

# A Murine Toolbox for Imaging the Neurovascular Unit

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## ABSTRACT

The neurovascular unit (NVU) coordinates many essential functions in the brain including blood flow control, nutrient delivery, and maintenance of BBB integrity. These functions are the result of a cellular and molecular interplay that we are just beginning to understand. Cells of the NVU can now be investigated in the intact brain through the combined use of high-resolution *in vivo* imaging and non-invasive molecular tools to observe and manipulate cell function. Mouse lines that target transgene expression to cells of the NVU will be of great value in future work. However, a detailed evaluation of target cell specificity and expression pattern within the brain is required for many existing lines. The purpose of this review was to catalog mouse lines available to cerebrovascular biologists and to discuss their utility and limitations in future imaging studies.

**KEY WORDS:** cerebral blood flow, capillary, arteriole, cre recombinase, pericyte, endothelium, astrocyte, vascular smooth muscle, two-photon microscopy, vasculature

**Abbreviations used:** AAVs, adeno-associated virus; BBB, blood-brain barrier; Chat, choline acetyltransferase; ChR2, channelrhodopsin-2; CSPG4, chondroitin sulfate proteoglycan 4; Cx40, connexin 40; eGFP, enhanced green fluorescent protein; FRET, fluorescence resonance energy transfer; GABA, gamma-aminobutyric acid; GECIs, genetically encoded calcium indicators; GFAP, glial fibrillary acidic protein; IRES, internal ribosome entry site; MLCK, myosin light chain kinase; NG2, neural/glial antigen 2; NO, nitric oxide; NPY, neuropeptide Y; NVU, neurovascular unit; PDGFR $\beta$ , platelet-derived growth factor receptor- $\beta$ ; PV, parvalbumin; SMC, smooth muscle cell; smMHC, smooth muscle myosin heavy chain; SST, somatostatin; TPLSM, two-photon laser-scanning microscopy; VIP, vasoactive intestinal peptide; vWF, von Willebrand factor; YFP, yellow fluorescent protein;  $\alpha$ -SMA,  $\alpha$ -smooth muscle actin.

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## INTRODUCTION

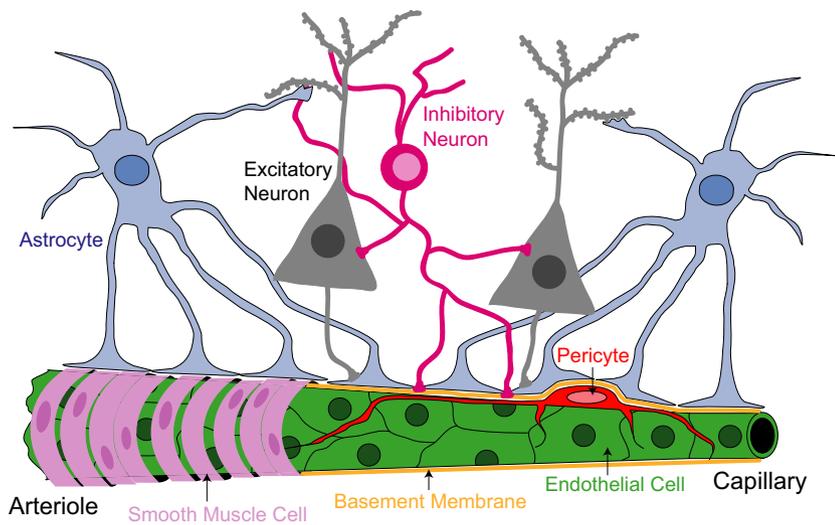
The cerebrovascular endothelium is ensheathed in several layers of cells including pericytes, vascular SMCs, astrocytes, and perivascular neurons (Figure 1). This assembly of cells, collectively referred to as the neurovascular unit (NVU), works in concert to serve many purposes including regulation of cerebral blood flow, nutrient delivery, maintenance of BBB integrity, and vascular remodeling after injury. The mechanisms by which NVU cells communicate to perform these tasks remain poorly understood.

*In vivo* TPLSM enables precise measurement of cellular and vascular activity in the intact rodent brain and is thus a key tool for dissecting the specific functions of NVU components [34,66,116]. Since TPLSM is a fluorescence-based technique [31], the presence of fluorescent molecules is a prerequisite for its use in chronic preparations [124]. Future *in vivo* studies will therefore rely heavily on an expanding catalog of transgenic mice, many of which arise

from crossings between promoter-specific Cre lines and reporter lines that express bright fluorescent proteins [78], GECIs [139] and optogenetic actuators [77]. The field of cerebrovascular biology will see great advances as these tools become better characterized and more widely adopted by researchers. In this review, we discuss existing transgenic mouse lines useful for labeling cells of the NVU (Table 1). We further highlight key *in vivo* imaging studies and address the potential limitations and opportunities that come with non-specific expression.

## PRIMER FOR GENETIC MOUSE TOOLS

Many transgenic mice carry ectopic genes, i.e., transgenes, driven by specific promoters to limit expression to specific cell types of interest. The generation of these mice falls into two general categories [56]. In the knock-in approach, transgenes can be spliced directly into the coding sequence of an endogenous gene, effectively hijacking its promoter to



**Figure 1.** Schematic of the NVU at the arteriole and capillary level. The NVU is an assembly of cells, comprised of endothelial cells (green) and pericytes (red) ensheathed by a basement membrane (yellow), vascular smooth muscle cells (lilac), surrounded by astrocytic endfeet (blue) that make contact with excitatory neurons (black). Inhibitory neurons (magenta) have been reported to synapse directly onto the basement membrane [127].

express another product. This approach tends to reproduce the expression pattern of the native gene, but can potentially lead to unwanted side effects due to loss of target gene function. Some lines avoid this issue by using an IRES so that the endogenous gene and transgene can be expressed bicistronically from the same mRNA, but potentially at the cost of reduced expression efficiency. A second approach involves use of a bacterial artificial chromosome to insert the transgene along with its own promoter sequence and cis-acting elements at a random locus within mouse genome. This method allows insertion of large DNA cassettes, but may lead to substantial variability between founder lines due to modulation of expression by additional *cis*-acting elements near the insertion site as well as variation in transgene copies inserted [36].

