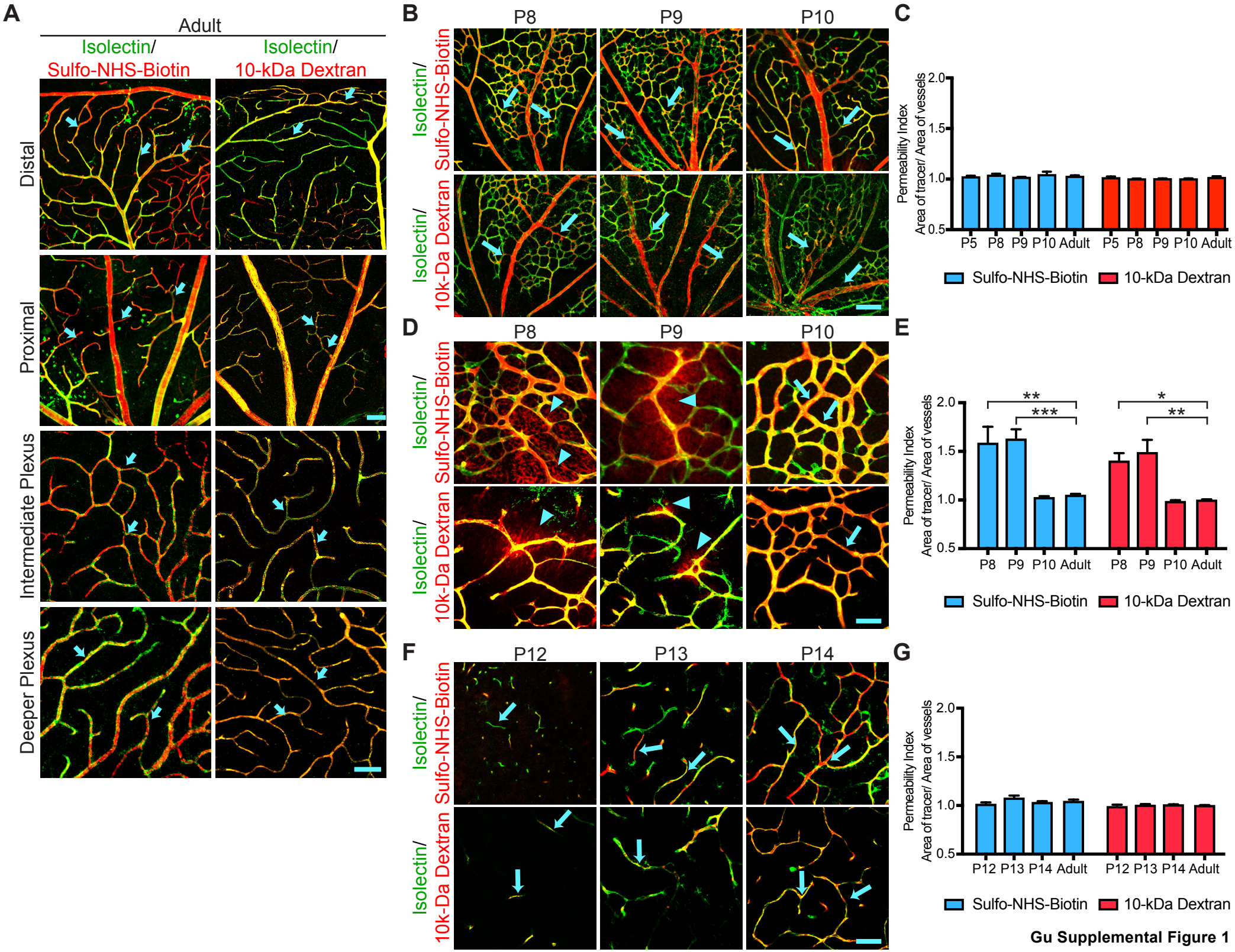
