Supplementary figure legends:

Extended Data Figure 1| Diagram illustrating two unique BBB properties of CNS endothelial cells. Compared to the endothelial cells from the rest of the body, CNS endothelial cells which possess a BBB are characterized by (1) highly specialized tight-junctions sealing the space between adjacent cells, and (2) an unusually low rate of transcytosis from the vessel lumen to the brain parenchyma.

Extended Data Figure 2| Spatial and temporal BBB maturation across brain regions and cortical regions.

a-b, Embryonic BBB develops in a caudal to rostral spatial pattern. 10-kDa dextran-tracer injection revealed that the BBB is already functional in the hindbrain (a) and midbrain (b) at E14.5, a time-point at which cortical BBB is still leaky. Epifluorescence (low magnification) and confocal (high magnification) images of brain sections from

injected embryos are shown. As illustrated in both (a) and (b), tracer was confined to blood vessels (arrows).

c-e, Cortical BBB develops in a ventro-lateral to dorso-medial spatial pattern. **c,** Diagram of the embryonic cortex indicating dorsal-medial and ventral-lateral cortical regions illustrated in d and e. **d,** At E13.5, the BBB of the dorsal cortex (upper panels) is not fully formed, as evidenced by little tracer inside the blood vessels (arrow) and tracer-filled parenchymal cells (arrowheads). In the ventral cortex (lower panels), capillaries were better sealed, showing more tracer within the lumen (arrow) and less tracer in the brain parenchyma (arrowhead). **e,** At E14.5, the capillaries in the dorsal cortex (upper panels) were still leaky, with little tracer inside the capillaries (arrow) and tracer-stained surrounding parenchyma (arrowheads). At the same age, the BBB of the ventral cortex (lower panels) was already functional, with all tracer confined to the capillaries (arrow). Confocal images are representative of results from at least six embryos across three different litters for each age. Green: lectin, Red: 10-kDa tracer. n=6 embryos from 3 litters for each age.

Extended Data Figure 3| Expression profile comparison of cortex (BBB) and lung (non-BBB) endothelial cells.

a, Pan-endothelial markers were highly expressed in endothelial cells isolated from both cortex and lung of the endothelial specific *Tie2-GFP* reporter mouse at E13.5. **b,** Genes involved in the transport of molecules across the BBB, known as adult BBB markers, were highly enriched in brain vs. lung endothelial cells. Data are mean of 4 biological replicates (4 litters).

Extended Data Figure 4| MFSD2A protein is selectively expressed in BBB-containing CNS vasculature of both embryos and adults.

Immunohistochemical staining of MFSD2A protein demonstrating its specific expression in BBB-containing CNS vasculature. Red: MFSD2A; green: PECAM (endothelium); blue: DAPI (nuclei). **a,** At E13.5, the time of BBB establishment, MFSD2A expression was detected in the brain (upper panels) but not in the lung vessels (lower panels) - confirming the microarray data. **b,** At E15.5,the first developmental time point of BBB functionality, MFSD2A expression was detected in spinal cord (upper panels) but not in lung (middle panels) or liver vessels (lower panels). **c,** MFSD2A selective expression in BBB-containing vessels persists in adult mice (P90) as shown in brain vessels (upper panels) but not in lung or liver (lower panels). **d,** MFSD2A is expressed in cerebral vessel (arrow) but not in pial vessels (arrow heads). Low (upper panel) and High magnification (lower panel) of pial-cerebral boundary (dotted line) of P5 dorsal cortex (wild-type mice). n=3 embryos from 3 litters for each age.

Extended Data Figure 5| The leaky BBB phenotype in *Mfsd2a*$^{-/-}$ mice persists after birth and is not restricted to carbohydrate-based tracers.

a,b, Injection of two non-carbohydrate-based tracers with different molecular weight and different molecular compositions at postnatal day (P) 4 revealed a persistent leaky barrier phenotype in mice lacking *Mfsd2a*. **a,** The small molecular weight tracer sulfo-NHS-biotin (~550 Da), was confined to vessels in wild-type controls (upper panels), whereas leaked out of the vessels (arrowheads) in *Mfsd2a*$^{-/-}$ mice (lower panels). Green: lectin,