While several mouse lines described in Table 1 directly express a transgene for cellular monitoring or modulation, the vast majority express Cre recombinase, a powerful tool for genetic manipulation [89]. Cre recombinase deletes, inverts, or translocates segments of DNA between short, specific nucleotide sequences called *loxP* sites. When crossed with “reporter” lines, Cre activates transgene expression by removing floxed (flanking *loxP* sites) STOP codons that gate expression of the coding sequence. To achieve more control over when Cre activity is initiated, some lines express a variant of Cre recombinase that is fused to the estrogen receptor, which prevents the entry of Cre into the nucleus where recombination must occur. Several versions of the Cre-estrogen receptor fusion protein exist, Cre-ER<sup>T2</sup> being the most common [35]. Post-natal administration of tamoxifen, an estrogen receptor ligand, then permits Cre to enter the nucleus and initiate recombinase activity.

Viruses also serve as an efficient method to deliver transgenes to neurons and astrocytes of the brain [75]. In

this case, specificity of expression can be achieved by using minimal promoters also carried by the virus, or by gating expression with *loxP* sequences that can only be activated when the virus is injected into Cre lines. While not discussed in this review, *in utero* electroporation of DNA vectors is also being rapidly adopted for transgene delivery to neurons and astrocytes [111].

## ENDOTHELIAL CELLS

Vascular endothelial cells serve many important functions in the brain including BBB formation and selective metabolite trafficking to and from the blood [51]. Several transgenic mouse lines have been generated to target the vascular endothelium, the most common of which uses the promoter for Tie2, an endothelium-specific receptor tyrosine kinase that binds angiotensin-1 [41]. In Tie2-GFP mice, GFP fluorescence is uniformly distributed throughout the cerebrovascular endothelium [131]. These mice were crossed with SMC and pericyte-labeled NG2-DsRed mice (see below) to discern between subsurface arterioles and venules in studies on CSF flow within the paravascular space [58]. Similarly, these mice have been used in conjunction with exogenous astrocyte-specific dyes to visualize the layers of the vascular wall *in vivo* [81]. Tie2-GFP mice can be advantageous over injectable plasma-labeling dyes when the goal is to chronically image vascular structure as it avoids problems associated with dye leakage [116]. These mice have been used to track angiogenesis during development [131] and in response to hypoxia [79], as well as to elucidate mechanisms of microvascular embolus extravasation [70]. Variants of this mouse line include a Tie2-claudin-GFP line, in which the tight-junction protein claudin is fused with enhanced green fluorescent protein (eGFP), and has been used to track early BBB changes in stroke [67]. Tie2-Cre [64] and inducible

**Table 1.** NVU-specific mouse lines expressing cre or fluorescent proteins

Promoter	Confirmed vascular cell types	Notes	Repository and stock number (if commercially available)
Tie2-GFP [85] Tie2-Cre [64] Tie2-CreER <sup>T2</sup> [39]	EC	Tie2-GFP is well characterized, specific and widely used for imaging the brain endothelium. Tie2-Cre crossed with reporter mouse produces specific, widespread endothelial cell labeling in brain [92]. Brain expression with the Tie2-CreER <sup>T2</sup> line is not well characterized	GFP: 003658 Cre: 008863 CreER <sup>T2</sup> : EMMA:00715 Mugen: M201002
Ephrin-B2-H2BGFP [30]	EC	Predominantly expressed in endothelium of cerebral arterioles and capillaries with faint expression in veins [86]. Individual endothelial cell nuclei can be observed	007843
hVWF-Cre [26]	EC	Not characterized in detail. Cross with Z/EG reporter showed expression only in brain endothelium	
VE-Cad-Cre [3] VE-Cad-CreER <sup>T2</sup> [83]	EC	Not characterized in detail. Crossing of the inducible line with a LacZ reporter mouse reveals widespread expression in cerebral vasculature [14]	Cre: 006137
PDGFB-iCreER <sup>T2</sup> [25]	EC	Cross with fluorescent reporter leads to widespread labeling of cerebral microvessels [21]	
Cx40-GCaMP2 [120]	EC	First generation genetically encoded calcium sensor exhibits widespread endothelium expression. Has not been used in brain	
Alk1-GFP-Cre [91]	EC	Not characterized in detail. Extensive labeling of brain vasculature when crossed with a lacZ reporter mouse	
NG2-DsRed [141] NG2-mEGFP [57] NG2-Cre [141] NG2-CreER <sup>TM</sup> [142] NG2 <sup>+YFP</sup> (knock-in) [62]	PC, SMC, N	NG2-DsRed most commonly used to image individual pericytes in brain, though NG2-CreER <sup>TM</sup> mice label a similar but less complete profile of cells when crossed with fluorescent reporters. Constitutive NG2-Cre mice reveal extensive neuronal labeling when crossed with Ai14, and may not be useful for imaging (Figure 2). Oligodendrocyte precursors are also targeted by the NG2 promoter	DsRed: 008241 mEGFP: 022735 Cre: 008533 CreER <sup>TM</sup> : 008538
B-actin-GFP [98]	PC, EC	Brain pericyte and endothelial cells are broadly labeled for <i>in vivo</i> imaging of microvascular walls [37]. Identity of other labeled cell types unclear	003291
Rgs5 <sup>+GFP</sup> (knock-in) [93]	PC	Evidence of selective labeling of individual brain pericytes [99]. Homozygous mouse shows altered hemodynamics	
PDGFR $\beta$ -Cre [27]	PC, SMC, EC, N	Targeting of SMCs is well established. Pericytes, endothelial cells, and some neurons are known to be targeted, but extent of expression is not well characterized (personal communication with V. Lindner).	
$\alpha$ -SMA-GFP [137] $\alpha$ -SMA-RFPCherry [104] $\alpha$ -SMACre [133] $\alpha$ -SMACreER <sup>T2</sup> [130] $\alpha$ -SMA-exMLCK [59]	PC, SMC	RFPCherry variant shows widespread labeling of arterial SMCs in brain, but pericytes of classical morphology were not reported. The GFP variant has not been evaluated in the brain. The inducible Cre line showed SMC expression when crossed with reporters, but other cell types were not examined in detail. The exMLCK line expresses a FRET-based Ca <sup>2+</sup> -sensor, and has not been used to study cerebral vasculature	
smMHC-Cre [107] smMHC-Cre-GFP [134] smMHC-CreER <sup>T2</sup> [132] smMHC-GCaMP2 [60]	PC, SMC, A, N	Crossing the Cre-GFP line with Ai14 reporters reveals sparse labeling of individual pericytes, astrocytes, and neurons, in addition to widespread but incomplete labeling of SMCs (A. Shih and D. Kleinfeld, unpublished). The GCaMP2 expressing line has been used to visualize SMC calcium dynamics <i>in vivo</i> , and appears to only label SMCs [65]	Cre-GFP: 007742 CreER <sup>T2</sup> : 019079
	SMC		Cre: 004746