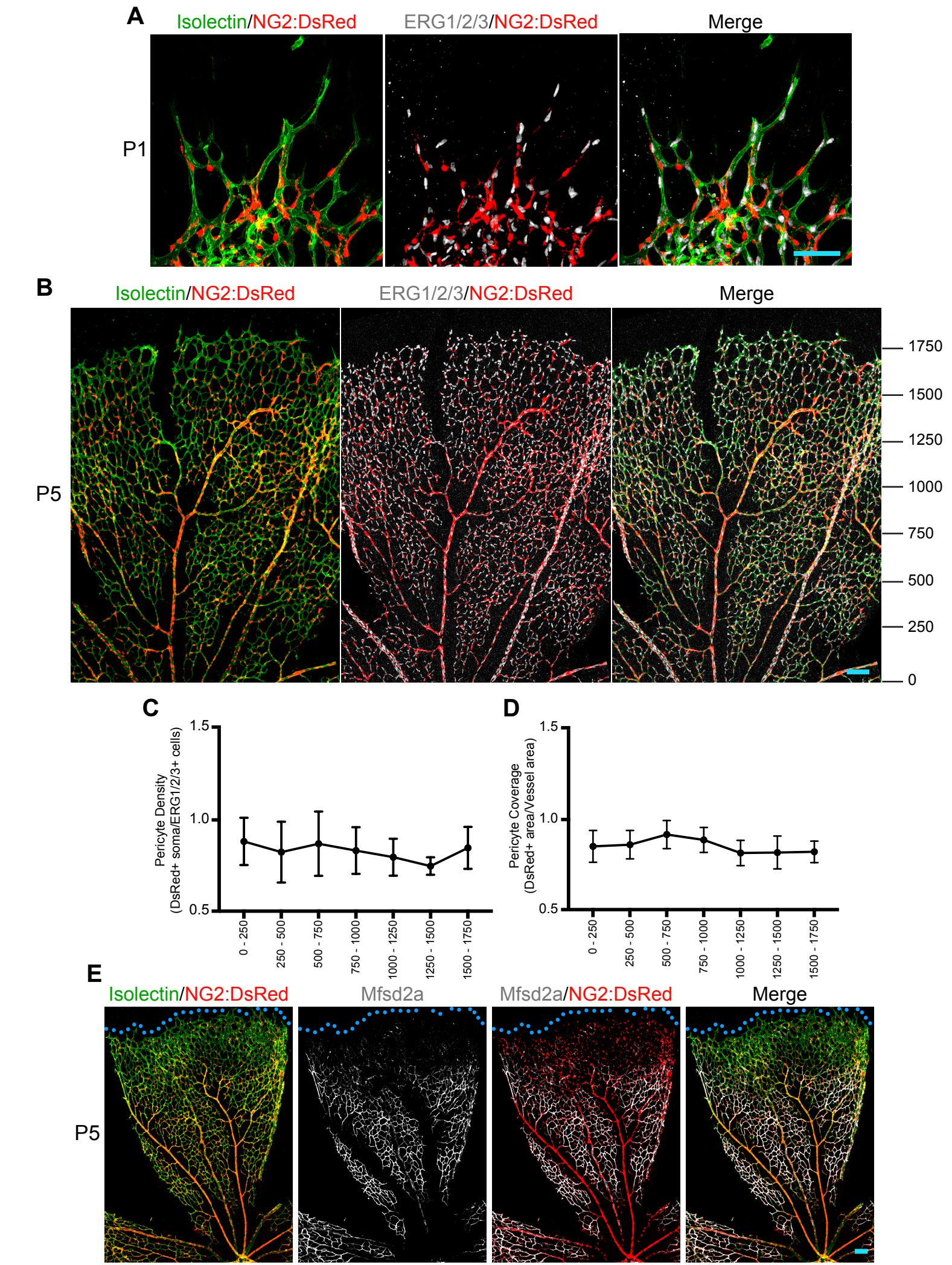
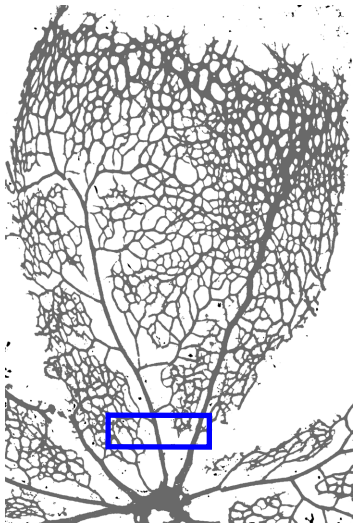
