altered astroglial differentiation during development. We show that during development of FGF-2 deficient mice the astroglial contacts with cerebral blood vessels are delayed compared to wild-type animals. However, this delay did not result in changes to the permeability properties of the blood brain barrier as assessed by exclusion of either small or larger sized molecules at this interface. In addition cerebral vessels were positive for tight-junction proteins and we observed no difference in the ultrastructure of the tight-junctions. The results indicate that the direct contact of astroglia processes to cerebral blood vessels is not necessary for either the formation of the tight-junctions or for basic permeability properties and function of the blood-brain barrier.

Keywords: FGF-2, Astrocyte, Perivascular endfeet, Blood-brain barrier, Tight-junction

INTRODUCTION

The blood-brain barrier is the general term used to describe an array of barrier systems that regulate the exchange of solutes between the blood and the central nervous system. Well-maintained and functional barrier mechanisms are essential for normal brain homeostasis; however, in pathological conditions they present a complex obstacle for delivery of many potentially beneficial therapeutics (Habgood and Ek, 2010). Being able to manipulate and precisely regulate blood-brain barrier function would therefore be a key to improving applications of novel neuropharmacotherapies. Studies of blood-brain barrier development have provided much understanding of its induction and regulation (Daneman et al., 2010; Saunders et al., 2012; Bauer et al., 2014). In recent years new insights into the signalling systems that are involved in blood-brain barrier formation and regulation have been obtained.

Pericytes have been shown to be crucial for aspects of normal blood-brain barrier development (Armulik et al., 2010; Daneman et al., 2010) and astrocytes are thought to be able regulate and maintain blood-brain barrier function by the secretory factor sonic hedgehog (Alvarez et al., 2011). However, the development of astrocytic end-feet at the neurovascular unit and their influence on permeability properties of the blood-brain barrier is still unclear. To further this understanding we have made use of FGF-2 deficient mice that previously have been shown to have dysregulation of astroglial differentiation. In earlier studies using both single and double FGF-2/FGF-5 mutants it was shown that FGF-2 is a major regulator of glial fibrillary acidic protein (GFAP) in astrocytes (Reuss et al., 2003; Irmady et al., 2011). In addition, double knockout animals showed a disruption of the blood-brain barrier but the formation of astrocytic end-feet was not examined in these animals. In the present study we can confirm the previous observation showing an altered differentiation of astrocytes during development in FGF-2 deficient mice. Using electron microscopy as well as immunohistochemistry we show that in the FGF-2 deficient mice contacts between the astrocytic end-feet and the endothelial cells at the neurovascular unit develop later than in wild-type animals. However, this developmental delay did not result in any change in the presence of tight-junctional proteins claudin-5 and ZO-1 in the endothelial cells of the brain barrier nor in any change in its permeability properties. These results show that in the developing brain the astrocytic end-feet are not necessary for the formation of tight-junctions that restrict intercellular passage of even small solutes across the blood-brain barrier interface.

METHODS

Animals

FGF-2 null single knock-out mice used for the present study were of the strain generated by Dono et al. (Dono et al., 1998). Wild-type littermates (+/+) were used as control animals. Animals were maintained in accordance with institutional guidelines for animal care.

Immunohistochemistry

Wild-type and FGF-2 null mice at P1, P20 and Adults (n=5–6 per group; mixed litters) were terminally anaesthetised with inhaled halothane, perfuse-fixed using a syringe pump via the heart with cold heparinised phosphate buffered saline for 2 min (pH7.3) followed by 10 min perfusion of cold 4% paraformaldehyde in phosphate buffer (0.1M, pH 7.3). Flow rate was set to 0.08 mL/min/gram animal to minimise risk of damage to blood vessels. Brain tissue was