Table 1. Continued

Promoter	Confirmed vascular cell types	Notes	Repository and stock number (if commercially available)
SM22 $\alpha$ / transgelin-Cre [54, 73] SM22 $\alpha$ / transgelin - CreER <sup>T2</sup> [69]		Constitutive Cre version may not label vasculature of the brain [40], but further characterization warranted. Inducible Cre version also not yet characterized or used in brain studies	
hGFAP-Cre [145] mGFAP-Cre [43] hGFAP-CreER [24] hGFAP-CreER <sup>T2</sup> [42, 53] hGFAP-eGFP [96, 144]	A	Crossing constitutive hGFAP-cre mice with reporters reveals neuronal expression, due to early Cre activity in neuronal progenitors [76]. Inducible forms appear to label individual astrocytes more specifically [12]. GFAP-eGFP mice label a subset of astrocytes in most CNS structures, and have been used to image individual astrocytes and their perivascular endfeet <i>in vivo</i> [58]. There is a labeling bias toward activated astrocytes, which express higher levels of GFAP	hGFAP-Cre: 004600 mGFAP-Cre: 012886 hGFAP-CreER <sup>T2</sup> : 012849 hGFAP-eGFP: 003257
GLASTCreER <sup>T2</sup> [84, 117, 129] GLAST-DsRed [106]	PC, A	Crossing Pflieger lab strain [117] with YFP reporter enabled imaging of individual pericytes in spinal cord that serve a scar-forming function [45]. Brain pericyte expression not reported. Götz and Nathans laboratory strains predominantly express Cre in protoplasmic astrocytes and in a minority of other cell types [84]. GLAST is expressed in some GFAP-negative cells, and extent of labeling can be varied by altering tamoxifen dose [106]. DsRed variant only expresses well in cerebellum [106]	CreER <sup>T2</sup> : 012586 (Nathans lab)
Glt1-eGFP [106]	A	Labels nearly all GFAP-positive cells in cortex. Has been used to label individual astrocytes and their perivascular endfeet [105]	
Aldh111-GFP [44] Aldh111-tdTomato [44] Aldh111-Cre [44]	A, N	Labels a greater number of astrocytes than do GFAP lines [8]. Aldh111-GFP line labels occasional oligodendrocytes and is more specific but less bright than Glt1-GFP animals [135]. Aldh111-Cre animals occasionally label some oligodendrocytes, neurons, and neural stem cells [38]. TdTomato variant has not been characterized	GFP: MMRRC: 011015-UCD tdTomato: MMRRC: 036700-UCD Cre: 023748
Cx30-CreER <sup>T2</sup> [117]	A	Crossing with a reporter enabled visualization of individual astrocytes in all brain regions. Appears specific to astrocytes	
FGFR3-iCreER <sup>T2</sup> [138]	A, N	Highly efficient, widespread labeling of astrocytes with occasional neurons and oligodendrocytes also labeled. The ability to image individual cells depends in part upon the reporter mouse used	
PV-Cre [52] PV-ChR2-eYFP [140]	N	Nearly all neurons expressing Cre were GABAergic but some glutamatergic were noted [125]. Optogenetically stimulating PV interneurons elicits vasoconstriction <i>in vitro</i> . A ChR2-eYFP line may be useful to translate these findings <i>in vivo</i>	Cre: 008069 ChR2-eYFP: 012355
5-HT3A-GFP [44]	N	Highly specific to 5-HT3a-expressing subtype of GABAergic neurons [72]. Individual cells were imaged in superficial cortex. These neurons release vasoconstrictive and vasodilatory molecules [100]	MMRRC: 000273-UNC
SST-ires-Cre [122] SST-CreER [122]	N	Targets somatostatin-expressing interneurons. The CreER version exhibits low recombination efficiency compared to the constitutive driver	Cre: 013044 CreER <sup>T2</sup> : 010708
VIP-ires-Cre [122]	N	Targets a small subset of VIP-expressing interneurons	010908
nNOS-CreER <sup>T2</sup> [122]	N	Labels both Type I and II neuronal NO synthase-expressing interneurons when crossed with Ai9 reporters	014541
	N		