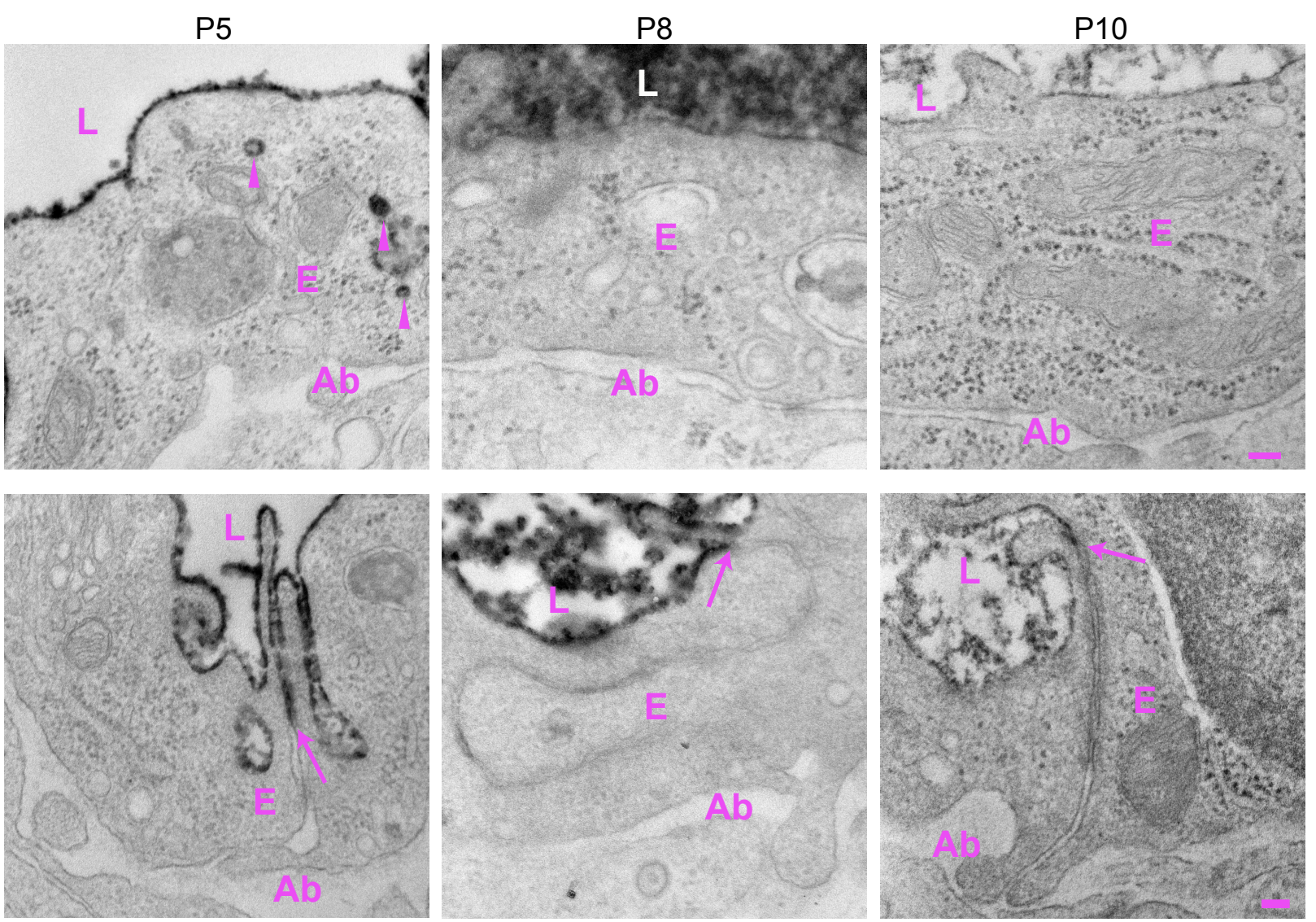