further fixed for 24 hours in Bouin's fixative. Tissue was embedded in paraffin and 5 μ m coronal sections were cut and mounted on glass slides. For immunohistochemistry, tissue sections were dewaxed, cleared with Histolene (Fronine, Australia) and rehydrated through decreasing concentrations of ethanol. For labelling using DAB method, selected sections were first blocked with Peroxidase and Protein Blockers (DAKO) for 2 hours each and incubated in the primary antibodies (see below) overnight at 4°C. Primary antibodies were: rabbit anti rat plasma proteins (1:2000, Sigma), which were used to assess permeability of the blood-brain barrier to endogenous plasma proteins (Stolp et al., 2005), rabbit anti-glial fibrillary acidic protein (GFAP, 1:200, DAKO) to measure astrocytes densities and mouse anti Claudin-5 (1:100, Chemicon) as well as rabbit anti ZO-1 (1:100, Invitrogen) to assess endothelial tight-junctions. Secondary antibodies were swine anti-rabbit (1:200, DAKO) or rabbit anti-mouse (1:200, DAKO) and tertiary antibodies were rabbit-PAP (1:200, Sigma) or mouse-PAP (1:200, DAKO) as appropriate. All sections were developed with the DAB (DAB+ kit, DAKO). Control sections were treated in the same way except that the primary antibody was omitted and remained blank. The anatomical relationship between astrocytes and cerebral blood vessels was studied. For this, sections were dewaxed, treated with Protein Blocker (DAKO) for 2 hours and incubated in a mixture of rabbit anti-Glial fibrillary acidic protein (1:150, DAKO) and biotinylated Griffonia (bandeiraea) simplicifolia lectin 1 isolectin B4 (GSA, 1:200, Vector Laboratories). After washing, sections were incubated in a mixture of swine anti-rabbit conjugated with TRITC (1:50, DAKO) and streptavidin-FITC (1:40, DAKO). Additionally, sections were incubated with mixture of rabbit anti-ALDH1L1 (1:10,000, Abcam) and goat anti-CD31 (1:200; R&D Systems) followed by incubation with Alexafluor-488/594 conjugated antibodies against appropriate species (1:1000; Vector). This also included an antigen retrieval step by 10min gentle boiling of sections in citrate buffer (pH6). Claudin-5 immunoreactivity was further examined. Sections were pre-processed as above and incubated with mouse anti-claudin-5 (1:100) mixed with tomato-lectin (2mg/mL; Vector Laboratories, Australia) overnight. Sections were then incubated with alexafluor-594 conjugated donkey anti-mouse antibodies (1:500) together with alexafluor-488 conjugated streptavidin (1:500). All sections were examined with an Olympus BX-50 fluorescence or a Zeiss LSM700 confocal microscope.

Assessment of GFAP immunoreactivity

Photographs were taken with an Olympus BX-50 microscope at x20 magnification of each immunostained section. Measurements of immunoreactivity were carried out on three tissue

sections: from the middle of the corpus callosum and one at 100 μ m at either side. Using ImagePro plus the corpus callosum was outlined and automatic dark object recognition used to outline GFAP positive areas of the sections and the percentage GFAP positive area in the corpus callosum was calculated for each section. The average of the three sections was calculated for each animal (n=5 animals at each age group).

Electron microscopic examination

Brain tissue from FGF-2 null and wild-type mice at P1 and adulthood (n=3-4 for each group; mixed litters) was prepared for electron microscopic examination. Animals were killed with inhalation of isoflurane (>10%) and were immediately perfused through the aorta with cold heparinised phosphate buffered saline (0.1M, pH 7.3) for 90 seconds and then for 10 min with a mixture of paraformaldehyde and glutaraldehyde (1.5% each) in 0.1M phosphate buffer (pH 7.3) using a syringe pump as above and left in fixative overnight at 4°C. Tissue was rinsed in 0.1M cacodylate buffer, treated with 2% osmium for 1 hour followed by uranyl acetate for 30 min, dehydrated in increasing concentrations of ethanol and embedded in Epon. Ultrathin sections were cut from grey matter in cortex using a Reichart ultramicrotome, contrasted with uranyl acetate and lead citrate and examined and photographed under a Phillips CM10 electron microscope. The examination was focused on cortical blood vessels and the morphology of the neurovascular unit including the endothelial tight junctions. In addition, the coverage of astrocytic end-feet around the cortical blood vessels was estimated with astroglial processes discerned on the criteria outlined by Caley and Kruger (1965) as well as Caley and Maxwell (1970). From each animal 20-30 capillaries from at least two different areas of the cortex were examined in detail under the microscope and each vessel was given a number by the examiner on a scale between 0-10 based on the proportion of the blood vessel that was surrounded by astrocytic processes (1=10%, 2=20% up to 10=100%). The examiner was blinded to the nature of the tissue.