Table 1. Continued

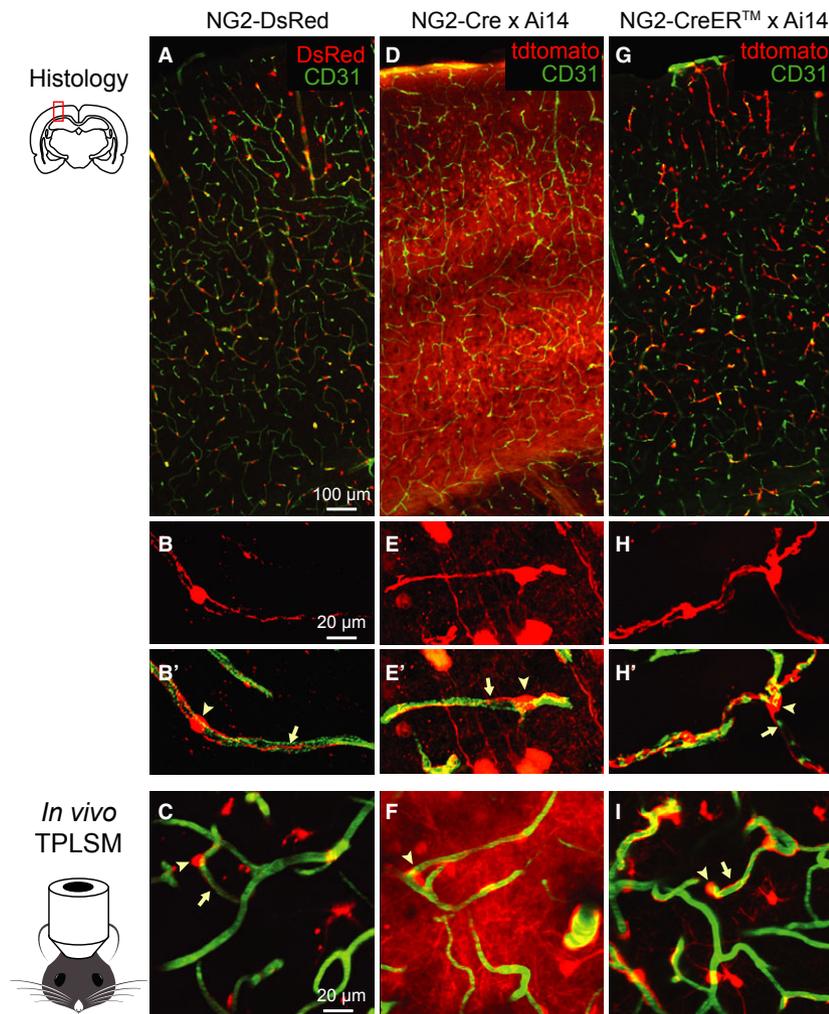
Promoter	Confirmed vascular cell types	Notes	Repository and stock number (if commercially available)
Chat-ires-Cre [108] Chat-CreER [109] Chat-eGFP [121] Chat-mhChr2-YFP [140]		Targets cholinergic neurons of basal forebrain, cortex, striatum, and other brain structures. The cortices of mhChr2-YFP and eGFP mice are densely packed with transgene expressing fibers projecting from the basal forebrain. The inducible CreER mice exhibit sparse recombination enabling visualization of full axon and dendritic arbors of cholinergic neurons. These complementary tools have not yet been used to study cholinergic modulation of cerebral blood flow <i>in vivo</i>	Cre: 006410 CreER: 008364 eGFP: 007902 mhChr2-YFP: 014546
Thy1-YFP-H [36] Thy1-GFP-M [36] Thy1-GCaMP3 [22] Thy1-ChR2-YFP [4] Thy1-NpHR 2.0-YFP [123]	N	Specific for projection neurons in CNS. Labeling in cortex for Thy1-YFP-H and primarily restricted to layer 5 neurons, and sparse layer 2/3 neurons. Thy1-GFP-M exhibits very sparse labeling that permits individual neurons and processes to be observed. Thy1-GCaMP3 mice are useful for calcium imaging. Thy1-ChR2-YFP mice have been used to optically evoke hemodynamic responses	YFP-H: 003782 GFP-M: 007788 GCaMP3: 017893 ChR2-YFP: 007612 NpHR 2.0-YFP:- 012334
<b>Reporter lines</b>			
<i>Fluorescent reporters</i> Ai 3/6/9/14 [78] Z/EG [97] mT/mG [87] RCE:dual [118]		Cre-dependent expression of fluorescent proteins is possible by placing a floxed transcriptional STOP sequence upstream of a fluorescent protein coding sequence and downstream from a strong, universal promoter such as that of chicken $\beta$ actin (CAG) or Rosa26. This is the genetic basis for nearly all mouse reporter lines. Some reporter lines such as Z/EG and mT/mG have the additional feature that cells will switch from the expression of one transgene to another when Cre-recombinase is active. There are many generations of fluorescent reporter mouse lines (reviewed in [1]), the most recent of which come from the Allen Institute, which offer the brightest and most universal labeling to date [78]. The Ai9/Ai14 reporters are bright, high sensitivity reporters expressing tdTomato, whereas Ai6 expresses eGFP and Ai3 expresses YFP. RCE:dual mice carry two STOP codons which can be removed independently by Cre of <i>Flo</i> recombinases, leading to eGFP expression	Ai3: 007903 Ai6: 007906 Ai9: 007909 Ai14: 007914 Z/EG: 003920 mT/mG: 007576 RCE:dual: MMRRC 032036-JAX
<i>Cell activator or inhibitor</i> Ai27/Ai32 [77] Ai35/Ai39 [77]		These lines are useful to target optogenetic actuators to NVU cells. A27 (ChR2-tdTomato) and Ai32 (ChR2-YFP) drive the expression of light-gated ion channel ChR2, to depolarize target cells. Ai35 (Arch3-GFP) and Ai39 (NpHR3.0-EYFP) express a light-gated proton pump and chloride pump, respectively, for hyperpolarizing target cells	Ai27: 012567 Ai32: 012569 Ai35: 012735 Ai39: 014539
<i>Cell activity sensors</i> Ai38 [139] Ai95/Ai96 Ai78D		The Ai38 line enables expression of the genetically encoded calcium sensor GCaMP3 in target cells. A new variant, GCaMP6, exhibits superior sensitivity to older versions and a reporter mouse line was being developed at the time of writing. Lines optimized for fast (GCaMP6f, Ai95) and slow calcium transients (GCaMP6s, Ai96) will be available. The Ai78D line enables expression of the FRET-based voltage-sensitive fluorescent protein Butterfly 1.2 that has been used for single-cell resolution of voltage fluctuations <i>in vivo</i> and was being developed at the time of writing [2]	Ai38: 014538 Ai95: 024105 Ai78D: 023528