Red: tracer. **b,** The high molecular weight protein tracer - horseradish peroxidase (HRP, ~44-kDa) was confined to vessels in control mice at P4 (left), while in mice lacking *Mfsd2a* (right), tracer was diffusing into the brain parenchyma (arrowheads). Brown: visualization of HRP in light microscopy by DAB reaction. Stainings were done on 100 μm cortical sections of tracer-injected animals. n=4 pups per genotype from 3 litters. **c**,**d,** BBB leakage of 70 kDa molecular weight tracer is observed in postnatal *Mfsd2a*$^{-/-}$ mice. Green: Lectin or PECAM (vessels), Red: tracer. The 10-kDa (a) and 70-kDa (b) tracers fluoro-Ruby-Dextran were confined to vessels in control mice (upper panels), but not in *Mfsd2a*$^{-/-}$ mice (lower panels), where tracer was taken up by brain parenchymal cells (arrowheads). Stainings were done on 12 μm cortical sections of tracer-injected animals. n=3 pups per genotype from 3 litters.

Extended Data Figure 6| Perinatal and adult mice lacking *Mfsd2a* do not display changes in cerebrovascular network properties or signs of vascular degeneration.

a, No abnormalities were found in cortical capillary density and vascular branching (upper panels) as well as capillary diameter (lower panels) of postnatal (P4, left) and adult (P70, right) *Mfsd2a*$^{-/-}$ mice. Images of vascular staining in coronal cortical sections and high magnification images of capillaries cross section profiles (green: PECAM) and quantification. Data are mean±s.e.m. n=3 animals per genotype, 20 sections per animal. **b,** Immunostaining for smooth muscle actin (arterial identifier, arrows) revealed no abnormalities in arterial distribution and specification in *Mfsd2a*$^{-/-}$ mice. Images of coronal cortical sections (green: PECAM, red: SMA, left) and quantification (right). Data are mean±s.e.m. n=3 animals per genotype, 20 sections per animal. **c,** Electron

microscopic examination of older *Mfsd2a*$^{-/-}$ mice did not reveal signs of cerebrovascular degeneration. **Left,** The overall capillary structure (e.g. cell size, shape of the nucleus, thickness of the vessel wall, basement membrane integrity and pericyte attachment) did not differ between wild-type and mutant mice. **Right,** At higher magnification, normal features, as pericyte (*) attachment within a normal basement membrane (between arrows), could be observed in mice lacking *Mfsd2a.*

Extended Data Figure 7| Vesicular activity and transcytosis events in the brain endothelial cells of *Mfsd2a*$^{-/-}$ mice are dramatically increased.

a, Quantification of the vesicular density (both total and individual type of vesicles) in E17.5 control and mutant endothelium. Mean vesiclar density was calculated from the number of vesiclar types per μm of luminal membrane (luminal type I and type II vesicles), per μm^2 of cytoplasm (cytoplasmic vesicles), and per μm of abluminal membrane (abluminal vesicles). **b,** Quantification of HRP luminal up-take in P90 HRP injected mice. No HRP-filled vesicles were found in wild-type mice. Data are mean±s.e.m. from 4 controls and 4 mutants (10 vessels per animal, 2 images at 12,000X per vessel). **P < 0.01, ***P < 0.001 in Student's *t*-test.

Extended Data Figure 8| Pericyte coverage, attachment and ultrastructure are normal in *Mfsd2a*$^{-/-}$ mice.

a,b, *Mfsd2a*$^{-/-}$ mice exhibit normal pericyte coverage. Co-staining of endothelium (claudin5, red) and pericytes (CD13 in a and PDGFRβ in b, green) revealed no overt difference in pericyte coverage of dorsal cortex vessels between wild-type (upper row)

and *Mfsd2a*$^{-/-}$ (bottom row) mice at P5. Quantification of vascular coverage in both a and b showed no significant difference between wild-type and *Mfsd2a*$^{-/-}$ samples (P>0.5). Data are mean±s.e.m. n=3 pups per genotype, 20 sections per animal. **c,** E17.5 *Mfsd2a*$^{-/-}$ mice exhibit normal pericyte-endothelial attachment. High magnification images of capillary cross sections co-staining for endothelium (Glut1, red) and pericytes (PDGFRβ, green) revealed no difference in pericyte-endothelial relationships between wild-type (upper panels) and *Mfsd2a*$^{-/-}$ (lower panels) mice (single asterisk – endothelial nucleus, double asterisk – pericyte nucleus). **d**, Electron micrographs of longitudinal capillary sections revealed that pericytes had normal appearance and were well positioned on the vessel walls in *Mfsd2a*$^{-/-}$ adult mice – pericytes were adjacent to endothelial cells and shared a common basement membrane. P: pericyte, L: lumen.