Measurements of blood-brain barrier function

Blood-brain barrier permeability to small water-soluble molecules was measured using radiolabelled sucrose (342Da). Permeability to plasma proteins was assessed immunocytochemically, see above. Results from FGF-2 null animals were compared with wild-type animals.

Sucrose permeability experiments were conducted in mice at P1, P20 and on adults. Animals received an intraperitoneal injection of 2 μCi ^{14}C -sucrose (Amersham) in sterile saline (6 μL injection per gram body weight). Experiments were conducted in un-anaesthetised animals. Three or 30 minutes after the injection, animals ($n=5-7$ per group; mixed litters) were terminally anaesthetised with inhaled halothane ($>10\%$), samples of blood were collected by cardiac puncture and centrifuged to separate plasma. We did not serially sample blood in animals due to the small size of P1 and P20 animals and to keep a standard protocol across all ages. The brain was removed from the skull and divided into cortex, deeper grey matter (striatum/thalamus), cerebellum and hindbrain. All samples were weighed. Brain samples were frozen overnight and then dissolved using Soluene-350 (Packard, USA). The radioactivity in each sample was determined by scintillation counting and expressed as dpm/mg sample. The 3 min brain/plasma concentration ratios were used to determine the amount of trapped blood in brain regions since it can be expected that in this short time the marker only distributes within the vascular space (Ek et al., 2001). Data are presented as brain/plasma concentration ratios for 30 min exposures to ^{14}C -sucrose and are corrected for residual vascular blood space yielding true concentration in brain parenchyma (Ek et al., 2001).

Statistics

Data were analyzed using unpaired two-tailed t-tests using SPSS software ($\alpha=0.05$).

RESULTS

Astrocytes and astrocytic foot processes

Estimation of immunoreactivity of GFAP was carried out on tissue sections from P1 and P60 animals of both groups (Fig. 1A). At P1 significant staining of astrocytes in wild-type mice was detectable in the corpus callosum while other regions of the brain showed faint sporadic staining. Immunoreactivity in FGF-2 null P1 brains was much weaker or not detectable (Fig. 1B and Suppl. Fig. 1 for lower power images). By P60 positive immunoreactivity for GFAP was clearly visible in both groups of animals. Positive immunoreactivity in corpus callosum was quantified using automatic detection of dark regions (immunopositive) with Image ProPlus software and calculated as a ratio to the whole corpus callosum. The proportion of the corpus callosum stained in P1 animals was significantly higher in wild-types compared to FGF-2 null mice ($2.52 \pm 0.4\%$ and $0.04 \pm 0.02\%$, respectively, $p < 0.001$). In contrast to adult

(P60) where the proportion area stained was similar in wild-type and FGF-2 null animals ($2.68 \pm 0.52\%$ and $2.27 \pm 0.34\%$, respectively, $p > 0.05$).

To visualise both astrocytes and blood vessels in tissue sections, double labelling was carried out using anti-GFAP antibodies/GSA-lectin or anti-ADLH1L1/CD31 antibodies. In P1 wild-type animals both GFAP and GSA-lectin were detectable in the corpus callosum (Fig. 1C).

Astrocytic foot processes were visible in close association of many blood vessels although there were also vessels that were not surrounded by astrocytic processes. In contrast, no astrocytic processes could be detected in association with blood vessels in P1 FGF-2 null mice. In adult animals, astrocytic processes were abundant around brain blood vessels in both wild-type and FGF-2 null animals (Fig. 1). ADLH1L1, an astrocyte marker, labelled cells in in most parts of the brain with most prominent staining towards the base of the brain.