A, astrocyte; EC, endothelial cell; N, neuron; PC, pericyte. Repository stock numbers are from The Jackson Laboratory, unless otherwise noted. Other repositories include (1) Integrated Functional Genomics in Mutant Mouse Models as Tools to Investigate the Complexity of Human Immunological Disease (Mugen), (2) European Mutant Mouse Archive (EMMA), and (3) Mutant Mouse Regional Resource Centers (MMRRC). If a mouse line is not commercially available, the principal investigator whose lab developed the mouse may offer assistance.

Tie2-CreER<sup>T2</sup> mice [39] will be useful to drive transgenes for observing and manipulating endothelial cell activity *in vivo*, though reports of their use in brain are currently scarce.

Mouse lines that provide sparse cell labeling may be useful when intending to track or count individual endothelial cells. While VE-cadherin-Cre mice have not been widely used for live imaging, histological evidence

suggests that a sparse labeling of endothelium can be achieved when crossed with YFP reporter mice [88]. Similarly, heterozygous ephrinB2-H2B-GFP knock-in mice [30] appear to have sparse labeling of endothelial cell nuclei, and have been used to study growth of arteriovenous malformations resulting from hyperactive Notch4 signaling [86].



**Figure 2.** NG2 mice for visualizing pericytes *in vivo*. Images taken from adult brains of three different mouse lines: NG2-DsRed (left column); NG2-Cre crossed with Ai14 tdTomato reporter mice (middle column); and NG2-CreER<sup>TM</sup> crossed with Ai14 and induced with tamoxifen in adulthood (right column). Tamoxifen was administered intraperitoneally at a dose of 100 mg/kg dissolved in corn oil:ethanol (9:1), every 24 hours for five consecutive days. Mice were killed for histology within two weeks after the last injection of tamoxifen. Brain sections (50  $\mu$ m thick) from all three lines were stained with anti-CD31 antibody to label the endothelium and imaged with wide field fluorescence (A, D, G), and confocal microscopy (B, B', E, E', H, H'). Confocal images (maximal projections of 20- $\mu$ m-thick stacks) are shown in the red channel alone to highlight fluorescent protein expression (B, E, and H), and with CD31 to show perivascular location of pericytes (B', E' and H'). Using confocal microscopy of histological slices, ovaloid pericyte cell bodies (arrowheads) are visible in all lines. Fine pericyte processes that radiate along the microvasculature, however, are most easily visualized with an NG2-CreER<sup>TM</sup>  $\times$  Ai14 cross (arrow). *In vivo* images (maximal projections of 50  $\mu$ m stacks taken 25–75  $\mu$ m below the pial surface) were collected through a PoRTs window using TPLSM (C, F and I) [116]. The vascular serum (green) is labeled with intravenous FITC-dextran (2 MDa) [116]. Pericytes are difficult to discern in the NG2-Cre x Ai14 line due to excessive background neuronal labeling. In comparison, NG2-CreER<sup>TM</sup>  $\times$  Ai14 and NG2-DsRed animals exhibit very sparse labeling of pyramidal neurons, and possible interneurons. Neurons appear distributed independent of cortical layer, and their number increases with time after tamoxifen induction. All animals label oligodendrocyte precursors and vascular SMCs.

In order to observe calcium transients in arteriolar endothelial cells, the first generation GECI GCaMP2 was placed under transcriptional control of Cx40, a gap junctional protein [120]. We are not aware of any evidence showing that Cx40-GCaMP2 is expressed in the brain of these mice, but if so, this line may represent an opportunity to investigate how endothelial activity is coordinated with the activity of other NVU cells. To make use of new, more

sensitive GCaMP variants, endothelial Cre driver lines may also be crossed with the Ai38/Ai95 (GCaMP3/GCaMP6) reporter lines to achieve a similar type of expression pattern (Table 1) [139].

A number of endothelial Cre lines require further characterization for *in vivo* imaging, but appear to be useful driver lines based on histological data. PDGFB-iCreER<sup>T2</sup> animals show remarkably complete labeling of the brain

endothelium when crossed with reporter mice [21]. The Alk1-GFP-Cre knock-in mouse exhibits widespread Cre activity in cerebral arteries and veins [91]. Finally, vWF-Cre mice exhibit endothelial expression in the brain but not other organs [26].

## PERICYTES

Pericytes play important roles in vascular development [28], BBB integrity [5], and regulation of blood flow [48]. Deficiencies in pericyte function accelerate neurodegeneration secondary to vascular degradation [11,110], providing an impetus to advance our understanding of pericyte action in the NVU.

