

# Ablation of astrocytic laminin impairs vascular smooth muscle cell function and leads to hemorrhagic stroke

Zu-Lin Chen, Yao Yao, Erin H. Norris, Anna Kruyer, Odella Jno-Charles, Akbarshakh Akhmerov, and Sidney Strickland

Laboratory of Neurobiology and Genetics, The Rockefeller University, New York, NY 10065

**A**strocytes express laminin and assemble basement membranes (BMs) at their endfeet, which ensheath the cerebrovasculature. The function of astrocytic laminin in cerebrovascular integrity is unknown. We show that ablation of astrocytic laminin by tissue-specific Cre-mediated recombination disrupted endfeet BMs and led to hemorrhage in deep brain regions of adult mice, resembling human hypertensive hemorrhage. The lack of astrocytic laminin led to impaired function of vascular smooth muscle cells (VSMCs), where astrocytes have a closer association with VSMCs in small arterioles, and

was associated with hemorrhagic vessels, which exhibited VSMC fragmentation and vascular wall disassembly. Acute disruption of astrocytic laminin in the striatum of adult mice also impaired VSMC function, indicating that laminin is necessary for VSMC maintenance. *In vitro*, both astrocytes and astrocytic laminin promoted brain VSMC differentiation. These results show that astrocytes regulate VSMCs and vascular integrity in small vessels of deep brain regions. Therefore, astrocytes may be a possible target for hemorrhagic stroke prevention and therapy.

## Introduction

The integrity of blood vessel walls is essential for proper functioning of the circulatory system and for preventing hemorrhage. The vascular walls in the central nervous system (CNS) are composed of an endothelial cell layer, basement membranes (BM), vascular smooth muscle cells (VSMCs) or pericytes, and astrocytic endfeet. The vascular BM is composed of laminin, collagen type IV, nidogen, thrombospondin, and various proteoglycans (Mohan and Spiro, 1986; Yurchenco and Patton, 2009). BM proteins play important roles in proliferation, migration, differentiation, survival, and function of cellular components of the vascular wall (Eble and Niland, 2009; Wagenseil and Mecham, 2009). Mutations in collagen type IV that affect vascular BMs can lead to intracerebral hemorrhage (ICH) in both mice and humans during development (Gould et al., 2005,

2006). Direct damage to the BM under pathological conditions such as ischemic stroke can also cause blood brain barrier (BBB) breakdown and hemorrhage (Wang and Shuaib, 2007; Zhang et al., 2007; Zhao et al., 2007; del Zoppo, 2009; Lo and Rosenberg, 2009).

Laminin, a major component of all BMs, are heterotrimeric molecules composed of  $\alpha$ ,  $\beta$ , and  $\gamma$  chains (Timpl and Brown, 1994; Miner and Yurchenco, 2004). Two laminin isoforms, laminins-111 and -211, which are produced by astrocytes, are found in the vasculature of the CNS (Jucker et al., 1996; Sixt et al., 2001). In addition, laminins-411 and -511 are expressed in the endothelial BM of most tissues (Frieser et al., 1997; Sorokin et al., 1997; Sixt et al., 2001).

The laminin  $\gamma$ 1 chain is an essential subunit of most laminin isoforms (Timpl and Brown, 1994; Hallmann et al., 2005; Durbeej, 2010), and global knockout of laminin  $\gamma$ 1 leads to early embryonic lethality (Smyth et al., 1999). To study its function, we generated a laminin  $\gamma$ 1 chain conditional knockout

Correspondence to Sidney Strickland: strickland@rockefeller.edu

Abbreviations used in this paper: BBB, blood brain barrier; BM, basement membrane; BVSMC, brain vascular smooth muscle cell; CD31, cluster of differentiation 31; CNS, central nervous system; EGFP, enhanced green fluorescent protein; EM, electron microscopy; GFAP, glial fibrillary acidic protein; ICH, intracerebral hemorrhage; LN, laminin; LN $\gamma$ 1-KO mice, laminin  $\gamma$ 1 knockout mice; PDGFR- $\beta$ , platelet-derived growth factor receptor- $\beta$ ; PLL, poly-L-lysine; SM1, smooth muscle myosin heavy chain 1; SM2, smooth muscle myosin heavy chain 2; SM22, smooth muscle protein 22  $\alpha$ ; SMA, smooth muscle actin  $\alpha$ ; VSMC, vascular smooth muscle cell.

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mouse line using the Cre/LoxP system (Chen and Strickland, 2003). Using this mouse line, our studies in the nervous system demonstrated the importance of this subunit in the expression and assembly of laminin isoforms and the BM (Chen and Strickland, 2003; Yu et al., 2005, 2007, 2009; Chen et al., 2008).

The expression of astrocytic laminin in CNS blood vessels suggests that it is important for BBB formation and CNS vascular integrity. To explore the specific roles of astrocytic laminin in the cerebrovasculature, we crossed our floxed laminin  $\gamma 1$  mice with a nestin-Cre transgenic mouse line (Tronche et al., 1999), producing a line that does not express laminin in glial cells and neurons (referred to as LN $\gamma 1$ -KO). In this transgenic mouse line, Cre is active in neural precursor cells that give rise to both neurons and glia as early as embryonic day 10.5, but Cre is not expressed in endothelial cells (Graus-Porta et al., 2001). This Cre line has been used to distinguish the role of endothelial (tie2-Cre) and nonendothelial (nestin-Cre, used in this study)  $\beta 8$  integrin in brain vascular development (Proctor et al., 2005).

The LN $\gamma 1$ -KO mice showed disruption of astrocytic laminins in the brain and presented with spontaneous hemorrhagic stroke in adulthood in deep brain regions such as the basal ganglia, which are most affected in human patients. Detailed analysis showed that ablation of astrocytic laminin disrupted VSMC/astrocyte interaction, down-regulated VSMC contractile protein expression, and weakened vascular integrity in deep brain regions, leading to hemorrhage. These results revealed a novel function of astrocytes in regulating VSMC function and vascular integrity in the brain. It is noteworthy that this phenotype is similar to that observed in patients with cerebral autosomal dominant arteriopathy with subcortical infarcts and leucoencephalopathy (CADASIL), caused by Notch 3 mutations (Hervé and Chabriat, 2010); these patients have VSMC degeneration often leading to ICH (Viswanathan et al., 2006; Oide et al., 2008).

ICH is a severe form of stroke with high rates of death and long-term disability (Qureshi et al., 2009). Unfortunately, the cellular and molecular mechanisms of how and why hemorrhage occurs are unclear (NINDS ICH Workshop Participants, 2005; Steiner et al., 2011). The spontaneous brain region-specific hemorrhagic phenotype presented by the LN $\gamma 1$ -KO mice suggests an involvement of astrocytic laminin in human hemorrhagic stroke. Therefore, this knockout mouse line may be a useful animal model for studying the mechanisms underlying hemorrhagic stroke. Furthermore, astrocytic laminin may be a novel target in the development of preventive and therapeutic strategies for ICH.

## Results

### Spontaneous hemorrhagic stroke in LN $\gamma 1$ -KO mice

LN $\gamma 1$ -KO mice were born at the expected Mendelian ratio and were smaller than their littermate controls. They began to present with spontaneous ICH in adulthood: 20% of LN $\gamma 1$ -KO mice had ICH by 2–3 months of age, and 60% had ICH by 6 months (Fig. 1 A). These results indicate that the ICH phenotype worsened

as LN $\gamma 1$ -KO mice aged. Most hemorrhages occurred in small arterioles in deep brain regions such as the basal ganglia, thalamus, and hypothalamus (Fig. 1 B). Analysis of 16 hemorrhagic LN $\gamma 1$ -KO mice revealed that 94% of hemorrhages were associated with the striatum, with 38% having hemorrhage only in striatum, 50% in both striatum and thalamus, and 6% in striatum, thalamus, and hypothalamus. Hemorrhage was not observed in the cerebral cortex or hippocampus of LN $\gamma 1$ -KO mice (Fig. 1 E). The severity of hemorrhage varied between mice—in the most severe cases, the temporal lobes were filled with blood (Fig. 1 B), but in other cases, the hemorrhage was constrained to the vicinity of blood vessels. Littermate control mice, either homozygous for the floxed allele but lacking nestin-Cre or heterozygous for the floxed laminin  $\gamma 1$  allele and also hemizygous for the Cre transgene, showed no signs of hemorrhage (Fig. 1, C and D).

### Laminin expression changes in the brain blood vessels of LN $\gamma 1$ -KO mice

Because LN $\gamma 1$ -KO mice showed a prominent ICH phenotype, we analyzed changes in laminin expression in the brain vasculature by immunohistochemistry and Western blot. Laminin  $\gamma 1$  was expressed in both control and LN $\gamma 1$ -KO brain tissues, but its expression was decreased in LN $\gamma 1$ -KO mice compared with controls (Fig. 2, Ab vs. Aa). Low-level expression of laminin  $\gamma 1$  was expected in the LN $\gamma 1$ -KO mice because nestin-Cre would not be expressed by endothelial cells (Tronche et al., 1999; Proctor et al., 2005) and they would therefore still express laminin  $\gamma 1$ .

Endothelial cells and astrocytes assemble their own BM. Under basal conditions, these two layers of BM are fused together and cannot be distinguished by light microscopy (Sixt et al., 2001). Interestingly, endothelial cells and astrocytes express different laminin  $\alpha$  chains (Sixt et al., 2001). We have shown in Schwann cells of the PNS that disruption of laminin  $\gamma 1$  expression results in concurrent lack of expression of the  $\alpha$  and  $\beta$  laminin chains (Yu et al., 2005), presumably due to an inability of the cell to assemble the laminin trimers in the absence of  $\gamma 1$ , resulting in intracellular retention or degradation of the  $\alpha$  and  $\beta$  chains. Based on these observations, we sought to determine whether astrocytic laminin was specifically depleted. We used antibodies targeting astrocyte- and endothelia-specific laminin  $\alpha$  chains to assess which  $\alpha$  chain was affected. Laminins  $\alpha 1$  and  $\alpha 2$ , which are expressed in astrocytes (Sixt et al., 2001), were dramatically decreased in blood vessels of LN $\gamma 1$ -KO mice (Fig. 2, Ad and Af vs. Ac and Ae). However, levels of laminin  $\alpha 4$ , which is expressed in endothelial cells (Fig. S1), were similar between KO and control mice (Fig. 2, Ag and Ah). Laminin  $\alpha 2$  was deposited at the astrocytic endfeet (stained by S100) ensheathing blood vessels in control mice, but was absent from these structures in LN $\gamma 1$ -KO mice (Fig. S2 A). Western blot analysis and quantification confirmed that expression of laminin  $\gamma 1$ ,  $\alpha 1$ , and  $\alpha 2$ , but not  $\alpha 4$ , were significantly decreased in LN $\gamma 1$ -KO mice (Fig. 2 B). The decrease in  $\alpha 1$  and  $\alpha 2$  in LN $\gamma 1$ -KO mice appeared more dramatic by immunohistochemistry than by Western blot, probably due to partial intracellular retention of these chains in the absence of  $\gamma 1$  chain in astrocytes,