However, cortex showed only scant immunoreactivity with the exception of the most the lateral parts as well as distinct reactivity just beneath pia mater corresponding to the pial endfeet (Nakazawa and Ishikawa, 1998), see Suppl. Fig 2. Processes from ADLH1L1⁺ cells in the hypothalamus of wild-type newborn mice were seen touching many CD31⁺ blood vessels while less common in FGF-2 null mice (Fig. 1D). In order to further explore the astrocytic processes at cerebral vessels, tissue sections were examined under the electron microscope. The coverage of astrocytic end-feet around the neurovascular unit was estimated in the cortex of P1 and adult wild-type and FGF-2 null mice. The results are presented in Fig. 1E and vessels illustrated in Fig. 1F. This shows that in P1 wild-type mice the coverage of astrocytic end-feet was $63.7 \pm 14.5\%$ (mean $\pm$ SD) whereas it was significantly lower in P1 FGF-2 null mice ($22.1 \pm 8.8\%$, $p < 0.001$). In adults the astrocytic end-feet covered almost the whole capillary ($\sim 98\%$) in both wild-type and FGF-2 null animals. It was also noticed that in both wild-type and FGF-2 null P1 mice, pericytes were commonly seen around cortical blood vessels although their numbers were not quantified.

Blood-brain barrier permeability

Sucrose permeability: Brain/plasma concentration ratios of ¹⁴C-sucrose after 30 min exposure to the isotope were calculated as a measure of blood–brain barrier permeability to small lipid-insoluble molecules (Stolp et al., 2005). Ratios are presented in Fig 2A and data have been corrected for residual blood space (Ek et al., 2001) in each individual brain region. The data show no significant difference in permeability of the blood-brain barrier to sucrose between wild-type and FGF-2 null animals at any developmental age tested (P1, P20 and adults) or in

any of the brain regions measured (cortex, striatum/thalamus, cerebellum, hindbrain). For instance, the blood/plasma concentration ratios in the cortex was 1.74 ± 0.14 in P1 wild-type versus 2.15 ± 0.27 in P1 FGF-2 null animals ($n=6$ each; $p=0.16$), 4.82 ± 0.59 in P20 wild-type versus $4.96.15 \pm 0.57$ in P20 FGF-2 null animals ($n=6$ and 7 , respectively; $p=0.24$), 4.08 ± 0.28 in adult wild-type versus 3.32 ± 0.48 in adult FGF-2 null animals ($n=5$ each; $p=0.21$). It should be noted that these ratios were from single terminal blood and brain samples at 30 min and should not be compared between different developmental ages as the only comparisons can be made between wild-type and FGF-2 null animals within the same age group (see Discussion).

Plasma protein: Blood–brain barrier permeability to endogenous blood plasma proteins, as markers of barrier permeability to large lipid insoluble markers, was assessed immunocytochemically using antibodies to whole rat plasma (see Methods). In all sections from all age groups of both the wild-type and FGF-2 null animals, plasma protein immunoreactivity was exclusively confined to the blood vessels and was not detected in the parenchyma of the brain, indicating that blood brain barrier to protein was not compromised in the brains from FGF-2 null animals (Fig. 2B and Suppl. Fig 3 for lower power images).

Tight-junction integrity

The tight-junctions of the cerebral blood vessels provide a physical barrier by restricting the paracellular pathway. Two methods were used to investigate the integrity of cerebral blood vessel tight junctions: cellular distribution of claudin-5 and ZO-1, and the ultrastructural integrity. Distribution of the tight-junctional protein claudin-5 and ZO-1 was carried out in tissue sections using immunocytochemical detection. The analysis of stained tissue sections showed that in wild-type and FGF-2 null mice claudin-5 and ZO-1 immunoreactivity was detected in the cell membranes of cerebral blood vessels in all regions of the brain (Fig 3A). No difference was apparent in either the claudin-5 or ZO-1 immunoreactivity between wild-type and FGF-2 null mice in any age groups. Lower magnification images of claudin-5 shows consistent staining of blood vessels in brain (Suppl. Fig. 4A). This was further validated with double labelling of claudin-5 and tomato-lectin showing that blood vessels were positive for claudin-5 in both wild-type and FGF null mice (Suppl. Fig 4B). Under the electron microscope tight junctions are only visible at high-power towards the luminal side of the intra-endothelial cleft as a series of fusion points of the two cell membranes (Ek et al., 2006). The structure of the tight-junctions was examined in cortical vessels of wild-type and FGF-2 null mice. A minimum of 50 vessels were examined at each age. The analysis showed that tight-junctions with multiple fusion points were present at all ages and did not appear