The most common promoter used to drive transgene expression in pericytes is that of CSPG4, a gene that codes for the transmembranous proteoglycan NG2. One mouse line driving expression of DsRed, a red fluorescent protein, under the NG2 promoter (Figure 2A–C) (NG2-DsRed) was recently used to visualize pericyte location along the brain microvasculature using TPLSM. This enabled studies on the role of pericytes in cortical blood flow control [48]. Due to the promiscuity of the NG2 promoter, oligodendrocyte precursors and SMCs are also labeled in these mice [94], but pericytes remain easily distinguishable based on their morphology and apposition to the microvasculature. An NG2-Cre driver line is available to express other transgenes in pericytes [141]. In practice, crossing constitutive NG2-Cre mice with the tdTomato reporter line Ai14 [78] leads to labeling of the same cells seen in the NG2-DsRed, but also a large number of neurons, which makes detection of pericytes difficult *in vivo* due to high background fluorescence (Figure 2D–F). The use of inducible NG2-CreER<sup>TM</sup> mice circumvents this problem by initiating Cre activity after a time when neural precursors are no longer NG2-positive [142]. NG2-CreER<sup>TM</sup>-Ai14 bigenic mice may be more useful than the NG2-DsRed mice for visualizing the fine structure of pericytes *in vivo* because tdTomato exhibits brighter fluorescence than DsRed (Figure 2G–I) [114]. Finally, YFP has also been knocked in downstream of the endogenous mouse promoter in NG2<sup>+/YFP</sup> mice, providing another means to visualize pericytes with fluorescence [62]. However, the effect of losing an NG2 allele has not been investigated.

Promoters for other pericyte markers have been used to drive Cre or fluorescent protein expression. For instance, regulator of G protein signaling 5 knock-in GFP mice (RGS5<sup>+/GFP</sup>) appear to have selective brain pericyte labeling based on histology [99]. However, homozygous RGS5<sup>GFP/GFP</sup> mice show a reduced mean arterial pressure in comparison to the wild-type, and this should be considered if investigating hemodynamics. While GLAST-CreER<sup>T2</sup> mice are predominantly used in the study of cerebral astrocytes [117] (see below), crossing these mice with R26R-YFP reporter mice

selectively labels pericytes in the spinal cord. These pericytes co-express accepted pericyte markers PDGFR $\beta$  and CD13, and show ultrastructural features of pericyte morphology [45]. With regard to other potential pericyte-targeting lines, PDGFR $\beta$ -Cre mice exhibit efficient targeting of brain SMCs, but brain pericyte expression has not been reported in detail [27]. Additionally,  $\alpha$ -SMA, has been used as a marker for brain and peripheral pericytes [37,136]. Alpha-SMA-RFP-cherry mice were used to study pericytes in peripheral tissues [104], but expression in brain pericytes remains unclear. Similarly,  $\alpha$ -SMA-CreER<sup>T2</sup> mice are useful for targeting SMCs, but their specificity for brain pericytes remains unknown [130].

## SMOOTH MUSCLE CELLS

Vasoreactivity during functional hyperemia is mediated by relaxation and constriction of SMCs [90], making these cells a critical player in the regulation of blood supply. SMCs also have important signaling and transport roles in the brain, the disruption of which is involved in pathologies such as arteriovenous malformations and cerebral amyloid angiopathy [10,143].

Alpha smooth muscle actin, PDGFR $\beta$ -, and NG2-Cre driver mice, as described above for pericyte targeting, are known to target SMCs. Additional SMC targeting promoters include (smMHC or Myh11) and SM22 $\alpha$ /transgelin. There are two independent mouse lines utilizing an smMHC promoter to drive Cre [107,134], both of which were reported to have Cre expression in cerebral arteries based on crosses with R26R-LacZ reporter mice. The smMHC-Cre-GFP line from the Kotlikoff lab expresses a bicistronic transgene consisting of both Cre and eGFP, enabling direct visualization of SMCs by fluorescence imaging. However, crossing these mice with reporter lines (i.e., Ai14) will reveal other cell types including sparsely distributed neurons, astrocytes, and pericytes (A. Shih and D. Kleinfeld, unpublished findings). The same laboratory also generated a line expressing GCaMP2 under the smMHC promoter offering the ability to visualize relative calcium changes within SMCs *in vivo* [60,65]. With a similar goal, the  $\alpha$ -SMA promoter was used to drive the expression of MLCK fused with a construct containing CFP and YFP linked by a Ca<sup>2+</sup>-calmodulin binding site. This enabled FRET-based measurements of SMC calcium that are more robust to movement artifacts and can be potentially calibrated *ex vivo* to obtain absolute calcium concentrations [80]. Three different SM22 $\alpha$ /transgelin mouse lines exist, though only one has been crossed with a reporter mouse. This cross with the R26R-LacZ reporter line revealed Cre activity in some brain structures, [40,54] but cerebrovascular labeling was not described. Crossing these mice with bright fluorescent reporter lines may be necessary to more thoroughly evaluate vascular expression.

Central nervous system expression of Cre in the other SM22 $\alpha$  mouse lines remains undetermined [69,73].

## ASTROCYTES

Astrocytes are a heterogeneous population of cells in the CNS with specialized functions ranging from synaptic signaling to blood flow regulation. Here we will focus on a subset of Cre lines that have been used for *in vivo* imaging studies of the NVU. For comprehensive reviews on the numerous Cre drivers for astrocyte targeting, we refer the reader to previous literature [74,102].

The promoter of human and mouse GFAP, a classic astrocyte marker, has been used to generate Cre mouse lines [43,145]. With the hGFAP promoter, Cre is expressed in embryonic radial glia [145], which gives rise to Cre-expressing neurons in the adult [53]. Greater astrocyte specificity may be achieved by using one of the many available inducible hGFAP-CreER<sup>T2</sup> lines [24,42,53], or by using Cre lines driven by the murine GFAP promoter [47].

Two independently generated hGFAP-GFP mouse lines [96,144] have been used for *in vivo* studies on NVU function. Adult hGFAP-GFP mice exhibit relatively sparse labeling of cortical astrocytes, but not neurons, allowing individual cells to be distinguished during imaging [96]. Calcium-sensitive dyes were used in conjunction with the Messing lab GFAP-GFP mice in the discovery that odor detection leads to concurrent astrocyte calcium elevation and vasodilation in the olfactory glomerulus [101]. Using a traumatic brain injury model, the edematous swelling of astrocytic endfeet and its relationship with local capillary flow could be addressed in the mice generated by the Kettenmann laboratory [119]. Unlike the GFAP-GFP line, which labels the relatively small percentage of astrocytes with high GFAP expression, it has been reported that GFP is visible in all cortical astrocytes in Aldh1l1-GFP mice, including astrocytes near microvessels [8].