Extended Data Figure 9| *Mfsd2a* gene expression and MFSD2A protein levels are down-regulated in mouse models of reduced pericyte coverage.

a, Analysis of micro array data[6] showed high levels of *Mfsd2a* expression in wild-type adult brain microvasculature, but a significant decrease in levels of *Mfsd2a* expression in mice that have reduced pericyte coverage at the BBB. *Pdgfb*$^{ret/ret}$ mice (mouse model 1), where PDGF-B binding to heparan sulphate proteoglycans was disrupted, exhibited a major loss of pericyte coverage (74% reduction)[5] and showed a dramatic decrease in *Mfsd2a* expression (74% reduction) compared to that of littermate controls. Similarly, *Tie2*^{Cre}*/R26*$^{P+/0}$*, pdgfb*$^{-/-}$ mice (mouse model 2) in which *Pdgfb* null alleles were complemented by one copy of human *PDGF-B* transgene showed a less dramatic loss of pericyte coverage (60% reduction)[5] and a smaller decrease in *Mfsd2a* expression (53%

reduction). ** (P=0.004), *** ($P=1X10^{-5}$). Bars reflect normalized signal of the *Mfsd2a* probe (1428223_at) in adult brain or cortex microvascular fragments (a.u). Data are mean±s.d. of 4 biological replicates. **b,** MFSD2A immunostaining in cerebrovasculature of postnatal $Pdgfb^{ret/ret}$ mice and littermate controls ($Pdgfb^{ret/+}$) revealed reduced MFSD2A protein expression in endothelial cells that are not covered by pericytes. Cross section, Red: MFSD2A; green: Claudin5 (endothelium); blue: DAPI (nuclei); gray: PDGFRβ (pericyte). Reduced MFSD2A signal was observed in endothelial cells of capillaries not covered with pericytes in $Pdgfb^{ret/ret}$ mice (arrow - lower panels), while strong MFSD2A signal was apparent in pericyte-covered capillaries in $Pdgfb^{ret/+}$ mice (arrow - upper panel). Inset in **b'** demonstrates in high magnification that MFSD2A expression is restricted to endothelial cells (co-localization with Claudin5 staining) and absent in pericytes (none co-localization with PDGFRβ staining). **c,** Additional example of the reduction in MFSD2A expression in endothelial cells of $Pdgfb^{ret/ret}$ mice (longitudinal section). **d,** Quantification of mean fluorescence intensity per vascular profile showed significant reduction of MFSD2A signal in $Pdgfb^{ret/ret}$ capillaries compared to controls. Data are mean±s.e.m. n=2 animals per genotype, 60 images quantified of at least 600 vascular profiles per animal.

Extended Data Figure 10| Immuno-EM reveals the subcellular localization of MFSD2A on the plasma membrane and vesicles, but not in tight junctions of cerebral endothelial cells.

a, Electron micrographs showing silver-enhanced immunogold labeling of MFSD2A in cerebral cortex capillaries from wild-type (*left*) but not in $Mfsd2a^{-/-}$ mice (*right*), demonstrating staining specificity. **b,** Upper panels (*i-iii*): examples of MFSD2A

localization on the plasma membrane (arrows) and in the cytoplasm (arrow-heads), but not in tight-junctions (asterisk). Lower panels (*iv*-*vi*)*:* high magnification examples of MFSD2A localization on the luminal plasma membrane (arrows), associated with luminal invaginating vesicles (*iv*,*v*) and with cytoplasmic vesicles (arrow-heads). All samples are of cortical vessels from adult mice (P30-P90). n=2 for each genotype. L: lumen.