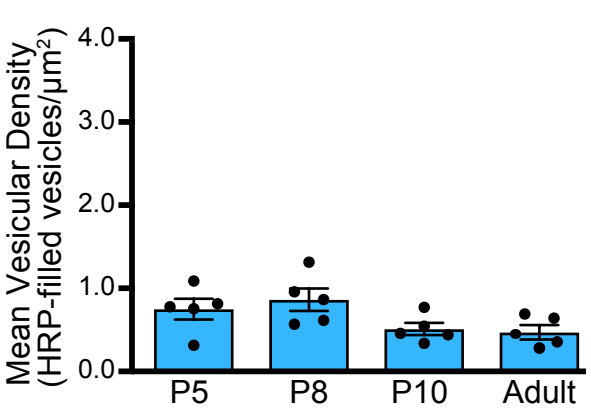
Gu Supplemental Figure 1

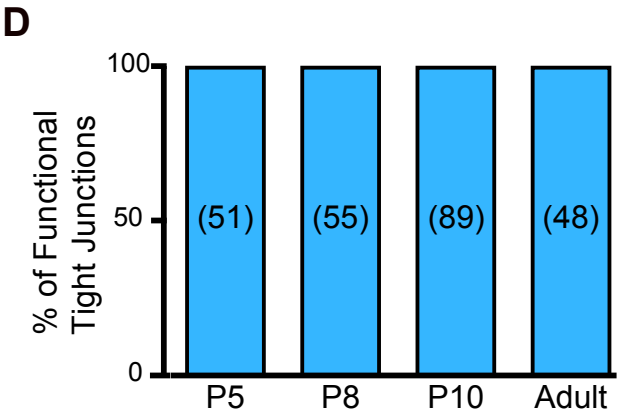


Gu Supplemental Figure 2

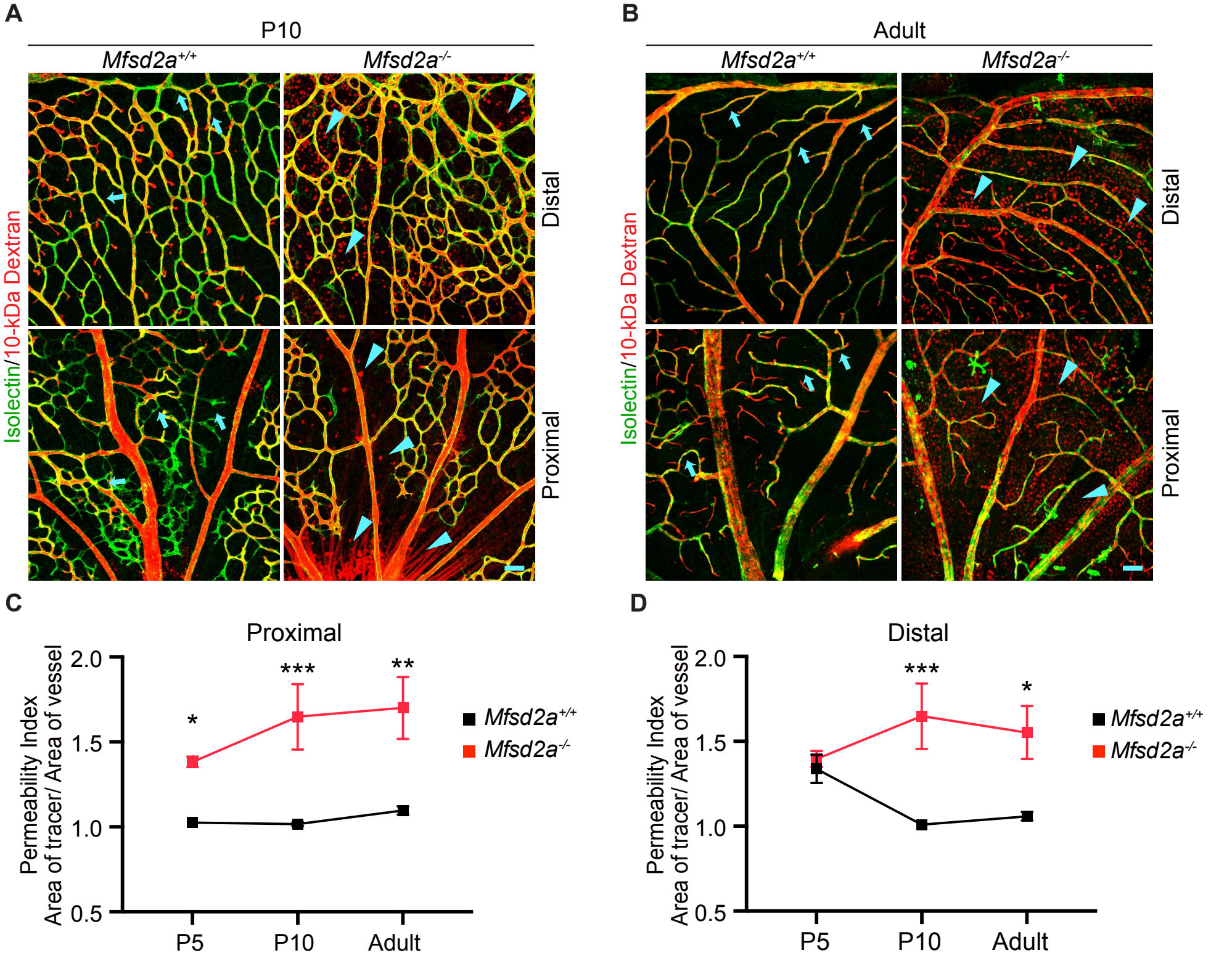








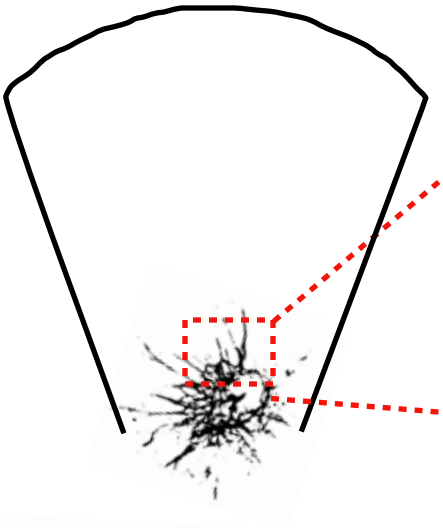
Gu Supplementary Figure 3