GLAST-CreER<sup>T2</sup> mice, which drive expression of inducible Cre under the promoter of an astrocyte-specific glutamate transporter, predominantly targets protoplasmic astrocytes when crossed with reporter mice, but also label occasional oligodendrocyte precursors and neurons [84]. Importantly, there is some control over labeling density as a varying percentage of astrocytes are visible depending on the dose of tamoxifen administered [84]. GLAST-CreER<sup>T2</sup> mice have enabled studies on astrocyte migration and proliferation in perivascular niches following cortical stab wound injury [8]. The promoter for Glt1, a gene that encodes another astroglial glutamate transporter, is active in a different subset of astrocytes than the GLAST promoter, and appears to have a very similar distribution of expression as GFAP [106]. Astrocytic endfeet are clearly labeled in Glt-1-GFP mice [105]. Newer mouse lines are also now available such as

Cx30-CreER<sup>T2</sup> and Fgfr3-CreER<sup>T2</sup> that show highly specific astrocyte labeling throughout the brain [117,138], making them promising tools for future imaging studies.

Calcium transients within astrocytes are thought to mediate activity-induced vasodilation and/or vasoconstriction [7]. However, there remains some debate on this idea [13,95]. The newly engineered calcium sensor GCaMP6 exhibits increased sensitivity over the organic dye Oregon Green Bapta 1-AM, and greatly increases the ability to observe small, restricted signaling events that may be important for blood flow control [23]. The Khakh laboratory further engineered Lck-GCaMP3 (and more recently Lck-GCaMP6) to tether the sensor to the plasma membrane, which unveils a greater diversity of calcium transients in astrocytic processes and endfeet [50,115]. These tools have been efficiently targeted to astrocytes using adeno-associated virus (AAVs) driving expression under a minimal hGFAP promoter.

## NEURONS

The mechanisms responsible for neural activity-induced changes in blood flow are still being elucidated, but it is generally accepted that neurons can alter blood flow directly, through release of vasoactive neurotransmitters onto the vasculature [17,127], or indirectly through astrocytic vasoactive pathways [7]. We briefly discuss mouse and viral strategies that have been used to study neuronal modulation of blood flow. For comprehensive reviews on tools and genetic strategies to monitor and manipulate neuronal populations *in vivo* we refer the reader to excellent resources [6,49,56,122].

There is evidence that certain interneuron subtypes directly contact the basement membrane of brain microvessels and modulate vessel flux by releasing the vasoactive substances NO, NPY, SST, VIP, and gamma-aminobutyric acid (GABA) [18,68]. However, the role that these interneuron subtypes play in blood flow control remains poorly understood. PV-Cre mice have been used to selectively express the light-gated cation channel ChR2 [15] in GABAergic fast-spiking interneurons by transducing the brain with AAVs carrying floxed ChR2. Optically activated PV interneurons were found to elicit a positive BOLD signal surrounded by zone of negative signal, suggesting that both dilatory and constrictive vascular changes were evoked [33,71]. Consistent with this, it was found that optical activation of ChR2-expressing PV neurons consistently led to arteriole constriction in brain slices [125]. Inhibitory neuron activity is thus involved in shaping of the hemodynamic response. The PV-Cre line appears highly specific for PV expressing interneurons, though a small fraction of pyramidal neurons may also be targeted [52,125]. Interneurons expressing the ionotropic serotonin receptor 5HT<sub>3</sub>AR, are

preferentially located near penetrating arterioles [100] and are capable of mediating vasodilation or vasoconstriction via NO or NPY, respectively, in response to serotonergic input. Somatostatin and VIP-expressing interneurons have been shown to influence arteriolar diameter in brain slices [18]. The *in vivo* role of these interneuron populations in blood flow regulation can be tested with an array of cell-specific, knock-in Cre driver mice [122] bred with reporter lines to express optogenetic tools [77]. An important strategy will be to optically silence interneuron subtypes with halorhodopsins to determine their role in NV coupling.

Cholinergic influences on cortical blood flow can be studied using Chat-Cre driver lines [108], as well as lines with expression of optogenetic actuators driven by the Chat promoter [61]. Release of acetylcholine from projections of the nucleus basalis of Meynert, e.g., generates global blood flow increase in cortex independent of local metabolism [112]. The axons of cholinergic neurons terminate near the vicinity of cerebral arterioles and microvessels, consistent with their strong effect on blood flow [126]. In addition to the basal forebrain, cholinergic interneurons of cortex, striatum, and other brain structures also express Cre-recombinase. These Chat transgenic mice have not been widely used in cerebral blood flow studies.

With respect to targeting excitatory neurons, the majority of recent studies have been performed using Thy1-ChR2-YFP mice. These animals exhibit strong ChR2-YFP expression in cortical layer 5 pyramidal neurons [4], enabling one to directly stimulate localized cortical areas with light from implanted optical fibers or collimated lasers, and observe the resulting hemodynamic response by TPLSM [32,113]. Conversely, Thy1-driven halorhodopsin lines such as the Thy1-eNpHR 2.0 mouse may be useful for selectively silencing brain regions [6]. In future work, a diverse catalog of Cre lines with improved targeting of specific neuronal subclasses and cortical layers will be useful for dissecting the cellular and anatomical basis of cortical blood flow control [49].

