Supplemental Data:

Spatio-temporal maturation of functional BBB across brain regions

We observed a spatial pattern in the development of BBB functionality. At E14.5, although cortical vasculature did not yet exhibit a functional barrier (Fig. 1b, middle panel), midbrain and hindbrain vasculature was already capable of preventing 10-kDa dextran leakage (Extended Data Fig. 2a,b). Spatial differences were also apparent in the cortex (Extended Data Fig. 2c-e). At E13.5, most of the injected tracer escaped vessels in the dorsal-medial cortex but was largely confined inside of the vessels in the ventral-lateral cortex (Extended Data Fig. 2d). Similarly, in the ventral-lateral cortex, the BBB was already fully sealed at E14.5 while still leaky in the dorsal-medial cortex (Extended Data Fig. 2e). Therefore, BBB formation exhibits a spatio-temporal pattern in the developing cortex from ventral-lateral to dorsal-medial, similar to patterns observed in other neurodevelopmental processes such as the tangential migratory pathway of inhibitory neuro-progenitors, deposition of extracellular matrix components, and cortical plate expansion[32, 33].

Expression profiling identifies genes involved in BBB formation

Cortical and lung endothelial cells were isolated from *Tie2-GFP* expressing mouse embryos at E13.5 using fluorescence-activated cell sorting (FACS). We then compared the expression profiles of BBB (cortex) and non-BBB (lung) endothelium, using an Affymetrix array. As expected from a comparison between these two endothelial populations, a great majority of the genes analyzed showed little difference in the relative representation of their transcripts (Fig. 2a, black dots), with overall enrichment of

endothelial-specific genes and de-enrichment of neuronal-, astrocyte- or pericyte-specific transcripts (Fig. 2b and table at Extended Data Fig. 3a). We identified a small fraction of transcripts with significantly higher representation in the cortical endothelium than in the lung endothelium (Fig. 2a, red dots).

Six months old *Mfsd2a*$^{-/-}$ mice display no sign of cerebrovascular degeneration

In this study, the oldest mice we had access to were 6 six month old. We examined their vascular integrity at the ultrastructural level using TEM. At this age, brain capillaries from *Mfsd2a*$^{-/-}$ mutant mice exhibit normal features (e.g. cell size, shape of the nucleus, thickness of the vessel wall, overall basement membrane structure and pericyte attachment (Extended Data Fig. 6c)).

Subcellular localization of MFSD2A by immuno-EM

Examination at the ultrastructural level using immuno-EM in adult brains revealed that MFSD2A protein is found in the luminal plasma membrane as well as associated with vesicular structures in brain endothelial cells, but not in tight-junctions (Extended Data Fig. 10). This observation suggests that MFSD2A is at a relevant location to directly regulate transcytosis.

Supplemental Discussion

Based on both our genetic loss of function and MFSD2A subcellular localization results, we hypothesize that MFSD2A serves as a cell surface molecule to directly regulate transcytosis. Although we cannot formally exclude the possibility that the elevated levels

of transcytosis in *Mfsd2a* mutant mice was due to some form of cellular stress, this seems very unlikely because cellular stress response is an acute cellular state by which, in response to external or internal stress inducers, the cell either resolves the circumstances and returns to homeostatic conditions, or in extreme conditions is driven into cell death[27]. In *Mfsd2a* mutant mice, the increased transcytosis and BBB leakage phenotype persist from embryonic stage to adulthood, and up to six month old these mice exhibit no sign of vascular degeneration (Extended Data Fig. 6c). Interestingly, increased numbers of pinocytotic vesicles have been observed in human pathological conditions[9] and transcytosis up-regulation was also observed following acute exposure to stress inducers (such as acute hypertension and bicuculline or electrically induced seizures) in animal models[34-36]. It will be interesting to examine whether MFSD2A is involved in these pathological and acute assault situations.

Supplementary References

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