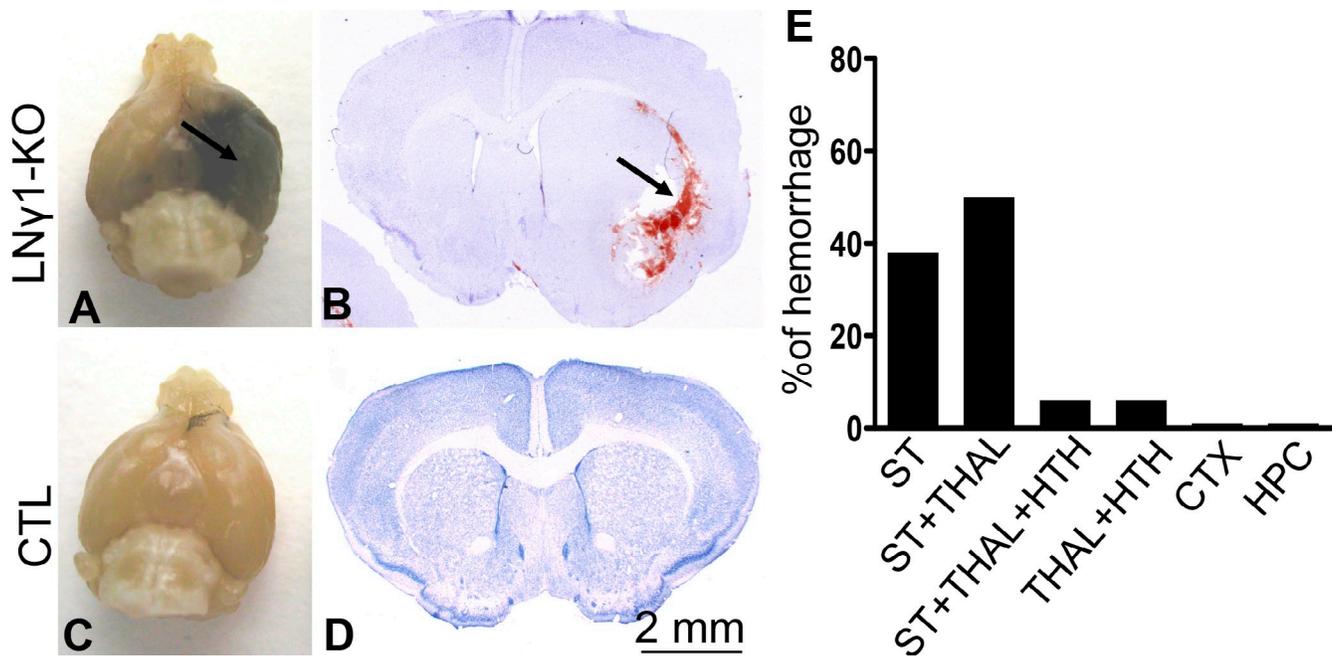


Figure 1. **LN $\gamma$ 1-KO mice present with ICH in adulthood.** Brains of 4-mo-old LN $\gamma$ 1-KO (A) and control littermate (C) mice. The LN $\gamma$ 1-KO mouse brain shows ICH in the temporal lobe (A, arrow), whereas the control mouse is normal. Hematoxylin-stained brain sections of LN $\gamma$ 1-KO (B) and control (D) mice. There is evidence of hemorrhage in the LN $\gamma$ 1-KO (B, arrow) but not in the control mouse brain sections. Brains of LN $\gamma$ 1-KO mice were enlarged near sites of hemorrhage, likely due to edema. Analysis of hemorrhages in different brain regions of LN $\gamma$ 1-KO mice showed that most hemorrhages occurred in striatum and thalamus (E;  $n = 16$  mice). CTX, cortex; HPC, hippocampus; HTH, hypothalamus; ST, striatum; THAL, thalamus. Bar, 2 mm.

which can still be detected by Western blot but not by immunohistochemistry. These results show that astrocytic endfeet-associated laminins are specifically disrupted in LN $\gamma$ 1-KO mice.

Because neurons also produce laminin  $\gamma$ 1 and nestin-Cre is expressed in neuronal progenitor cells that give rise to neurons and glia (Graus-Porta et al., 2001), neuronal laminin could also contribute to the vascular BM. To investigate whether knocking out neuronal laminin  $\gamma$ 1 could affect vascular laminins, we analyzed the expression of different laminin chains ( $\gamma$ 1,  $\alpha$ 2, and  $\alpha$ 4) in CaMKII-Cre/laminin  $\gamma$ 1 conditional KO and littermate control mice (neuron-specific laminin  $\gamma$ 1 KO; Chen et al., 2008). There was no change in laminin expression in the vasculature between control and CaMKII-Cre/laminin  $\gamma$ 1 KO mice (Fig. S2 B), indicating that the disruption of laminin  $\alpha$ 1 and  $\alpha$ 2 expression in LN $\gamma$ 1-KO mice is due to ablation of laminin  $\gamma$ 1 in astrocytes and not neurons or endothelial cells. We also analyzed Cre expression patterns in the nestin-Cre mouse line by crossing this Cre line with the ROSA26-enhanced green fluorescent protein (ROSA26-EGFP) reporter transgenic mice (Mao et al., 2001). Our results showed that Cre is active in the parenchyma cells (astrocytes and neurons), but not in VSMCs in the nestin-Cre mouse line during postnatal development (P14; unpublished data) and in adulthood (Fig. S3). These results indicate that the ICH phenotype observed in LN $\gamma$ 1-KO mice is likely due to the disruption of astrocytic laminin.

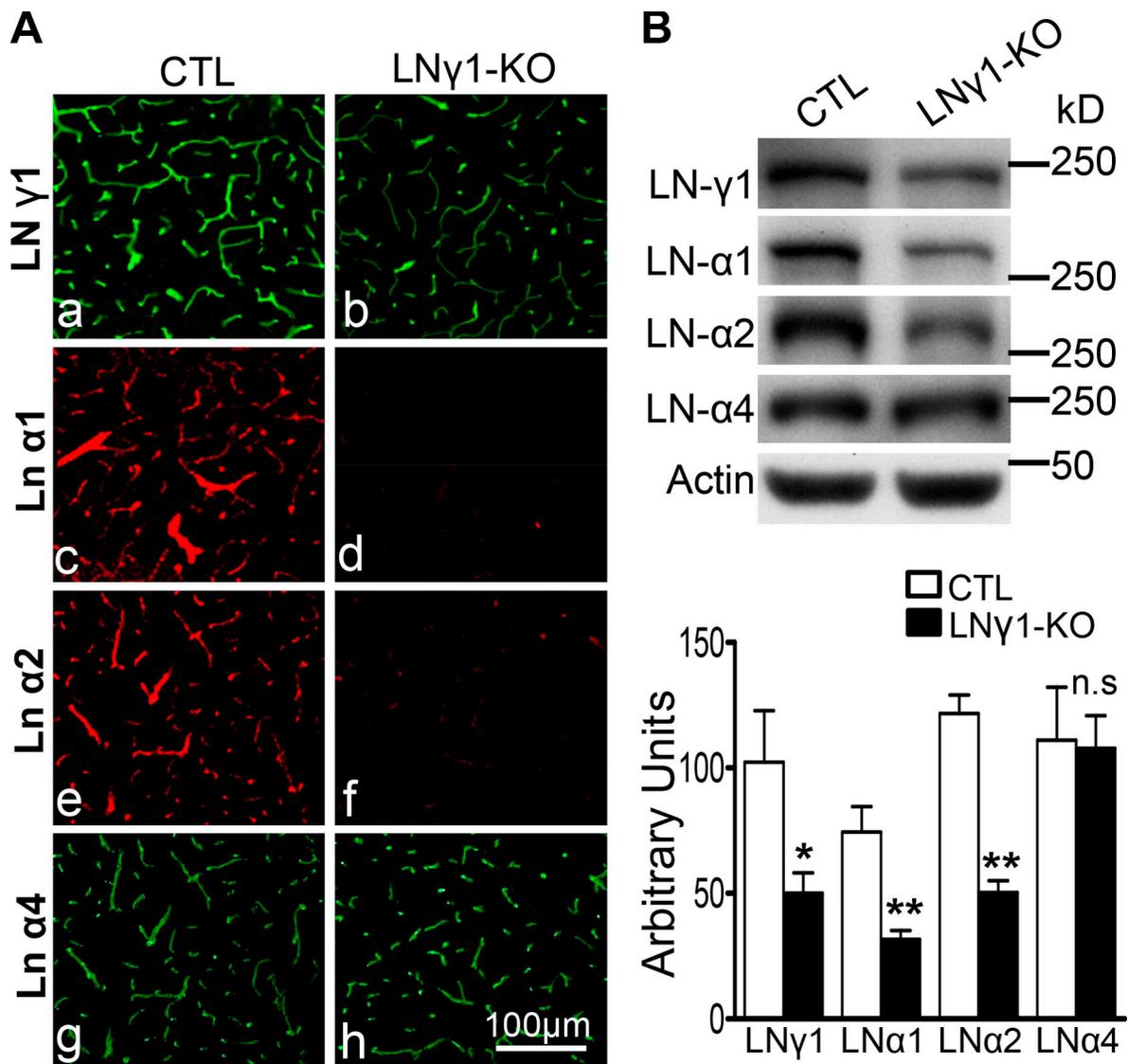
#### Brain vascular wall changes in LN $\gamma$ 1-KO mice

To investigate the pathological changes that may be responsible for hemorrhage in LN $\gamma$ 1-KO mice, we examined structural changes in the vascular walls of LN $\gamma$ 1-KO mouse striatum, the

most affected region. We used cell type-specific markers for the cellular components of CNS vasculature: cluster of differentiation 31 (CD31) for endothelial cells; platelet-derived growth factor receptor- $\beta$  (PDGFR- $\beta$ ) for pericytes; glial fibrillary acidic protein (GFAP) for activated astrocytes; and smooth muscle  $\alpha$ -actin (SMA) for VSMCs. CD31 staining showed a similar pattern and intensity in LN $\gamma$ 1-KO and control mice (Fig. 3, Aa and Ab). PDGFR- $\beta$  staining was also similar between LN $\gamma$ 1-KO and controls as it covered most of the blood vessels in both mouse lines (Fig. 3, Ac and Ad). GFAP expression was similar in both mouse lines in basal ganglia (Fig. 3, Ae and Af), and activated astrocytes associated with large blood vessels (Fig. 3, Ae and Ag). However, there was a dramatic decrease in SMA expression in LN $\gamma$ 1-KO mice compared with controls (Fig. 3, Ag and Ah). GFAP-positive astrocytes were associated with SMA expression in control blood vessels (Fig. 3, Ae and Ag), but GFAP staining was not associated with SMA staining in LN $\gamma$ 1-KO mice (Fig. 3, Af and Ah). Western blot analysis and quantification showed expression levels of CD31, PDGFR- $\beta$ , and GFAP were not significantly changed in LN $\gamma$ 1-KO mice compared with controls (Fig. 3 B). However, SMA expression was significantly decreased in LN $\gamma$ 1-KO mice compared with controls. These results indicate that VSMCs were affected in LN $\gamma$ 1-KO mice.

#### Vascular smooth muscle cell changes in LN $\gamma$ 1-KO mouse brains

Because SMA was decreased in the blood vessels of LN $\gamma$ 1-KO mouse striatum (Fig. 3) and hemorrhages were mostly observed in the same regions (Fig. 1), we reasoned there might be a correlation between VSMC changes and region-specific hemorrhages.