structurally different between wild-type and FGF-2 null mice (see Fig. 3B). In addition, the number of fusion points of the junctions were counted in 10-12 vessels of each newborn mice which showed little difference in the number of fusion points between wild-type and FGF-2 null mice (3.67 ± 1.24 vs 3.35 ± 1.30 , respectively; Mean $\pm$ SD; $p=0.32$).

DISCUSSION

The fundamental structural basis for the barrier at cerebral blood vessels (the blood brain barrier) is the tight-junctions that block the paracellular route for diffusion of blood solutes. These junctions, together with other barrier mechanisms, compartmentalise the brain enabling it to function within its own environment. Understanding when and how these junctions form during development is of fundamental importance to developmental neuroscience but may also lead to new insights into blood-brain barrier regulation and manipulation. The involvement of astrocytes in the induction of the blood-brain barrier has been implicated in many studies (Abbott et al., 2006), but it has been unclear whether the formation of contacts between astrocytes and endothelial cells is a direct generative event of the blood-brain barrier during development. In this report we have studied the putative involvement of astrocytes in the formation of contacts to cerebral vessels and the induction of endothelial cell tight-junction formation and blood-brain barrier function. Overall our study indicates that these contacts do not determine the formation of endothelial tight-junctions at the blood-brain barrier or the permeability characteristics and exclusion of molecules across cerebral blood vessels.

Astroglia in the brain are generated from progenitor cells in either the ventricular zone (neuroepithelium) or the subventricular zone, though other origins have also been suggested (Levison et al., 2005). A recent study showed multiple lineages of astrocytes and distinct clonal clusters but the functional role of these different lineages and clusters is still unclear (García-Marqués and López-Mascaraque, 2013). This and other studies show that the astrocytes are a very heterogeneous cell population, however, one common characteristic of many of these glia cells is that they form intimate contacts with blood vessels within all parts of the central nervous system. In a series of experiments Zerlin and colleagues proposed a model of the development of these contacts in the rat neocortex (Zerlin et al., 1995; Zerlin and Goldman, 1997). Glial progenitors cells migrate along radial-glia, send simple processes to blood vessels, branching occurs of these processes and at about the same time contacts are also established with nearby neuronal cell bodies, which is the beginning of establishing a

neurovascular unit. The mature astrocyte may have several highly branched processes wrapping around blood vessels. At about the same time as these contacts form, astrocytes start producing intermediate filaments and this interaction has been suggested to be a starting point of astroglial differentiation (Zerlin and Goldman, 1997). Filamentous labelling has been a hallmark of astrocyte identification. However, the filaments present in astroglia seem diverse and no one filament labelling, such as GFAP, will label all astrocytes even the mature ones. Therefore the timing of contacts forming between astroglial processes and cerebral blood vessels are more reliably identified with ultrastructural examination. In the mouse these contacts are initiated at the same time as the large expansion of the astroglia cell population occurs, appearing a few days before birth with the process finished in the few weeks after birth in rodents (Caley and Maxwell, 1970; Xu and Ling, 1994; Lunde et al., 2014). Given this timeframe, we decided to investigate the effect of these contacts on the formation of tight-junctions in newborn and young adult mice making use of FGF-2 deficient mice, which have an altered astrocyte phenotype (Reuss et al., 2003; Irmady et al., 2011) and could possibly also be having an altered developmental timing of the end-feet contacts with cerebral blood vessels.