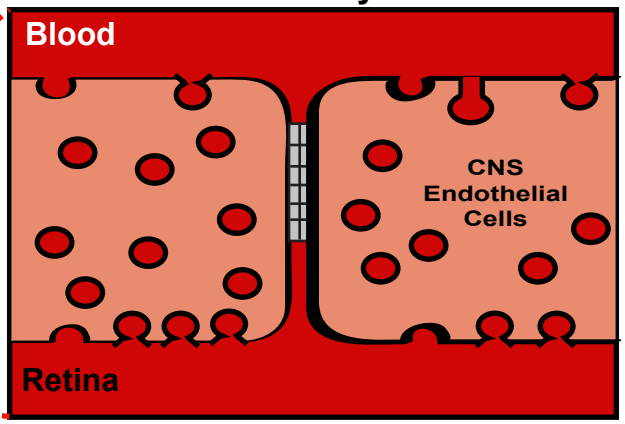
Gu Supplementary Figure 4

When vessels first enter the CNS

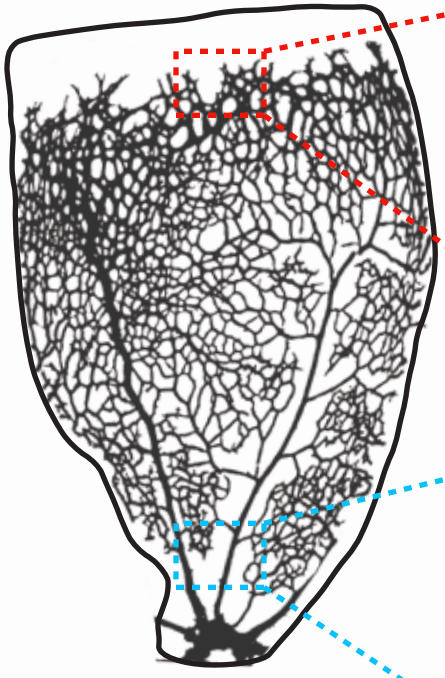
P1



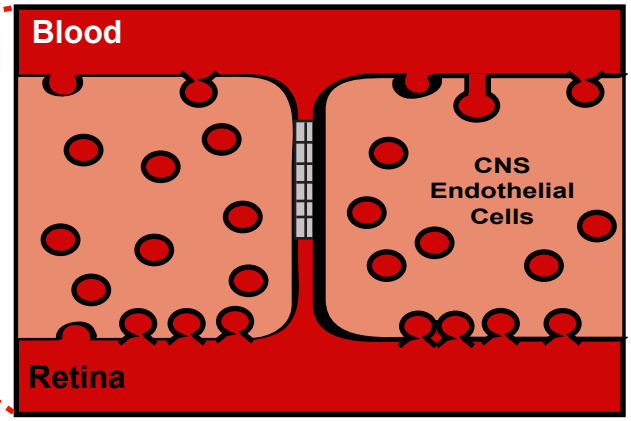
Bulk transcytosis



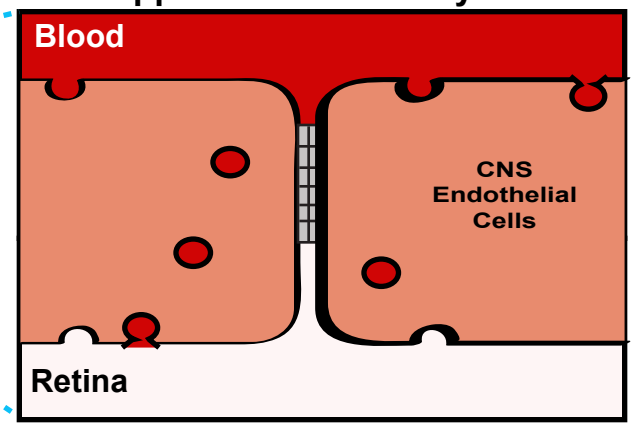