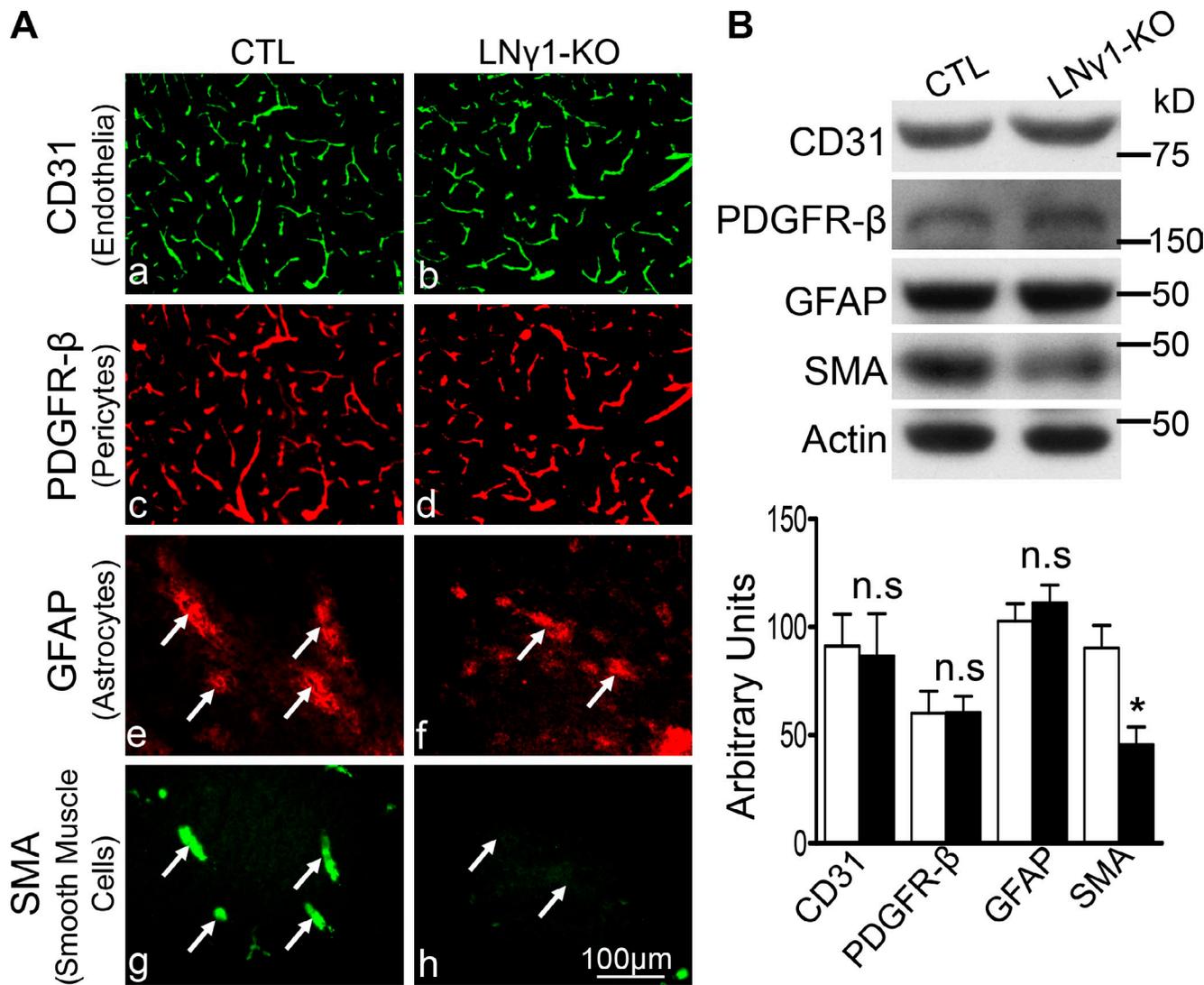


**Figure 2. Disruption of astrocytic but not endothelial laminins in LN $\gamma$ 1-KO mice.** (A) Immunohistochemical analysis showed vascular laminin  $\gamma$ 1 is expressed in both control and LN $\gamma$ 1-KO mice (a and b), but its expression level is decreased in LN $\gamma$ 1-KO mice compared with control (a vs. b). Expression levels of laminin  $\alpha$ 1 and  $\alpha$ 2 (astrocytic laminins) were dramatically decreased in LN $\gamma$ 1-KO mice (c and e vs. d and f). However, laminin  $\alpha$ 4 (endothelial) expression remained similar between control and LN $\gamma$ 1-KO mice (g vs. h). Bar, 100  $\mu$ m. (B) Western blot analysis and quantification of different laminin chain expression levels in the brains of control and LN $\gamma$ 1-KO mice showed laminin  $\gamma$ 1,  $\alpha$ 1, and  $\alpha$ 2 were significantly decreased in LN $\gamma$ 1-KO mice, but laminin  $\alpha$ 4 expression was similar between control and KO mice (Student's *t* test, *n* = 7 in each group). n.s.: not significant.

To address this question, we compared SMC contractile protein expression in different cerebrum regions between control and LN $\gamma$ 1-KO mice by immunohistochemistry and Western blot to investigate whether VSMCs were affected in hemorrhagic regions. Immunohistochemistry for SMA and smooth muscle myosin heavy chain 1 (SM1) showed that expression of these two proteins in the cerebral cortex was not affected (Fig. 4 A), but in the striatum their expression was decreased (Fig. 4 A). To quantify regional differential changes of VSMC contractile protein expressions, we performed Western blot analysis. Cerebrums were separated into three different regions: cerebral cortex, hippocampus, and the rest of the cerebrum (deep regions), which include basal ganglia, thalamus, and hypothalamus. Western blot analysis revealed that expression of both SMA and SM1 was significantly decreased in deep cerebral

regions of LN $\gamma$ 1-KO mice (Fig. 4 B). However, levels of SMA and SM1 were similar in the cerebral cortex and hippocampus in control and LN $\gamma$ 1-KO mice (Fig. 4 B). These results showed VSMC contractile protein expression was affected in deep brain regions where hemorrhages occurred but not in the cerebral cortex and the hippocampus where no hemorrhage was observed, indicating a possible involvement of VSMC in hemorrhages in LN $\gamma$ 1-KO mice.

Decreased VSMC marker protein expression in LN $\gamma$ 1-KO mice could be due to SMC death or impaired differentiation. To address this question, we performed TUNEL staining on brain sections from P0, P14, P28, and adult LN $\gamma$ 1-KO and control mice. Our results showed there was no significant difference in blood vessel-associated apoptotic cell death in LN $\gamma$ 1-KO mouse striatum compared with the controls during development



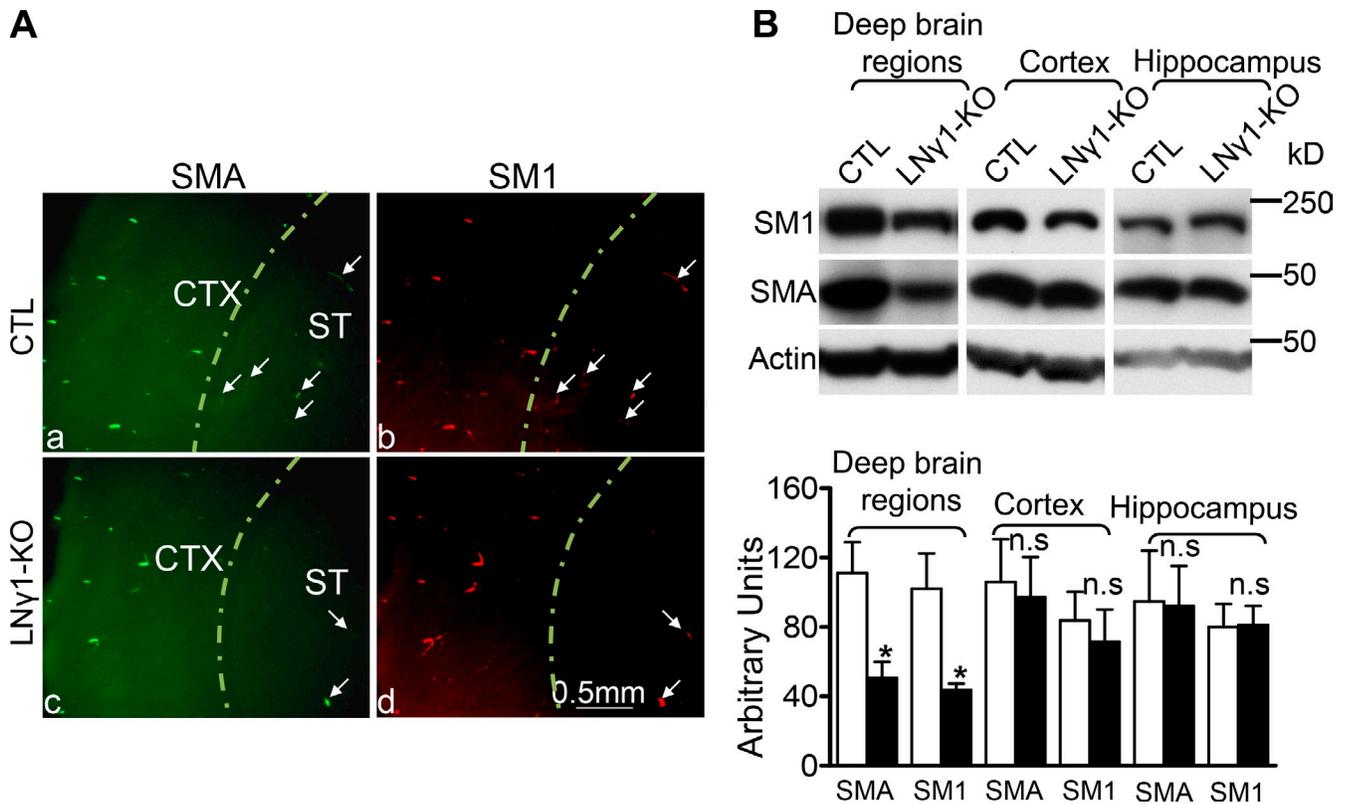
**Figure 3. Cerebral vascular wall changes in the striatum of LN $\gamma$ 1-KO mice.** (A) Immunohistochemistry analysis revealed expressions of CD31 (endothelial cell marker; Aa and Ab), PDGFR- $\beta$  (pericyte marker; Ac and Ad), and GFAP (astrocyte marker; Ae and Af) were similar between control and LN $\gamma$ 1-KO mice. However, expression of SMA (smooth muscle cell marker) was decreased in the striata of LN $\gamma$ 1-KO mice compared with control mice (Ag and Ah). GFAP expression was associated with SMA in the striata of control mice (Ae and Ag, arrows), but minimal SMA expression was observed in the striata of LN $\gamma$ 1-KO mice (Af and Ah). Bar, 100  $\mu$ m. (B) Western blot analysis and quantification showed CD31, PDGFR- $\beta$ , and GFAP expressions were not significantly changed in LN $\gamma$ 1-KO mice, but SMA expression was significantly decreased in LN $\gamma$ 1-KO mice compared with control mice (Student's *t* test, *n* = 7 in each group). n.s.: not significant.

stages and adulthood (Fig. 5, A and B). These results showed that decreased VSMC marker protein expression in LN $\gamma$ 1-KO mice was not due to VSMC death.

To examine whether there were SMCs in the arteries or arterioles in LN $\gamma$ 1-KO mouse striatum, we used EphrinB2 as a marker for arteries (Wang et al., 1998; Li et al., 2011). Although EphrinB2-positive arteries expressed high levels of SM1 in control mice (Fig. 5, Ca and Cc), EphrinB2-positive arteries expressed very low levels of SM1 in LN $\gamma$ 1-KO mice (Fig. 5, Cb and Cd). EphrinB2 expression was also decreased, indicating VSMCs were likely associated with blood vessels but their differentiation was impaired. Blood vessels with diameters between 8–20  $\mu$ m were most affected, and large diameter blood vessels (>25  $\mu$ m) were not affected in LN $\gamma$ 1-KO mouse striatum (not depicted). To further analyze VSMC differentiation in

LN $\gamma$ 1-KO mouse striatum, we performed Western blot for additional SMC differentiation proteins. Expression levels of smooth muscle myosin heavy chain 2 (SM2), smoothelin, and smooth muscle 22  $\alpha$  (SM22  $\alpha$ ) were all significantly decreased in LN $\gamma$ 1-KO mouse striatum (Fig. 5, D and E). These results further suggest that VSMC differentiation was impaired in the striatum of LN $\gamma$ 1-KO mice.