Firstly, to investigate developmental astrocyte differentiation in FGF-2 null mice, we measured the GFAP positive area in the corpus callosum in newborns and adults both in the mutant and in wild-type. We did not find a detectable signal from other areas of the brain in the newborn FGF-2 null mice (see Suppl. Fig. 1). Our results showed that the FGF-2 null newborn mice had smaller GFAP⁺ area than wild-type animals in corpus callosum but in the adult these differences were no longer present (Fig. 1A,B). Staining for ADHL1L which showed more widespread labelling of astrocytes than GFAP in newborns also displayed reduced staining in newborn FGF-2 null newborn mice (Fig. 1D). These findings confirm previous studies in FGF-2 deficient mice in that these mice have alterations in astrocyte differentiation (Reuss et al., 2003; Irmady et al., 2011). We also visualised blood vessels together with astrocytes by immunolabelling and showed that in the FGF-2 null mice fewer intimate contacts between astrocyte processes and blood vessels in the newborn were observed whereas in the adult there was no difference (Fig 1C,D). To further examine these contacts in a more quantitative way, we used transmission electron microscopy to investigate the astrocytic endfeet in the FGF-2 deficient mice estimating their coverage of capillaries in the cortex. This showed coverage of around 22% in newborn FGF-2 null mice whereas in wild-type mice newborns the coverage was higher at around 64% (Fig. 1E). In adult animals

of both FGF-2 null and wild-type groups an almost complete coverage of blood vessels was observed similar to previous studies in rats (Bertossi et al., 1993; Mathiisen et al., 2010). These data together demonstrate that FGF-2 deficiency leads to changes in astrocyte differentiation, confirming previous studies, and is associated with a delay in contacts formed between astrocytes and cerebral blood vessels.

We then went on to investigate whether the delay in astrocyte contacts with cerebral capillaries influenced morphological and permeability properties of the blood-brain barrier. The FGF-2 null mice showed no difference in immunoreactivity for claudin-5 or ZO-1, two key tight-junctional proteins (Nitta et al., 2003; Umeda et al., 2006), compared to normal wild-types in either newborns or adults (Fig. 3). To functionally assess the blood-brain barrier permeability we quantitatively measured transfer of sucrose (342Da) in newborn, 20-day old and adult mice. This showed that there were no differences in sucrose permeability between FGF-2 null mice and wild-types at early postnatal stages or in the adults (Fig. 2). It should be noted that the sucrose concentration ratios can only be compared within each age since there are likely to be differences in plasma sucrose concentrations during the experiments between the ages due to differences in rate of uptake into blood from intraperitoneal cavity and excretion rates through kidneys. For instance, the low ratios at P1 compared to adults can be explained by higher end (30 min) plasma sucrose concentrations in P1 animals (around double) than adults. We also assessed the blood-brain barrier permeability to larger molecules by investigating extravasation of plasma protein. This showed extensive presence of plasma protein within the lumina of brain blood vessels but no detectable immunoreactivity in the parenchyma in any brains investigated, whether FGF-2 null or wild-type animals. We then went on to assess ultrastructure of the tight-junctions in FGF-2 null mice compared to wild-type animals. Tight-junctions of mature appearance, with multiple fusion points of cell membranes could already be found in newborn wild-type mice and these were not altered in FGF-2 null mice (Fig. 3). The criteria we used to assess the permeability function of the blood-brain barrier therefore could find no differences between the FGF-2 null and wild-type mice even at P1. Our results suggest that a delay in astrocyte contacts with blood vessels is not per se a requirement for the formation of tight-junctions between cerebral endothelial cells or for the permeability restriction properties of the blood brain barrier.

Barrier development and barrier induction have been somewhat of a disputed subject. However, there is today much evidence that cerebral blood vessels acquire barrier function at an early stage in brain development shortly after they penetrate into brain from the perineural

plexus. For instance cerebral tight-junctional proteins are present in cerebral blood vessels soon after they are formed in mammals including humans (Virgintino et al., 2004; Ballabh et al., 2005; Ek et al., 2006; Daneman et al., 2010) and cerebral blood vessels have also been found to be functionally restrictive at an early stage (Ek et al., 2006; Daneman et al., 2010; Alvarez et al., 2011). It is important to point out though that this does not mean that the some blood-brain barrier mechanisms do not undergo subsequent developmental changes. Several studies have for instance shown that p-glycoprotein on cerebral blood vessels increases during development potentially making the developing brain to more vulnerability to neurotoxic compounds (Virgintino et al., 2008; Ek et al., 2010). In contrast, transport of some amino acids into the brain has been shown to be higher in the young animal (Cornford et al., 1982) and this correlates with higher expression of many Slc transporter genes for amino acids (Daneman et al., 2010; Saunders et al., 2012). We still know very little about functional aspects of many transport mechanisms in the developing brain and it is likely that with more studies we would find further differences between the developing and adult barrier mechanisms.