## LIMITATIONS AND OPPORTUNITIES

The density of cellular labeling is an important issue for *in vivo* imaging, as it becomes impossible to visually distinguish or optically activate individual cells when density of labeling is high. Furthermore, the ability to resolve fine structures, such as astrocyte processes, is limited because the excitation volume during TPLSM is typically larger than the fine structures under investigation. One example of how this can lead to ambiguous results is during calcium imaging of densely labeled tissue with organic calcium dyes. While cell bodies of individual astrocytes can be easily identified *in vivo*, the perceived somata are interweaved with neuronal processes that potentially contaminate the “astrocyte-specific” signal. Sparse cellular labeling is therefore necessary to reduce

signal contamination. An effective strategy to reduce labeling density is to use inducible driver lines, i.e., Cre-ER<sup>T2</sup> [35], which provides a “pulse” of Cre activation to induce, e.g., GECI expression after crossing with the Ai38 reporter. Finally, since the method of tamoxifen administration can influence the degree of recombination and thereby the density of labeling [84], it may be useful to reduce the concentration and/or number of repeated injections. Inducible Cre animals generally confer more specific labeling of target cells, albeit at a lower efficiency of recombination than lines with constitutively active Cre.

Another potential method to obtain sparse labeling is to use viruses with floxed transgenes [55]. Adeno-associated viruses are available as a variety of serotypes, each with a slightly different cell tropism, which can be diluted to transduce fewer cells [19,20]. One limitation, however, is that there are no reports of common viral vectors (i.e., AAVs, lentiviruses, adenoviruses) successfully infecting endothelial cells, pericytes, or SMCs *in vivo*. It is not clear whether these virus types lack the receptors to bind these cell types, or whether accessibility of the viral particle is limited by the adventitia, basement membrane, and astroglial endfeet. If viruses can be used to transduce a greater variety of vascular cell types *in vivo*, it would remove a significant bottleneck given the relative ease of swapping promoters and transgenes compared to generating new mouse lines.

Some experiments will require more complex genetic manipulations than binary recombination to ensure that transgenes are solely targeted to one cell type. For example, while endothelial cells appear to be selectively targeted by the Tie2 promoter, it is likely that specific expression of Cre in SMCs and pericytes will require intersectional strategies that place control under two promoters [56,75]. One possibility is to use the split Cre system, where Cre recombinase is expressed as two separate non-active proteins, each driven by a different promoter [16,128]. When both parts are expressed in the same cell, the proteins unite to form a functional recombinase. For example, more specific pericyte targeting may be achievable by expressing split Cre under the NG2 and PDGFR $\beta$  promoters, as was similarly carried out to target neural stem cells [9]. Another possibility is to use two recombinases, i.e., Cre-Flp, where both Cre (driven by promoter A) and Flp (driven by promoter B) would need to be active in the same cell to activate transgene expression [63,118,122]. This strategy requires dual reporter mice with two STOP codons preceding the coding sequence, one flanked by *loxP* and the other by *frt* sequences [82]. In some cases, the *frt* sequences flank the entire coding sequence to enable removal or replacement of the transgene [78]. This design is useful for subtraction strategies, where labeling specificity is increased by using a Flp driver to eliminate transgene expression in a subset of cells targeted by the Cre driver [56].

Absolute cell-specific transgene expression, however, is not essential for all experiments. If labeling is sparse overall, the cell of interest can still be distinguished based on morphology and/or location, as was the case with studies on pericyte regulation of blood flow [48]. Calcium imaging of isolated cells along the vasculature is also possible if they are well separated from other cells that may contaminate their signal. Optogenetic activation of isolated cells using spatially restricted two-photon excitation is becoming more efficient with new Chr2 variants [103].

Genetically targeting multiple NVU components for a single-imaging experiment would be valuable in determining the structural association of multiple components in 3-D space, or to observe flow of information from one cell type to another. Crossing multiple transgenic lines would thus be rewarding, but can be time-consuming and costly when using Cre strains because these usually require establishing stable Cre:reporter bigenic mice that are then bred with other mouse lines. Viruses on the other hand provide a rapid method to label astrocytes or neurons in an existing transgenic line [29]. For example, astrocytes can be easily transduced with AAVs carrying a minimal hGFAP promoter and used to drive opsins [46] or GECIs [50]. Viral transduction of mice, already expressing GCAMP3/6 in smooth muscle or pericytes through cross-breeding, might be a means to studying coupling of activity between neurons, astrocytes, and vascular cells during brain activity.

In summary, the maturation of several key technologies including *in vivo* TPLSM, a variety of reporter lines, and molecular tools to drive and observe cell activity provide unprecedented opportunities to dissect NVU function in the intact brain. However, the field would benefit with a critical re-evaluation of existing mouse lines that are potentially useful for such studies. We encourage researchers to (i) characterize “NVU” strains with a particular focus on transgene expression in cortical cerebrovascular system, and (ii) provide detailed reports on the outcome of new crosses that may be of utility to study neurovascular function *in vivo*, which includes at a minimum, information on the type of cells labeled in the adult mouse brain, the density of labeling, the breeding strategy and/or induction protocol to achieve consistent cellular labeling, and the age of the mice studied.

## PERSPECTIVE

There is an expanding array of new mouse and viral reporter tools available to observe and manipulate cells *in vivo*. To make use of these resources, a collective effort is necessary to better characterize existing “NVU” Cre driver lines for expression in the cerebrovasculature. A trusted toolbox of mouse lines that enables specific targeting of transgenes to individual cell types of the NVU will accelerate our understanding of neurovascular function in health and disease.

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## NOTE ADDED IN PROOF

An article by Lamantia, Tremblay and Majewska (Neurophotonics, 1(1), 011014, 2014) was recently published, but not yet available on Pubmed at the time of publication. This article provides an exemplary description of eGFP expression in the brains of Tg(Id3-EGFP)/FS137Gsat mice from GEN-SAT. Sparse expression with layer-dependent density was noted for astrocytes in the cortex of adult mice, which enabled *in vivo* imaging of fine astrocyte processes. Expression was also noted in the blood vessel wall, though it was not determined whether this was attributed to expression in endothelial cells, pericytes, astrocyte endfeet or all of the above. Animals with GECI expression driven under the Id3 promoter may be useful to study flow of information from astrocytes to blood vessels.

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