We then investigated whether decreased SMC contractile protein expression in adult LN $\gamma$ 1-KO mice was due to developmental changes by analyzing SMA and SM1 expression in postnatal stages. Cerebrums from control and LN $\gamma$ 1-KO mice at 4 wk of age were separated into three regions in the same way as the adult brains. Expression levels of SMA and SM1 in these three regions were compared between control and LN $\gamma$ 1-KO mice by Western blot. In deep brain regions both SMA and SM1



**Figure 4. Region-specific VSMC contractile protein expression changes in LN $\gamma$ 1-KO mice.** (A) Immunohistochemistry showed that SMA (a and c) and SM1 (b and d) expression in cerebral cortex were similar between control and LN $\gamma$ 1-KO mice, but decreased in striatum of LN $\gamma$ 1-KO mice compared with controls (arrows). (B) Western blot analysis and quantification showed that expression levels of SMA and SM1 were significantly decreased in deep cerebral regions of LN $\gamma$ 1-KO mice compared with controls. SMA and SM1 expression in cerebral cortex and hippocampus of LN $\gamma$ 1-KO mice were not significantly changed compared with the same regions of control mice (Student's *t* test, *n* = 7 in each group). n.s.: not significant.

showed a significant decrease in LN $\gamma$ 1-KO mice compared with the littermate controls (Fig. S4). However, there were no significant differences in SMA and SM1 expression in cerebral cortex and hippocampus between control and LN $\gamma$ 1-KO mice (Fig. S4). These results suggest that SMC contractile protein expression was already decreased in LN $\gamma$ 1-KO mice during postnatal development, indicating that the decreased expression of VSMC contractile proteins in LN $\gamma$ 1-KO mice was probably due to developmental defects.

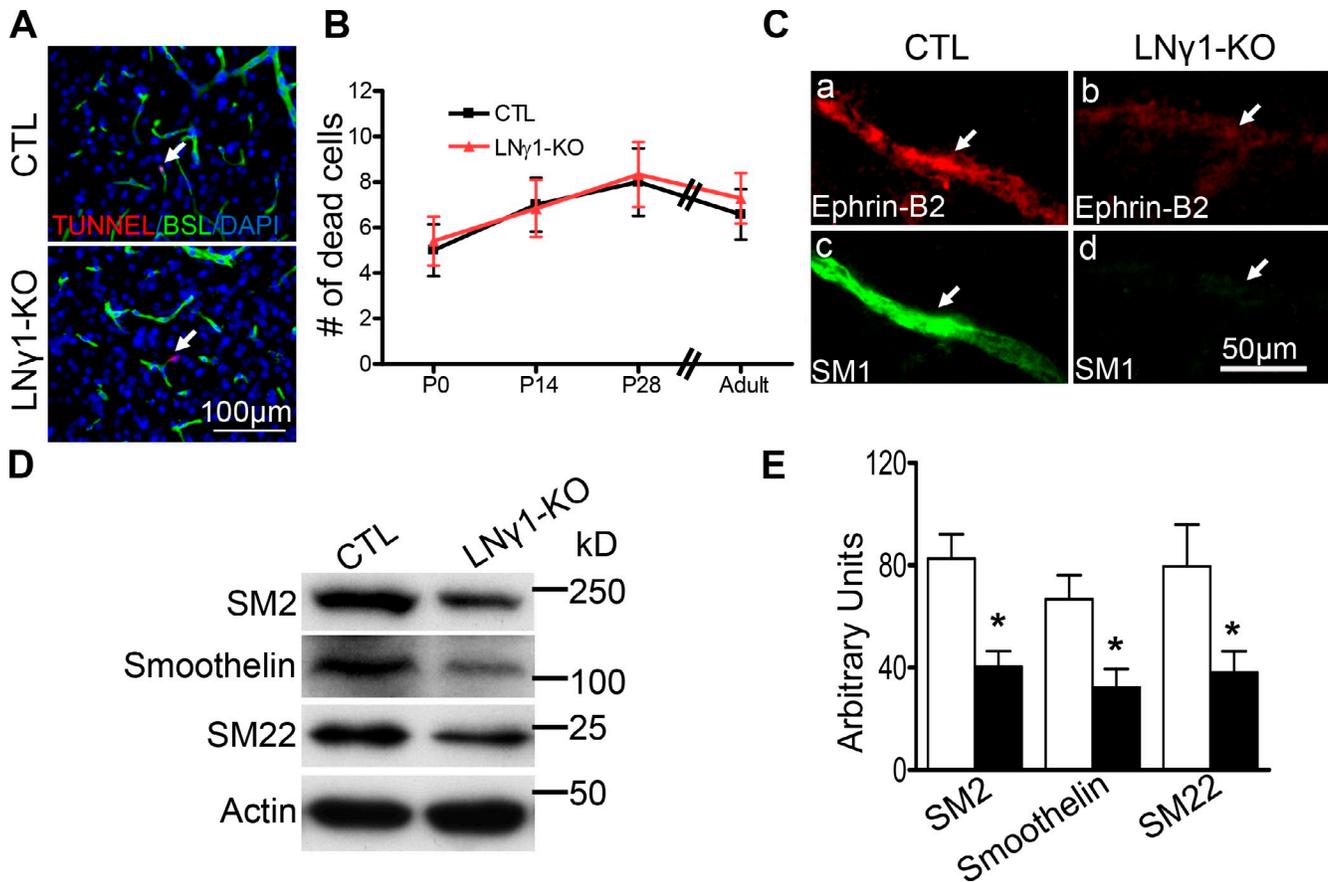
#### Ablation of astrocytic laminin in adult mice disrupted SMC contractile protein expression

To further analyze whether astrocytic laminin is necessary for the maintenance of VSMC differentiation in adult animals, we disrupted laminin expression in astrocytes of adult mice using adenovirus expressing Cre recombinase under the control of an astrocyte-specific promoter (ad-pGFAP-Cre). We injected ad-pGFAP-Cre into the striata of control and homozygous floxed laminin  $\gamma$ 1 allele mice (fLAM $\gamma$ 1). 7 d after injection, the mice were analyzed for adenovirus infection, astrocytic laminin expression, and VSMC differentiation. The ratio of astrocytes infected by adenovirus was  $86 \pm 8\%$ , and disruption of astrocytic but not endothelial laminin in fLAM $\gamma$ 1 mice was confirmed (unpublished data). In contrast to control animals, the fLAM $\gamma$ 1 mice showed significant decreases in SMA and SM1 expression

(Fig. 6). The large caliber blood vessels in control mice, shown by CD31 staining, still expressed SMA (Fig. 6, A and B, arrows) and SM1 (Fig. 6, F and G, arrows) after ad-pGFAP-Cre injection. However, these large caliber blood vessels in fLAM $\gamma$ 1 mice had decreases in SMA (Fig. 6, D and E) and SM1 (Fig. 6, I and J) after astrocytic laminin depletion by ad-pGFAP-Cre, and these decreases were statistically significant (Fig. 6, E and J). These results indicate that acute ablation of astrocytic laminin in adult animals can disrupt VSMC contractile protein expression, revealing its importance in the maintenance of brain VSMC differentiation.

#### Astrocytes and astrocytic laminins promote brain VSMC differentiation in vitro

Because disruption of astrocytic laminin impaired brain VSMC (BVSMC) contractile protein expression in vivo, we analyzed whether astrocytic laminins or astrocytes have direct effects on BVSMC differentiation in vitro. Astrocytes express laminins-111 and -211, so we compared SMA expression levels by BVSMCs cultured on plates coated with poly-L-lysine (PLL) or laminin-111 or -211 or a combination of laminins-111 and -211. BVSMCs cultured on laminin-111- or -211-coated plates showed significantly more SMA expression compared with PLL-coated plates (Fig. 7 A). Interestingly, BVSMCs cultured on plates coated with a combination of laminins-111 and -211 showed significantly



**Figure 5. Impaired VSMC differentiation but not cell survival in striatum of LN $\gamma$ 1-KO mice.** TUNEL staining showed blood vessel-associated apoptotic cell death was not significantly increased in LN $\gamma$ 1-KO mice compared with controls during development (P0, P14, and P28) and in adulthood (A and B; Student's *t* test,  $n = 5-7$  in each group at each time point). (C) Impairment of arterial SMC differentiation. Arteries labeled by EphrinB2 (a and b) expressed high level of SM1 in control (c) but very low level in LN $\gamma$ 1-KO (d) mice. Western blot analysis and quantification of VSMC differentiation protein expression in the striatum of control and LN $\gamma$ 1-KO mice. Expression of SM2, Smoothelin, and SM22 were significantly decreased in the striatum of LN $\gamma$ 1-KO mice compared with control mice (E). Bars: (A) 100  $\mu$ m; (C) 50  $\mu$ m.

more SMA expression than BVSMCs cultured on plates coated with laminin-111 or -211 alone (Fig. 7 A). Because astrocytes produce both laminins-111 and -211 and a combination of these laminin isoforms can additively promote BVSMCs differentiation, astrocytes may be well positioned to regulate BVSMCs differentiation.

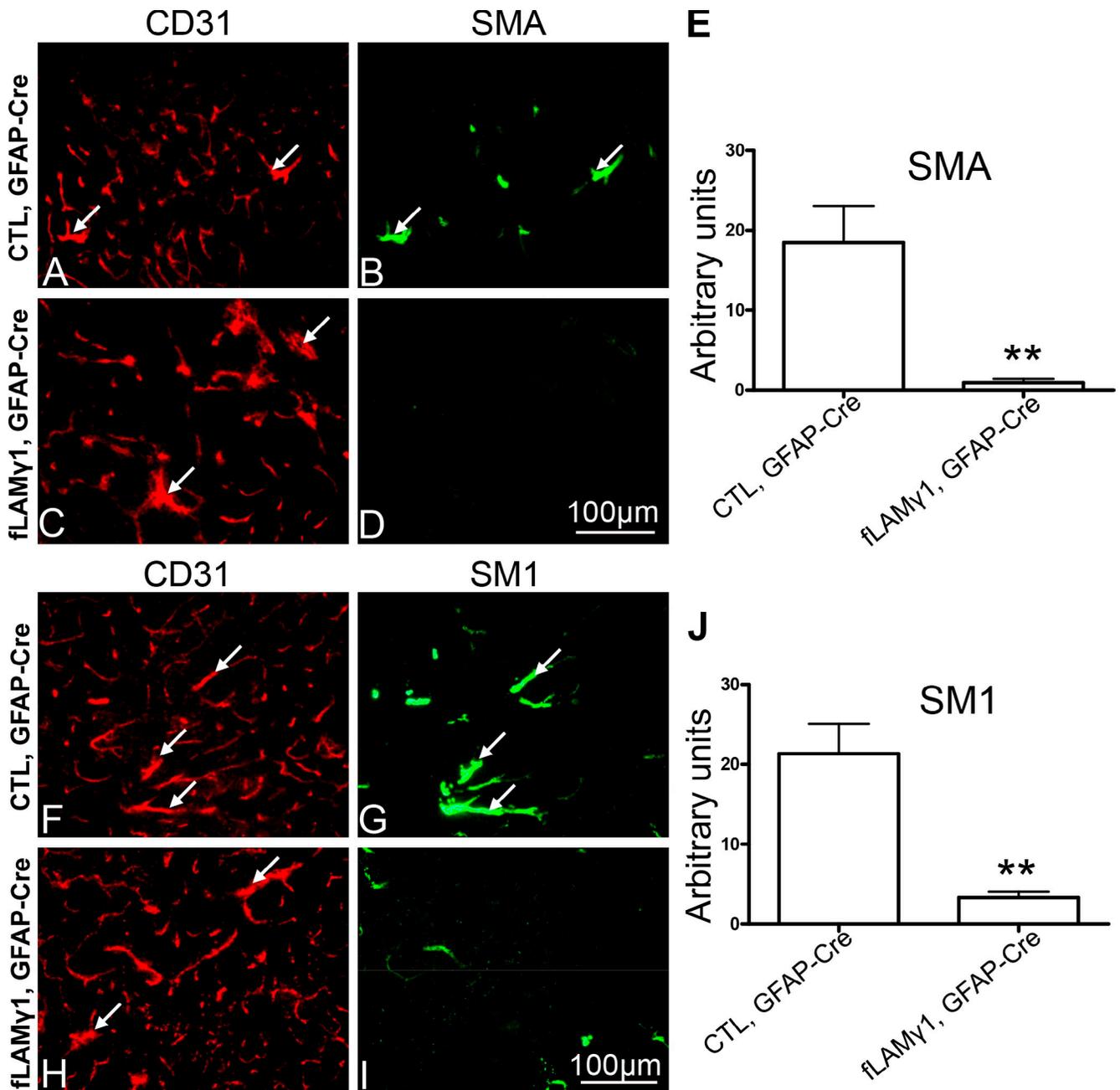
We also compared SMA expression levels by culturing BVSMCs on plates coated with laminin and collagen type IV, also a major component of the BM. BVSMCs cultured on laminin expressed significantly more SMA than cells cultured on collagen type IV (Fig. 7 B). These results indicate astrocytic laminin can directly promote BVSMC differentiation, and the effect of laminin on BVSMC differentiation is stronger than other components of the BM such as collagen type IV.