P5



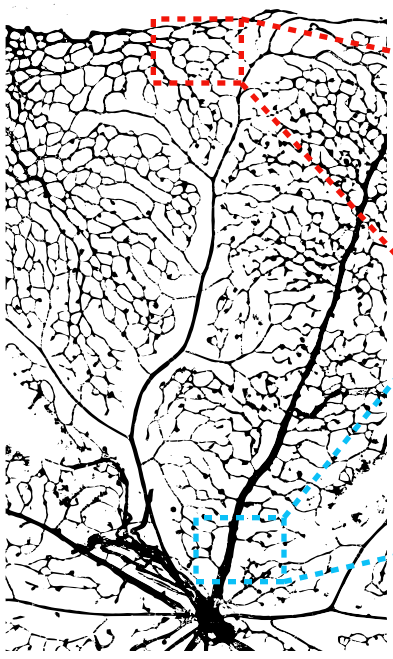
Bulk transcytosis



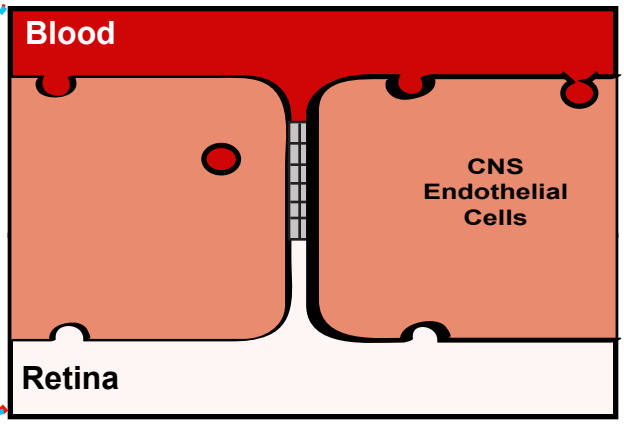
Suppression of transcytosis



P10



Suppression of transcytosis



Gu Supplemental Figure 5

Supplemental Figure Legends:

Figure S1. Characterization of functional BRB in adult retinal vasculature and developing proximal, deeper plexus and intermediate plexus vessels. Related to Figure 1. (**A**) In a functional adult BRB, the vessels (isolectin; green) of the primary (1st and 2nd row), intermediate (3rd row) and deeper plexus (4th row) completely confines (arrow) both the Sulfo-NHS-Biotin (left) and 10-kDa dextran (right) tracers (red). (**B**) Intravenous injections of Sulfo-NHS-Biotin (left) and 10-kDa dextran (right) tracer in postnatal mice demonstrate that the proximal vessels (green) already confine the tracer. (**C**) Quantification of the permeability index of the proximal vessels. Data are presented as mean ± s.e.m. (n = 5-8 pups per age, from 3 different litters). Statistical significance was determined by one-way ANOVA, followed by post-hoc Bonferroni multiple comparison adjustment, comparing the various neonatal ages with the adult in the respective Sulfo-NHS-Biotin and 10-kDa dextran group. No significance was observed in the comparison of the proximal vessels. (**D**) Intravenous injections of Sulfo-NHS-Biotin (left) and 10-kDa dextran (right) tracer in postnatal mice demonstrate that sprouting deeper plexus vessels exhibit tracer leakage at P8 and P9 but not at P10. (**E**) Quantification of the permeability index of the deeper plexus vessels. Data are presented as mean ± s.e.m. (n = 5-8 pups per age, from 3 different litters). Statistical significance was determined by one-way ANOVA, followed by post-hoc Bonferroni multiple comparison adjustment, comparing the various neonatal ages with the adult in the respective Sulfo-NHS-Biotin and 10-kDa dextran group. (**F**) Sprouting intermediate plexus vessels completely confine the tracer as early as P12. (**G**) Quantification of the permeability index of the deeper plexus vessels. Data are presented as mean ± s.e.m. (n = 5-8 pups per age, from 3 different litters). Statistical significance was determined by one-way ANOVA, followed by post-hoc Bonferroni multiple comparison adjustment, comparing the various neonatal ages with the adult in the respective Sulfo-NHS-Biotin and 10-kDa dextran group. No significance was observed in the comparison of intermediate plexus vessels. *, $P < 0.05$, **, $P < 0.01$. ***, $P < 0.01$. Scale bar represents 50 μm for all panels.

Figure S2. Pericyte coverage is constant throughout the retina, including distal leaky vessels during BRB development. Related to Figure 1.

(**A)** As early as vessel ingression at P1, pericytes (NG2:DsRed; red) are already ensheathing budding CNS vessels (isolectin; green and ERG for endothelial nuclei; white). (**B-D**) At P5, pericyte density (**C**) (NG2:DsRed+ soma/ ERG1/2/3+ cells) and (**D**) pericyte coverage (NG2:DsRed area/Isolectin area) is around 1:1 throughout the retinal vasculature, including the distal, nascent vessels. Data are presented as mean ± s.e.m. (n = 5). The ticks in (**B**) represent the distance in microns from the ONH. 0 is set slightly before the ONH. (**E**) Pericytes are present in nascent, distal vessels yet Mfsd2a is absent in P5 retinas. The angiogenic front is outlined in blue (determined by isolectin staining). Scale bar represents 100 μm for all panels.

Figure S3. EM analysis of Proximal Vessels at P5, P8, P10 and Adult. Related to Figure 2.

(**A)** P5 to P10 proximal endothelial cells already contain a negligible number of vesicles. **(B)** EM analysis of proximal vessels from P5, P8 and P10 retinas reveals tracer product halts abruptly at the tight junctions (arrows). (**C**) Quantifications of tracer-filled vesicular densities in the proximal endothelial cells at P5, P8 and P10. Data are presented as mean ± s.e.m. (n= 5 mice per age; each circle represents the average vesicular density from 15 – 20 vessels per mouse). Statistical significance was determined by one-way ANOVA, followed by post-hoc Bonferroni multiple comparison adjustment, comparing the proximal vessels of the various neonatal ages to the proximal vessels of the adult. No significant difference was observed in the comparisons of the proximal vessels. (**D**) Quantification of the number of tight junctions that halt the tracers at the luminal side without parenchymal leakage over the total number of tight junctions in proximal vessels. (n = 5 mice per age; 15-20 vessels analyzed per mouse; the number of tight junctions analyzed are displayed in the bars). The Adult data in Figure S2C and S2D is the same as Figure 2E and 2F. L – lumen, E – endothelial cells,

Ab – abluminal, RBC- red blood cell. ***, $P < 0.001$. Scale bar represents 100 nm for all panels.