Several authors have previously pointed out that barrier properties of cerebral vessels are present before astroglia come in contact with them and that it therefore appears unlikely these contacts are pivotal for barrier induction (Haseloff et al., 2005; Daneman et al., 2010; Saunders et al., 1999, 2014). Our results support this notion. However, astrocytes *in vivo* may still influence barrier formation by distant soluble factors, although this cannot be the case in the earliest stages of vascularisation of the embryonic brain as astrocytes mostly differentiate in the postnatal period (Caley and Maxwell, 1970; Daneman et al., 2010). Endothelial cells in culture appear to be much influenced by astrocytes even when they are not in direct contact with each other. From these co-culture studies of brain endothelial cells and astrocytes it seems that astrocytes produce a range of factors, such as TGF β and ANG1, which can influence endothelial barrier phenotype (Abbott et al., 2006). However, *in vivo* confirmation of these findings is still scarce and the overall role of the astrocytes *in vivo* at the blood-brain barrier is still not fully clear. The secretion of sonic hedgehog is one mechanism that has recently been proposed by which astrocytes can remotely promote barrier integrity (Alvarez et al., 2011). In many disease states there is also an association between disturbances to astroglia and blood-brain barrier dysfunction (Abbott et al., 2006). Endothelial cells are also much influenced by other cells within the neurovascular unit. The pericytes, which are linked to the cerebral blood vessels before the astrocytic endfeet form contacts with blood vessels, seem to

have an important role in barrier integrity in that they reduce transcytosis in the endothelial cells (Armulik et al., 2010; Daneman et al., 2010). These cells have been shown *in vitro* to be able to induce tight-junctional proteins in endothelial cells (Hori et al., 2004). Intimate interplay between astrocytes and pericytes has also recently been suggested in that astrocytic laminin, via affecting pericyte differentiation, is important for normal maintenance of barrier function (Yao et al., 2014). More comprehensive reviews on how these closely positioned cells influence each other and the signalling systems believed to be involved in barrier formation have been published (Bonkowski et al., 2011; Engelhardt and Liebner, 2014). Astrocyte contacts at the more mature cerebral vessels are believed to be involved in maintenance and regulation of blood-brain barrier function (Abbott et al., 2006; Engelhardt and Liebner, 2014).

In conclusion, in this study we confirm that FGF-2 deficiency leads to abnormal astrocyte differentiation and a delay in encirclement of cerebral blood vessels by perivascular astrocytic end-feet. However this delay does not appear to influence tight junctional integrity or blood-brain barrier permeability function. This together indicates that the basic permeability properties of the blood brain barrier seem to be established early in brain development and are independent of the formation of astrocytic end-feet at cerebral vessels.

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FIGURE LEGENDS

Figure 1. Astrocytes in wild-type and FGF-2 null mice.

A) Percentage area immunoreactive for GFAP (marker for astrocytes) in the corpus callosum in P1 and adult mice. At P1 GFAP immunoreactivity was significantly lower in FGF-2 null mice than in wild-types whereas in adults the area stained is very similar. Data mean $\pm$ SEM, n=5 for each age. ***p<0.001 (t-test).

B) Examples of GFAP immunoreactivity in corpus callosum (dashed area) from wild-type and FGF-2 null mice at P1 and P60. Scale bar=100 μ m.

C) GFAP (red, astrocyte marker) and GSA-lectin (green, blood vessel marker) double-labelling immunohistochemistry. In newborns (P1) astrocytes are in close association with some blood vessels in the corpus callosum of wild-type mice whereas in FGF-2 null mice there are fewer astrocytes visible in close association with the blood vessels. In the adult, however, astrocytic processes appear to surround the blood vessels of the cortex in both wild-type and FGF-2 null mice. Scale bar=25 μ m.