We then analyzed whether astrocytes can directly regulate BVSMC differentiation by co-culturing BVSMCs with astrocytes. BVSMCs were grown to 90% confluence. Different amounts of astrocytes were added into BVSMC culture, and they were co-cultured for 6 d. Compared with the controls, BVSMCs co-cultured with astrocytes showed significantly more SMA expression and was astrocyte number dependent (Fig. 7 C). However, astrocytes by themselves did not express SMA (Fig. 7 C).

We also performed SMA and GFAP immunocytochemistry to compare SMA expression in BVSMCs cultured with or without astrocytes. In BVSMC/astrocyte co-culture, SMA expression was elevated and astrocytes were positive for GFAP staining (Fig. 7 D). However, in BVSMCs cultured without astrocytes, SMA expression remained at basal levels and there was no GFAP expression, indicating the purity of the SMC (Fig. 7, Db). These results indicate that astrocytes could directly promote BVSMC differentiation.

#### Brain region-specific vascular changes in LN $\gamma$ 1-KO mice

Because most hemorrhages were observed in the deep brain regions and VSMCs were impaired in these same regions, we investigated why there was such a regional specificity. One possibility is that the regional heterogeneous expression of Cre recombinase resulted in more complete knockout of laminin in deep brain regions compared with cerebral cortex or hippocampus. Therefore, we compared astrocytic laminin expression between deep brain regions and cerebral cortex or hippocampus in LN $\gamma$ 1-KO mice. However, there was no significant difference (unpublished data). The regional differences could be due to different association between astrocytes and VSMCs. While

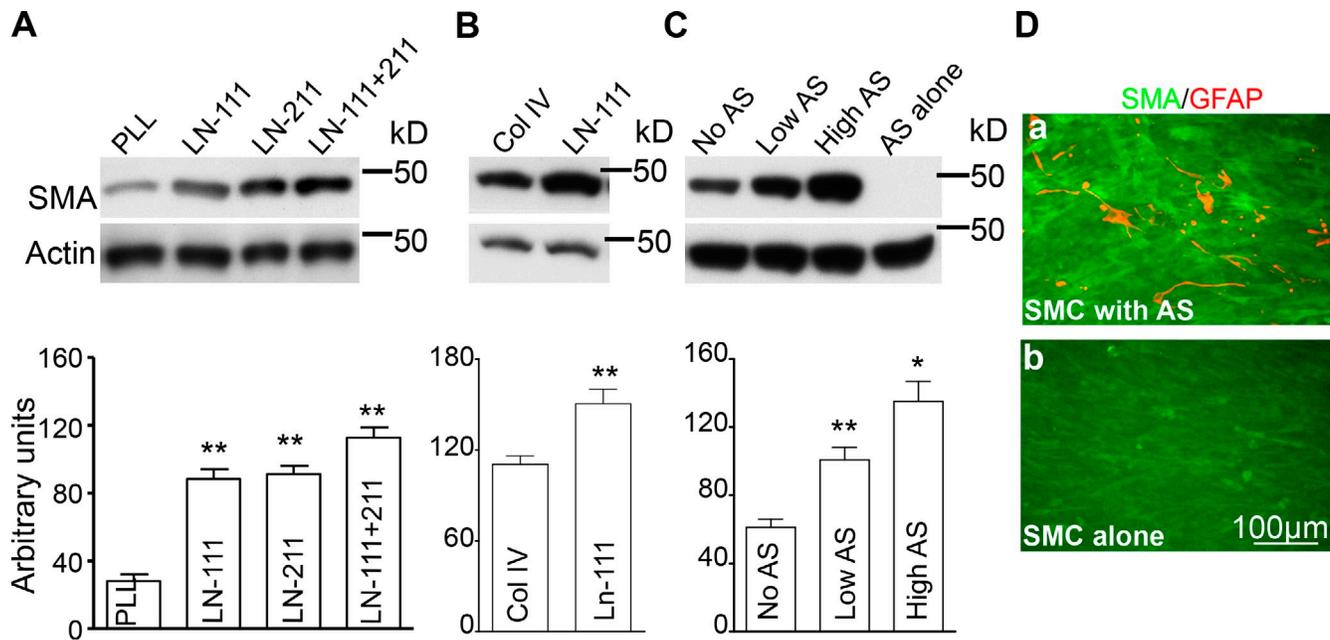


**Figure 6. Ablation of astrocytic laminin in adult mice disrupts VSMC contractile protein expression.** Adenovirus expressing Cre recombinase under the control of GFAP promoter (ad-pGFAP-Cre) was injected into the striata of control or fLAMY1 mice. 7 d after ad-pGFAP-Cre injection, both SMA (B and D) and SM1 (G and I) were dramatically decreased in fLAMY1 mice (D and I) when compared with control (B and G). Blood vessels were visualized by CD31 staining (A, C, F, and H). Large caliber blood vessels express SMA (A and B, arrows) and SM1 (F and G, arrows) in control mice, whereas expression of SMA (C and D, arrows) and SM1 (H and I, arrows) in fLAMY1 mice was decreased. The decreases in SMA and SM1 in fLAMY1 mice after ad-pGFAP-Cre injection were statistically significant when compared to control by Student's *t* test (E and J). Bar, 100  $\mu$ m.

arteries penetrate through the brain parenchyma, the pia meninges cover the blood vessels and directly contact VSMC in the media layer of arteries. Astrocytic endfeet cover the pia meninges that penetrate the arteries, and as big arteries branch into small arteries and small arteries ramify into arterioles, the pia meninges become thinner. At the capillary level, there are no pia meninges, and astrocytic endfeet directly contact pericytes or endothelial cells. One possible explanation for the effects of disruption of astrocytic laminin on VSMC in the deep brain regions could be that small arteries and arterioles in deep brain regions had

closer contact with astrocytes than those arteries in cerebral cortex or hippocampus and were therefore more influenced by astrocytes.

To investigate this possibility, we compared the relationship between astrocytes and VSMCs in small arteries or arterioles in striatum and cerebral cortex by electron microscopy (EM). In small arteries or arterioles in the striatum, there were regions where the BMs of VSMCs and astrocytes were fused together and astrocytes showed close contact with VSMCs (Fig. 8, A and B). However, this close relationship between



**Figure 7. Astrocytes and astrocytic laminins promote BVSMC differentiation in vitro.** (A) Western blot analysis showed BVSMCs cultured on plates coated with laminin-111 and/or -211 had increased SMA expression compared with cells cultured on PLL-coated plates by ANOVA ( $n = 18$  in each group). (B) BVSMCs cultured on plates coated with laminin-111 had significantly more SMA expression than cells cultured on collagen type IV-coated plates by Student's  $t$  test ( $n = 18$  in each group). (C) BVSMCs co-cultured with astrocytes had significant increases in SMA expression compared with controls and is astrocyte number dependent by ANOVA, whereas astrocytes did not express SMA ( $n = 18$  in each group). (D) Immunocytochemistry showed BVSMCs co-cultured with astrocytes had obvious SMA expression and astrocytes were GFAP positive (a), whereas BVSMCs cultured alone only maintained a basal low level SMA expression and there were no GFAP-positive cells (b). Bar, 100  $\mu$ m.

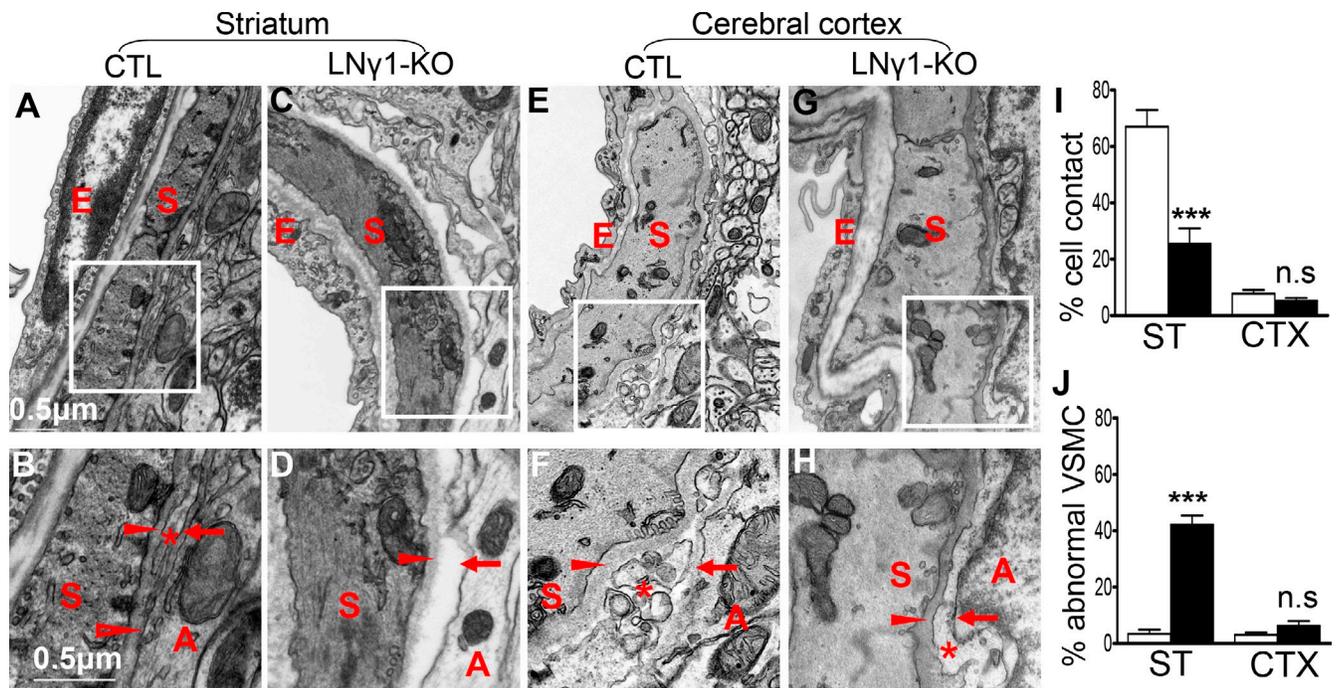
astrocytes and VSMCs was not observed in small arteries or arterioles in the cerebral cortex (Fig. 8, E and F). In LN $\gamma$ 1-KO mice, ablation of astrocytic laminin  $\gamma$ 1 disrupted astrocytic endfeet BM formation and contact between astrocytes and VSMC in small arteries and arterioles in the striatum, but the VSMC BMs were mostly intact (Fig. 8, C and D) due to the absence of Cre expression in this cell type in nestin-Cre mouse line (Fig. S3). However, in small arteries or arterioles in the cerebral cortex of LN $\gamma$ 1-KO mice, VSMCs were covered by pia meninges, located between VSMC and astrocytes, and loss of astrocytic endfeet BM did not affect the contact between VSMCs and pia meninges (Fig. 8, G and H). Interestingly, disruption of astrocyte/VSMC contact in the striatum of LN $\gamma$ 1-KO mice caused abnormal VSMC morphology with less contractile machinery (Fig. 8, C and D). We quantified the percentage of VSMCs (with diameter between 8 and 20  $\mu$ m) showing direct contact with astrocytes and percentage of VSMCs with abnormal morphology in different brain regions between control and LN $\gamma$ 1-KO mice. There was a decreased percentage of VSMCs showing direct contact with astrocytes in the striatum but not in the cortex in LN $\gamma$ 1-KO mice compared with controls (Fig. 8 I). VSMCs with abnormal morphology (less contractile machinery) were dramatically increased in the striatum, but not in the cortex in LN $\gamma$ 1-KO mice (Fig. 8 J). In control mice, many more VSMCs showed direct contact with astrocytes in striatum than in cortex (Fig. 8 I). These results indicate that astrocytes were more closely associated with VSMCs in small arteries and arterioles in striatum than in the cerebral cortex. Ablation of astrocytic laminin disrupted astrocytic endfeet BM and affected the