Figure S4. Mfsd2a is essential for functional BRB formation. Related to Figure 3.

(**A and B**) Intravenous injection of tracer in P10 (A) and adult (B) *Mfsd2a*$^{+/+}$ mice results in confinement of the tracer throughout the vasculature (arrows) whereas tracer injection in *Mfsd2a*$^{-/-}$ mice results in tracer leakage into the retinal parenchyma (arrowheads) at both distal and proximal regions of the retina. **(C-D)** Quantification of the permeability of the **(C)** proximal and **(D)** distal vessels at P5, P10, and adult ages. Data are presented as mean ± s.e.m. (n = 5,6, and 4 for wildtype P5, P10 and adult respectively and n=5, 5 and 4 for *Mfsd2a*$^{-/-}$ P5, P10 and adult respectively). P5 proximal and distal vessel data is the same data from Figure 4H. Statistical significance was determined by comparing *Mfsd2a*$^{-/-}$ and *Mfsd2a*$^{+/+}$ littermates at the respective age using two-way ANOVA, followed by post-hoc Bonferroni multiple comparison adjustment. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$. Scale bar represents 50 μm for all panels.

Figure S5. Illustration of the gradual suppression of transcytosis governs functional BRB formation. Related to Figure 1.

As early as vessel ingression at P1 (top), functional tight junctions are already formed to restrict paracellular flux between budding CNS endothelial cells. However, bulk transcytosis is present with many transcytotic vesicles in the endothelial cells, resulting in a leaky, immature barrier between the blood and retinal parenchyma. By P5 (middle), there is a spatiotemporal gradient of a functional BRB formation. The more mature, proximal vessels have both functional tight junctions and suppression of transcytosis to establish a functional barrier. However, the nascent, distal vessels have functional tight junctions but display bulk transcytosis, resulting in a leaky, immature barrier. By P10 (bottom), the entire retinal vasculature has functional tight junctions and suppressed transcytosis, resulting in a functional BRB throughout the vasculature.

Method S1:

```
//This Imagej macro quantifies the area of dye and the area of vessels in retina images
taken from Olympus FV1200 (.oib files) using 0.75 NA 20x Objective.
//The analysis may change accordingly if other microscopes and file types are used.

//initialize ImageJ
run("Colors...", "foreground=white background=black selection=yellow");
run("Options...", "iterations=1 count=1 black edm=Overwrite");

//choose folder with raw image files
rawdir=getDirectory("Choose Raw Data Folder");
//rchoose folder to save your dataset
resultdir=getDirectory("Choose Result Data Folder");
run("Set Measurements...", "area limit display redirect=None decimal=3");

//Batch process raw images
var f;

list=getFileList(rawdir);
for(f=0;f<list.length;f++)
 {
  open(rawdir+list[f]);
  process();
 }

//Saves the output as an excel file
selectWindow("Summary");
saveAs("Text", resultdir+"Result.xls");

//This macro assumes images are in z-stacks and asssumes "channel one" is blood
vessel and "channel two" is tracer
function process()
 {
  //max z project
  //split channels and rename
  //run("Stack to Images");
  run("Z Project...", "projection=[Max Intensity]");
  run("Subtract Background...", "rolling=100");
  run("Gaussian Blur...", "sigma=2");
  run("Split Channels");
  //Determine Vessel Area
  selectWindow("C1-MAX_"+list[f]);setAutoThreshold("Default
dark");setOption("BlackBackground", true);run("Convert to Mask");saveAs("Tiff",
resultdir+list[f]+"_Vessel_Mask.tif");run("Analyze Particles...", "size=10-Infinity
summarize");
```

```
  //Determine Tracer Area
  selectWindow("C2-MAX_"+list[f]);setAutoThreshold("Default
dark");setOption("BlackBackground", true);run("Convert to Mask");saveAs("Tiff",
resultdir+list[f]+"_Tracer_Mask.tif");run("Analyze Particles...", "size=10-Infinity
summarize");
  run("Close All");
}
```