D) ADLH1L1 (red, astrocyte marker) and CD31 (green, endothelial marker) immunohistochemistry in hypothalamus of newborn. Top, ADLH1L1⁺ cells are abundant and processes closely associated to blood vessels of wild-type mice whereas less evident in FGF-2 null mice. Bottom, 2D projections of Z-stacks obtained by confocal microscopy showing processes of ADLH1L1⁺ cells on the outside of blood vessels in newborn wild-type mice. Scale bar=50 μ m (top) and 25 μ m (bottom).

E) The area of blood vessels in the cortex surrounded by astrocytic processes in wild-type and FGF-2 null mice at P1 and in adults. In newborns the coverage was significantly lower in FGF-2 null mice (22%) than wild-types (64%) whereas in adults the end-feet covered almost the whole capillary. Data are mean $\pm$ SEM, n=3-4 for each age. **p<0.01 (t-test).

F) Electron micrographs illustrating typical cortical blood vessels. In newborn wild-type mice a larger portion of the capillary is surrounded by astrocytic processes in whereas in newborn FGF-2 null mice a smaller proportion of the capillary is encircled by astrocytic processes. In adults, astrocytic end-feet completely surrounded the capillaries in both wild-type and FGF-2 null mice. As – Astrocyte, p – pericyte, e – endothelial cell. Scale bar=5 μ m.

Figure 2. Blood-brain barrier permeability in wild-type and FGF-2 null mice.

A) Sucrose brain/plasma concentration ratios in P1, P20 and adults. Ratios have been corrected for residual brain blood space (see Methods). No difference was observed in concentration ratios between wild-type and FGF-2 null animals in any region of the brain. Data are mean $\pm$ SEM, n=5-7/group.

B) Barrier permeability to endogenous plasma proteins was assessed immunohistochemically using antibodies to whole rat plasma proteins developed with DAB method. Sections are from the cortex. This shows that in all animals the reaction product confined to blood vessels and no staining in brain parenchyma was visible, indicating no differences in brain barrier permeability between ages and wild-type/FGF-2 null animals. Scale bar=40 μ m.

Figure 3. Tight-junctions in wild-type and FGF-2 null mice.

A) Claudin-5 and zonula occludens-1 (ZO-1) protein immunoreactivity in cortical tissue sections of newborn (P1) mice. Blood vessels in cortex in both wild-type and FGF-2 null animals are positive for tight-junctional proteins indicating blood vessels with barrier properties. Scale bar=100µm.

B) Electron micrographs illustrating structure of typical tight-junctions in cortical capillaries of P1 and adult wild-type/FGF-2 null mice. The tight-junctions are visible towards the luminal cleft between the endothelial cells where the cell membranes are fused together at multiple points. Structurally similar tight-junctions were found between all endothelial cells at all ages (arrows). Scale bar=200nm.

Supplementary Figure 1. Lower power photomicrographs of GFAP immunoreactivity in newborn wild-type (A) and FGF2^{-/-} (B) mice. Scale bar=500µm.

Supplementary Figure 2. Immunoreactivity for ADLH1L1 (red) and CD31 (green) in hypothalamus (A) and medial parts of cortex (B) in wild-type mice. Arrow indicates ADLH1L1 immunoreactivity just beneath pia mater. Scale bar=200µm.

Supplementary Figure 3. Lower power photomicrographs of plasma protein staining from newborn and adult animals. Scale bar=100µm.

Supplementary Figure 4.

A) Lower power photomicrographs of claudin-5 staining from newborn and adult animals. Scale bar=100µm.

B) Co-localisation of tomato-lectin positive blood vessels with claudin-5 in newborn and adult animals. Scale bar=50µm.

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184x209mm (300 x 300 DPI)

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207x294mm (300 x 300 DPI)

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157x260mm (300 x 300 DPI)

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Author/s:

Saunders, NR;Dziegielewska, KM;Unsicker, K;Ek, CJ

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