contact between VSMC and astrocytes in striatum but not in the cerebral cortex. These regional differences may explain why striatum were mostly affected.

#### Disruption of astrocytic laminin impairs VSMC differentiation and leads to hemorrhagic stroke

We examined the relationship between astrocytic laminin expression, VSMC contractile protein expression, and hemorrhage. In control mice, blood vessels (identified by BSL, fluorescein-bandiraea simplicifolia lectin; Daneman et al., 2010) expressed laminin  $\alpha$ 2 (Fig. 9, Aa and Ac) and laminin  $\alpha$ 1 (Fig. S5 C). The expression of astrocytic laminin in large caliber blood vessels correlated with the expression of SMA (Fig. 9, Aa, Ac, and Ae; and Fig. S5, E and G). However, in LN $\gamma$ 1-KO mice, the large caliber blood vessels did not express laminin  $\alpha$ 2 (Fig. 9, Ad), laminin  $\alpha$ 1 (Fig. S5 D), or SMA (Fig. 9, Ab, Ad, and Af; and Fig. S5, F and H). Furthermore, analysis of hemorrhagic regions of LN $\gamma$ 1-KO mice showed that hemoglobin, indicating hemorrhage, was deposited outside the blood vessels that had dramatically decreased SM1 expression in the striatum (Fig. 9, Bb), whereas no hemoglobin was detected in control mouse brain (Fig. 9, Ba).

Ultrastructural analysis was performed on hemorrhagic blood vessels in the brains of LN $\gamma$ 1-KO mice. Ruptured blood vessels were identified by the presence of blood cells outside the vessels and in the parenchyma (Fig. 9, Ca). EM revealed that the vascular unit was disassembled and smooth muscle cells were fragmented (Fig. 9, Ca and Cb, arrowheads). Together, our results



**Figure 8. Region-specific vascular changes in LN $\gamma$ 1-KO mice.** (A and B) Relationship between astrocytes (A), VSMC (S), and pia meninges (\*) in small arterioles in striatum of control mice; there are some regions in which the BM of VSMC and astrocytes are fused together (arrow). (B) Higher magnification of the boxed area in A showed details of the BM of VSMC (arrowhead) and astrocytes (arrow) fused together and astrocyte showed close contact with VSMC (open arrowhead). In some regions the pia meninges (\*) lied between VSMC and astrocytes. (C and D) Relationship between VSMC and astrocytes in striatum of LN $\gamma$ 1-KO mice. Astrocytic endfeet detached from the wall of blood vessel. (D) Higher magnification of boxed area in C showed that ablation of astrocytic laminin  $\gamma$ 1 disrupted astrocytic endfeet BM formation (arrow) as well as the contact between astrocytes and VSMC (the space between them). However, SMC BMs still exist (arrowhead). (E and F) Relationship between VSMCs, astrocytes, and pia meninges in cerebral cortex in control mice. Pia meninges are found between VSMCs and astrocytes, and there is no direct contact between VSMCs and astrocytes. (F) Higher magnification in boxed area in E showed that the BMs of VSMCs and astrocytes were always separated, and the pia meninges are between them. (G and H) Relationship between VSMCs and astrocytes in cerebral cortex of LN $\gamma$ 1-KO mice. VSMC were covered by pia meninges (\*). (H) Higher magnification of the boxed area in G shows that ablation of astrocytic laminin  $\gamma$ 1 disrupted astrocytic endfeet BM formation (arrow), but the VSMCs were covered by pia meninges (\*) and the BM of VSMCs was intact (arrowhead). (I) Quantification of EM micrographs ( $n = 7$  mice per genotype) revealed that VSMC/astrocytic endfeet direct contact was dramatically decreased in the striatum in LN $\gamma$ 1-KO mice (black bar) compared with controls (white bar). There were dramatically more VSMCs showing direct contact with astrocytic endfeet in striatum than in cerebral cortex in control mice (I). There was no significant difference in percentages of VSMCs showing direct contact with astrocytic endfeet between control and LN $\gamma$ 1-KO cortex (I). There were significantly more VSMCs showing abnormal morphology in the striatum in LN $\gamma$ 1-KO (black bar) than in control mice (white bar), but there was no such difference between control and LN $\gamma$ 1-KO cortex (J). All statistical analyses were by Student's *t* test. A, astrocytes; CTX, cortex; E, endothelium; n.s.: not significant; S, smooth muscle cell; ST, striatum. Bars in A, C, E, and G are the same and is shown in A; Bars in B, D, F, and H are the same and is shown in B.

suggest that astrocytic laminin is important for the integrity of blood vessels in deep cerebral regions, and disruption of astrocytic laminin destabilizes the blood vessels and eventually leads to ICH.

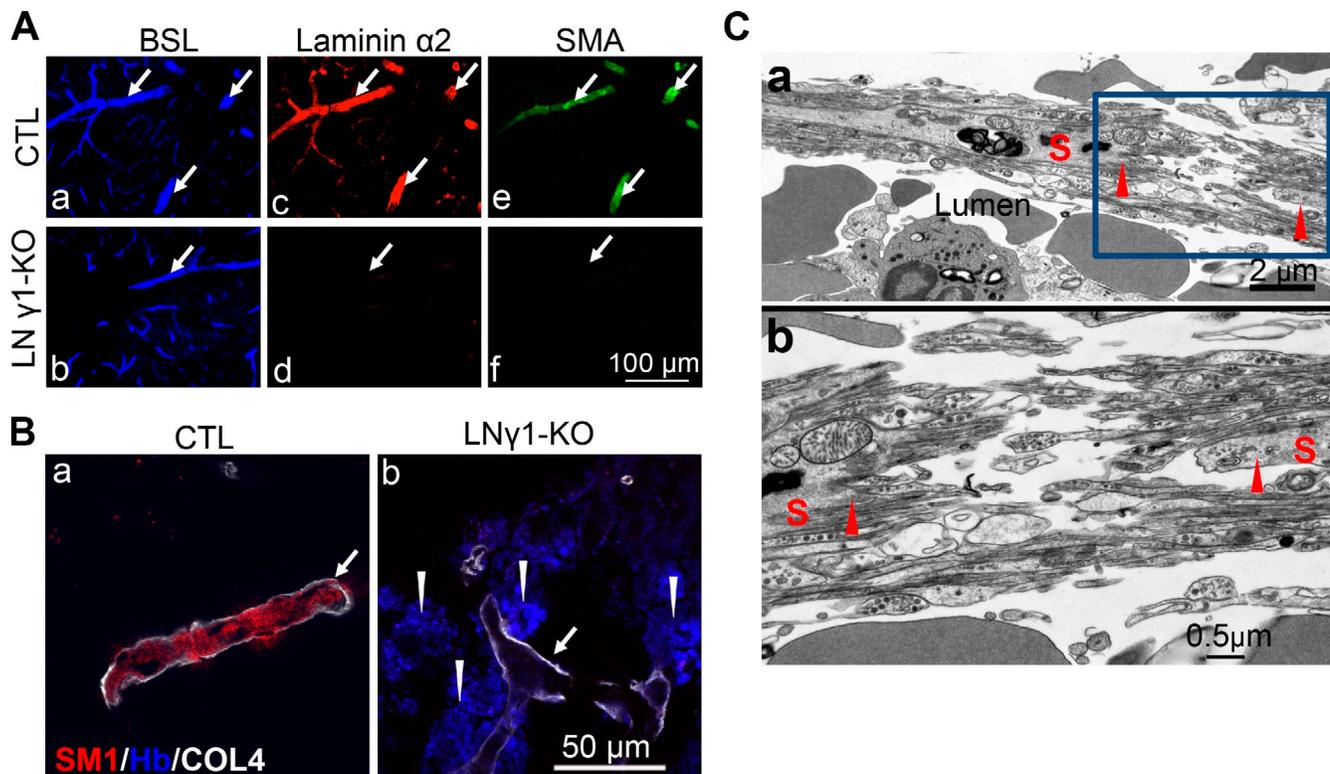
## Discussion

Astrocytes are the most abundant cell type in the CNS and are closely associated with the vasculature. They play important roles in regulating cerebral vascular development and function, maintaining BBB integrity and homeostasis of the CNS micro-environments, and neurovascular coupling (Abbott et al., 2006; Iadecola and Nedergaard, 2007; Zlokovic, 2008). Astrocytes interact with CNS vasculature through astrocytic endfeet where a BM is assembled. The astrocytic BM is different from the endothelial BM in that it contains different laminin isoforms. Our results showed that ablation of astrocytic laminin disrupted endfeet BM formation and impaired VSMC development in small arteries or arterioles in deep brain regions with decreased VSMC contractile protein expression. In these deep brain regions, astrocytes were more closely associated with VSMCs in small arteries or arterioles. While in the cerebral cortex or hippocampus

where astrocytes are not as closely associated with VSMCs as in the deep brain regions, VSMCs were not significantly affected by the loss of astrocytic laminin. We also showed that disruption of astrocytic laminin caused dissociation of the astrocytic endfeet from the vascular wall, therefore destabilizing the blood vessels. These blood vessels were eventually disassembled, which led to ICH. In adult mice, acute ablation of astrocytic laminin in the striatum also disrupted VSMC contractile protein expression, indicating astrocytic laminin was necessary for the maintenance of differentiation of this cell type. In vitro, both astrocytic laminin and astrocytes promoted BVSMC differentiation. Because the regulation of VSMC development and maintenance by astrocytes has not been reported, our studies reveal a novel function of astrocytes in the brain.

### Region-specific effects of disruption of astrocytic laminin on VSMC development and vascular integrity

Hemorrhages were only observed in the deep brain regions such as basal ganglia, thalamus, and hypothalamus, but not



**Figure 9. Disruption of astrocytic laminin impaired VSMC differentiation and led to ICH.** (A) Blood vessels were visualized by BSL (a and b). Laminin  $\alpha$ 2 was expressed in these blood vessels in control (c) but not LN $\gamma$ 1-KO (d) mice. Laminin  $\alpha$ 2 was coexpressed with SMA in large caliber blood vessels in control mice (e), but this correlation was absent in LN $\gamma$ 1-KO mice (f). (B) In control mouse striatum hemoglobin was not detected around large caliber blood vessels expressing SM1 (arrow). In LN $\gamma$ 1-KO mice, however, hemorrhagic regions (stained by hemoglobin, arrowheads) was associated with large caliber blood vessels expressing little SM1 (arrow, a representative image). (C) A ruptured blood vessel in LN $\gamma$ 1-KO mice (a) was identified, which shows that the wall of the vessel was disassembled and smooth muscle cells were fragmented (arrowheads). (b) Higher magnification of boxed area in panel a showed the details of fragmented smooth muscle cells (arrowhead). COL4, collagen type IV; Hb, hemoglobin. Bars: (A) 100  $\mu$ m; (B) 50  $\mu$ m; (Ca) 2  $\mu$ m; (Cb) 0.5  $\mu$ m.

in cerebral cortex or hippocampus of LN $\gamma$ 1-KO mice. Consistent with the region-specific hemorrhagic phenotype, expression levels of VSMC contractile proteins were only significantly decreased in the deep cerebral regions but not in cerebral cortex or hippocampus, indicating the involvement of VSMC changes in the hemorrhagic phenotype observed. Because the amount of astrocytic laminin was similar in different brain regions of LN $\gamma$ 1-KO mice, the region-specific phenotype was probably not due to more complete disruption of astrocytic laminin in the deep brain regions. Our further analyses revealed that astrocytes were more closely associated with VSMCs in small arterioles of the deep cerebral regions than those in the cerebral cortex or hippocampus. We showed by EM that the BMs of astrocytes and VSMCs were fused together in some areas, and knockout of astrocytic laminin resulted in dissociation of astrocytic endfeet from the vascular wall. However, in the cerebral cortex, astrocytes were less closely associated with VSMCs in small arterioles and the pia meninges almost completely covered the entire outmost surface of VSMCs. This different relationship between astrocytes and VSMCs could be one of the mechanisms responsible for regional differences of hemorrhage in LN $\gamma$ 1-KO mice.

In human hypertensive hemorrhagic stroke patients, the most affected regions are the deep brain regions such as basal ganglia and thalamus (Mayer and Rincon, 2005; Sutherland and Auer, 2006; Qureshi et al., 2009), the same regions that showed

hemorrhages in our LN $\gamma$ 1-KO mice. The reasons why these regions are more susceptible to ICH had been unclear. Hemodynamic forces in small arteries or arterioles in these regions probably accounts for one mechanism (Yen et al., 2005; Penn et al., 2011). Our results also reveal that abnormalities in astrocytes, such as astrocytic BM changes, lead to hemorrhages in the same brain regions as the human hypertensive hemorrhagic patients, indicating that astrocytic changes may play an important role in the pathogenesis of hemorrhagic stroke in humans.

#### Astrocytic laminins in BVSMC development and maintenance

Previous *in vitro* studies show that laminin induces and maintains contractile protein expression in VSMCs (Thyberg and Hultg rdh-Nilsson, 1994; Hultg rdh-Nilsson and Durbeek, 2007). However, the *in vivo* function of laminin in VSMC development and differentiation had not been investigated. The extracellular environment of VSMCs is rich in laminins. On the interfaces with endothelium, VSMCs interact with endothelial BM which contains laminins-411 and -511 (Sixt et al., 2001). On the outmost side, VSMCs contact pia meninges that likely contain laminin-111.

Furthermore, as our current study revealed, in some small arterioles in the deep brain regions, VSMCs may also interact with the astrocytic endfeet BM which contains laminins-111

and -211. Endothelium BM can promote VSMC maturation (Campbell and Campbell, 1986; Scheppke et al., 2012). The BM is composed of various extracellular matrix proteins, all of which may have different effects on VSMC differentiation, and the net effects of BM from each cellular source may differ. It is not clear whether astrocytic endfeet BM exhibits a different role in promoting VSMC maturation compared with endothelial BM. Our *in vitro* experiments showed that a combination of laminins-111 and -211, which are produced by astrocytes *in vivo* and are components of the astrocytic endfeet BM, showed stronger effects on BVSMC differentiation (Fig. 7 A) than these two laminin isoforms alone. It will be interesting to compare the effects of endothelial laminin (-411 and -511) and astrocytic laminin on BVSMC differentiation. Interestingly, co-culture of BVSMCs with astrocytes also promoted SMC differentiation, which may be due to the laminins or other factors expressed by astrocytes. VSMCs may also produce laminin and assemble BMs (Glukhova et al., 1993). However in nestin-Cre mice, Cre is not expressed in brain VSMC (Fig. S3), and VSMC BMs were mostly intact in LN $\gamma$ 1-KO mice. Because vascular cell death was not significantly changed during development stages and adulthood in LN $\gamma$ 1-KO mice, astrocytic laminin plays a role in VSMC differentiation, whereas laminins from other cell types may be important for cellular viability.

VSMC degeneration is a prominent pathological change in the cerebrovascular wall of hypertensive ICH patients (Qureshi et al., 2009). EM analysis of ruptured arteries in surgically removed brain samples from hypertensive ICH patients showed VSMC atrophy and fragmentation (Takebayashi and Kaneko, 1983), suggesting that one of the major mechanisms underlying hypertensive ICH is VSMC degeneration. However, it remains unclear as to why smooth muscle cells degenerate under hypertensive conditions. In the ruptured arteries of our LN $\gamma$ 1-KO mice, VSMCs also showed fragmentation (Fig. 9, Cb), similar to the ruptured arteries of hypertensive ICH patients. Other studies show the association between astrocytic endfeet swelling, BBB breakdown, and neuronal death in hypertensive rats (Tagami et al., 1990), suggesting an important role of astrocytes in regulating vascular function under hypertensive conditions. In LN $\gamma$ 1-KO mice, even though their blood pressure was normal (unpublished data), ablation of astrocytic laminin disrupted the astrocyte-vasculature interaction in deep brain regions, leading to impairment of VSMC maturation, vascular instability, and ICH. It is also possible that abnormalities in astrocytes may contribute to vascular wall changes in human hypertensive hemorrhagic stroke pathogenesis. Further analysis of samples from human hemorrhagic stroke patients would shed light on whether astrocytic abnormalities are involved in hypertensive hemorrhages.

#### Laminin receptors in mediating laminin function in BVSMC development and vascular integrity

Knockout of either integrin  $\alpha$ v or  $\beta$ 8 from the neuroepithelium leads to a defective association between cerebral microvessels and the cells of the surrounding brain parenchyma as well as the development of ICH at embryonic and postnatal stages

(McCarty et al., 2002, 2005; Proctor et al., 2005). In contrast to our LN $\gamma$ 1-KO mice that present with adult onset and age-dependent ICH, adult integrin knockout mice do not experience ICH (McCarty et al., 2005; Proctor et al., 2005). The differences between LN $\gamma$ 1-KO mice and integrin  $\alpha$ v or  $\beta$ 8 knockout mice are likely due to distinctive underlying mechanisms. The TGF $\beta$  pathway appears to be involved in integrin  $\alpha$ v or  $\beta$ 8 function in cerebral vascular development (Nguyen et al., 2011), whereas the major laminin receptors expressed in the neurovascular unit in postnatal stages or adulthood are integrin  $\beta$ 1 and dystroglycan (del Zoppo and Milner, 2006). However, knockout of integrin  $\beta$ 1 or dystroglycan from astrocytes did not show ICH during development or adulthood (Graus-Porta et al., 2001; Moore et al., 2002; Nguyen et al., 2011). Because our results indicate that disruption of astrocytic laminin and VSMC impairment is the likely mechanism responsible for ICH, laminin receptors expressed by VSMCs may play an important role in mediating this effect. Among laminin receptors, integrin  $\alpha$ 7 $\beta$ 1 seems to play an important role in VSMC development and vascular integrity. Knockout of integrin  $\alpha$ 7 in VSMCs results in reduced VSMCs and cerebral vascularization and cerebrovascular hemorrhage (Flintoff-Dye et al., 2005). Integrin  $\beta$ 1 receptors also play important roles in VSMC development and phenotype maintenance as knockout of integrin  $\beta$ 1 in VSMCs leads to SMC defects, vascular aneurysms, and neonatal or postnatal lethality (Abraham et al., 2008; Turlo et al., 2012). Dystroglycan, another laminin receptor, may also play an important role in VSMC development and function (Moiseeva, 2001; Hultg ardh-Nilsson and Durbeej, 2007). However, the role of laminin and its receptors in the development and function of VSMCs in cerebrovasculature is not well studied. The effects of astrocytic laminin on VSMC development in small arteries and arterioles in deep brain regions could be mediated by any of the laminin receptors expressed by VSMCs. It would be interesting to determine whether VSMCs in small arteries or arterioles in deep brain regions express different laminin receptors than those in other brain regions. Most of the studies on vascular development and maintenance thus far have focused on the vascular wall components themselves; however, vascular interaction with their environments may also play important roles in their development and function, as we showed here.

VSMCs play important roles in blood vessel integrity and strength. Abnormal changes of VSMCs are implicated not only in hypertensive ICH (Takebayashi and Kaneko, 1983; Pleşea et al., 2005), but also in hereditary cerebral hemorrhage with amyloidosis (Herzig et al., 2004; McCarron and Nicoll, 2004) and atherosclerosis, among others (Orr et al., 2010). Our results reveal a novel pathway that regulates cerebral VSMC differentiation and function. These results may provide the building blocks for developing new preventative and therapeutic strategies in hemorrhagic stroke and transformation in humans.

## Materials and methods

### Animals

To generate the nestin-Cre/laminin  $\gamma$ 1 knockout mice, homozygous floxed laminin  $\gamma$ 1 (fLAM $\gamma$ 1) mice on a C57BL6 background were mated with nestin-Cre transgenic mice on a C57BL6 background (Tronche et al., 1999).

The fLAM $\gamma$ 1 mice contain two loxP sites inserted into exons 1 and 2 of the *laminin  $\gamma$ 1* gene so that after Cre-mediated recombination, the allele is nonfunctional (Tronche et al., 1999; Chen and Strickland, 2003). The double heterozygous fLAM $\gamma$ 1/+;nestin-Cre/+ mice were mated with homozygous fLAM $\gamma$ 1 mice to obtain mice that are homozygous for the floxed laminin  $\gamma$ 1 allele and also carry the Cre transgene (LN $\gamma$ 1-KO mice). To generate the CaMKII-Cre/laminin  $\gamma$ 1 knockout mice, CaMKII-Cre transgenic mice, where Cre is expressed in neurons, were used to obtain mice that are homozygous for the floxed laminin  $\gamma$ 1 allele and also carry the CaMKII-Cre transgene (C57BL6 background; Chen et al., 2008). Littermates that were homozygous for the floxed laminin  $\gamma$ 1 allele but did not carry the Cre transgenes or heterozygous for the floxed laminin  $\gamma$ 1 allele and also carry the Cre transgenes were used as controls in all experiments. To monitor Cre expression patterns in nestin-Cre transgenic mice, ROSA26-EGFP reporter mice were used (C57BL6 background; Mao et al., 2001). Mice that were hemizygous for both the nestin-Cre and ROSA26-EGFP transgenes at P14 and P56 (adult) were used to determine Cre expression patterns in the brain. ROSA26-EGFP hemizygous mice without the nestin-Cre transgene were used as controls. Genomic DNA was analyzed by PCR for genotyping as described previously (Chen and Strickland, 2003).

#### Stereotaxic adenovirus injection

Adult (8–12 wk of age) control (C57BL6) or fLAM $\gamma$ 1 mice were anesthetized with atropine (0.6  $\mu$ g/g of body weight) and avertin (0.02 ml/g of body weight) intraperitoneally. After making an incision, a burr hole was drilled at stereotaxic coordinates of  $-1$  mm posterior to bregma and 2.5 mm lateral from midline. Then, 2  $\mu$ l Ad-pGFAP-Cre (a gift from Arturo Alvarez-Buylla, University of California, San Francisco, San Francisco, CA) was injected into the brain at a depth of 3.5 mm over 5 min. The needle was kept in place for 2 min to prevent reflux. 1 wk after injection, the mice were transcardially perfused and the brains were sectioned for analysis. We conducted all experiments in accordance with the guidelines of the US National Institutes of Health Guide for the Care and Use of Laboratory Animals and with approval from the Animal Care and Use Committee of The Rockefeller University.

#### Histology and immunohistochemistry

Histology and immunohistochemistry was performed as described previously (Chen et al., 2008). In brief, control and LN $\gamma$ 1-KO mice were anesthetized, the animals perfused, the brains removed, and cryo-sections prepared. For hematoxylin staining, mice were perfused with 4% PFA, and the brains removed and postfixed. Free-floating brain sections were prepared and stained with hematoxylin. Brain hemorrhages were counted in different brain regions of LN $\gamma$ 1-KO mice. For immunostaining, the sections were fixed in 4% PFA in 0.1 M phosphate buffer for 30 min. After washing in PBS, sections were blocked in PBS containing 0.3% Triton X-100 and 5% normal donkey serum. Primary antibodies used were against: laminin  $\alpha$ 2, S-100, and SMA (Sigma-Aldrich); laminin  $\alpha$ 1 and  $\gamma$ 1 (EMD Millipore); CD31 (BD); SM1 (Kamiya Biomedical Company); laminin  $\alpha$ 4 (R&D Systems); PDGFR- $\beta$  (eBioscience); GFAP (Dako); collagen type IV (Abcam); hemoglobin (Santa Cruz Biotechnology, Inc.); EGFP (Molecular Probes); and EphrinB2 (R&D Systems). The sections were incubated at 4°C overnight with primary antibody diluted in PBS containing 0.3% Triton X-100 and 3% normal donkey serum. After rinsing in PBS, sections were incubated with the appropriate secondary antibodies for 1 h. The sections were rinsed in PBS, cover-slipped, and examined using fluorescence microscopy (Axiovert 200; Carl Zeiss).

Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling assay (TUNEL) was performed using the In situ Cell Death Detection kit (Roche) according to the manufacturer's instructions. LN $\gamma$ 1-KO and control mice at P0, P14, P28, and adult were perfused with ice-cold saline, and fresh frozen brain sections were prepared on a cryostat (Leica). Brain sections were fixed in 4% PFA, and permeabilized in 0.1% Triton X-100/0.1% sodium citrate on ice for 2 min and stained with the kit. Blood vessels were visualized with BSL (Vector Laboratories) and nuclei were counterstained with DAPI. Total numbers of blood vessel-associated TUNEL and DAPI double-labeled nuclei were determined and counted in the striatum of LN $\gamma$ 1-KO and control mice (5–7 mice in each group at each time point), and the differences were analyzed by two-tailed Student's *t* test.

#### Western blot analysis

Control and LN $\gamma$ 1-KO mice were perfused with cold PBS ( $n = 7$ /group). Brains were collected, and the olfactory bulb and cerebellum were removed and then the right hemisphere of the cerebrum was separated into the cerebral cortex, hippocampus, and the rest of the deep brain regions

including basal ganglia, thalamus, and hypothalamus (collectively referred as to deep brain regions). The left hemispheres of the brain were used for brain section preparation and immunohistochemistry. The samples were frozen in dry ice. The tissues were homogenized on ice in 2% SDS, 95 mM NaCl, 25 mM Tris, pH 7.4, 10 mM EDTA, and protease inhibitor cocktail (Roche). After centrifugation, extracts were run on a reducing 8 or 10% SDS-PAGE, blotted to PVDF membrane (EMD Millipore), incubated overnight at 4°C in primary antibody (rat anti-laminin  $\gamma$ 1, Thermo Fisher Scientific; rat anti-laminin  $\alpha$ 1, EMD Millipore; mouse anti-laminin  $\alpha$ 2, Santa Cruz Biotechnology, Inc.; rabbit anti-laminin  $\alpha$ 4, Santa Cruz Biotechnology, Inc.; mouse anti-CD31, Abcam; rabbit anti-PDGFR- $\beta$ , Cell Signaling Technology; mouse anti-GFAP, Sigma-Aldrich; mouse anti-SMA, Sigma-Aldrich; rat anti-SM1, Kamiya Biomedical Company; mouse anti-SM2, Abcam; goat anti-smoothelin, Santa Cruz Biotechnology, Inc.; and rabbit anti-SM22, Gene Tex), and then incubated with HRP-labeled secondary antibody (Jackson ImmunoResearch Laboratory) for 1 h at room temperature. Protein expression was observed with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific). After stripping, the membranes were reblotted with mouse anti-actin antibody (Sigma-Aldrich). All Western blot results were normalized to actin.

#### Cell culture

Human brain vascular smooth muscle cells (BVSMCs; Schwartz et al., 1986; Braun et al., 1999) and human astrocytes (van der Laan et al., 1997; Hatton, 2002; Chen and Swanson, 2003), obtained from ScienCell Research Laboratory, were cultured in an incubator with 5% CO<sub>2</sub>/95% air at 37°C. The second or third passages of the cells were used for experiments. Plates were coated with poly-L-lysine (PLL, 2  $\mu$ g/cm<sup>2</sup>; ScienCell Research Laboratory), collagen type IV (4  $\mu$ g/cm<sup>2</sup>; Gibco), laminin-111 (Gibco), laminin-211 (4  $\mu$ g/cm<sup>2</sup>; a gift from Peter Yurchenco, Robert Wood Johnson Medical School, New Brunswick, NJ), or laminin-111 and -211 (2  $\mu$ g/cm<sup>2</sup> of each). The BVSMCs were plated with medium 231 (Gibco) with smooth muscle growth supplement (Gibco) and 5% fetal bovine serum (FBS). Once the cells reached 80–90% confluence, medium was changed to medium 231 with 0.5% FBS and replaced every other day. Human astrocytes were cultured on PLL-coated plates with astrocyte medium containing 2% FBS (ScienCell Research Laboratory). Once they reached 80–90% confluence, astrocytes were collected for co-culture with BVSMCs.

For BVSMC and astrocyte co-culture, BVSMCs were cultured on PLL-coated plates. Once they reached 80–90% confluence, astrocytes (1,000 or 4,000 cells/cm<sup>2</sup>) were seeded with BVSMCs, and they were co-cultured for 6 d in astrocyte medium. The medium was replaced every other day. In BVSMC sister control cultures, the culture conditions and media were exactly the same as the co-cultures but without astrocytes.

For Western blot analysis, the cells were washed with cold PBS three times and lysed in lysis buffer as described above. In BVSMC and astrocyte co-culture experiments, the total cell numbers of control cultures and BVSMC/astrocyte co-cultures were determined. The percentages of astrocytes in the co-culture were determined by GFAP (a marker of astrocytes) immunocytochemistry (number of GFAP-positive cells versus total number of DAPI). The same percentage of astrocytes was added to the BVSMC control cultures before lysing the cells to ensure the SMC numbers were similar between the two groups for comparison of their differentiation.

#### Richardson's staining and EM analysis

Mice were anesthetized and perfused with PBS and 2.5% PFA/2.5% glutaraldehyde in sodium cacodylate buffer. The brains were removed and immersed in the same fixative. Brain sections (100  $\mu$ m) were cut on a vibratome and collected in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, post-fixed in reduced osmium (1% osmium tetroxide, 1.6% K-ferrocyanide in 0.1 M cacodylate buffer), blocked, stained in 1% uranyl acetate, and embedded in resin. Semi-thin sections were cut and stained with Richardson's staining for proteins. For EM analysis, ultra-thin sections were cut on a microtome (UltraCut E; Reichert-Jung) and post-stained with uranyl acetate and lead. Sections were examined and photographed on a transmission electron microscope (100CXII; JEOL) at 80 KV. For analysis of percentages of VSMCs showing direct contact with astrocytes or with abnormal morphology in different brain regions in LN $\gamma$ 1-KO and control mice ( $n = 7$ /group), the analyzer was blind to the genotypes of the samples. The differences between LN $\gamma$ 1-KO and control mice were analyzed by Student's *t* test.

#### Imaging analysis

After immunohistochemistry or immunocytochemistry, brain sections were analyzed with a microscope (Axiovert 200; Carl Zeiss) equipped with Plan-Neofluar (1.25 $\times$  NA 0.03; 20 $\times$  NA 0.5, and 40 $\times$  NA 0.75) objective lenses at room temperature. The imaging medium was air for all the objective

lenses used. The AxioCam color camera (Carl Zeiss) and AxioVision software (Carl Zeiss) were used for image collection. Each set of stained sections was processed under identical gain and laser power settings. Each set of obtained images was processed under identical brightness and contrast settings using Adobe Photoshop. Figures were prepared using Microsoft PowerPoint. For quantification of fluorescence staining intensity after ad-GFAP-Cre injection, three brain sections containing the injection sites from three ad-GFAP-Cre-injected control and  $\beta$ 1 mice were used. The immunostaining signal intensity was analyzed using ImageJ (National Institutes of Health). The Western blot films were digitized, and the signal intensity of the bands was analyzed using ImageJ.

#### Statistical analysis

Prism 4 (GraphPad Software) was used for all statistical analysis as indicated in the figure legends by either Student's *t* test or one-way analysis of variance (ANOVA).

#### Online supplemental material

Fig. S1 shows that all endothelial cells express laminin  $\alpha$ 4 in both control and  $\beta$ 1-KO mice. Fig. S2 shows disruption of laminin  $\alpha$ 2 expression at the astrocytic endfeet of  $\beta$ 1-KO mice and that neuronal laminin does not contribute to the vascular matrix. Fig. S3 shows Cre expression patterns in nestin-Cre mouse line. Fig. S4 shows region-specific VSMC contractile protein expression changes in  $\beta$ 1-KO mice during development. Fig. S5 shows relationship between astrocytic laminin  $\alpha$ 1 and SMA expression. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.201212032/DC1>.

We thank Eleana Spichas and Kunihiro Uryu in the Electron Microscopy Resource Center at The Rockefeller University for technical support and assistance, Peter Yurchenco at UMDNJ for recombinant laminin-211, Arturo Alvarez-Buylla at UCSF for Ad-pGFAP-Cre, and GENSAT at The Rockefeller University for ROSA26-EGFP mice. We also thank Anita Ramnarain and all members of the Strickland laboratory for scientific support and advice.

This work was supported by NIH grant NS050537 and the Louis Herlands Fund.

Submitted: 7 December 2012

Accepted: 6 June 2013

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