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## Bidirectional control of CNS capillary diameter by pericytes

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### Abstract

Neural activity increases local blood flow in the CNS, which is the basis of BOLD and PET functional imaging techniques<sup>1–3</sup>. Blood flow is assumed to be regulated by precapillary arterioles, because capillaries lack smooth muscle. However, most (65%) noradrenergic innervation of CNS blood vessels is of capillaries rather than arterioles<sup>4</sup>, and in muscle and brain a dilatory signal propagates from vessels near metabolically active cells to precapillary arterioles<sup>5,6</sup>, suggesting that blood flow control is initiated at the capillary level. Pericytes, which are apposed to CNS capillaries and contain contractile proteins<sup>7</sup>, could initiate such signalling. Here we show that pericytes can control capillary diameter in whole retina and cerebellar slices. Electrical stimulation of retinal pericytes evoked a localised capillary constriction, which propagated at  $\sim 2\mu\text{m}/\text{sec}$  to constrict distant pericytes. Superfused ATP in retina, or noradrenaline in cerebellum, made pericytes constrict capillaries, and glutamate reversed the constriction produced by noradrenaline. Electrical stimulation or puffing GABA receptor blockers in the inner retina also evoked pericyte constriction. In simulated ischaemia, some pericytes constricted capillaries. Pericytes are likely modulators of blood flow in response to changes in neural activity, which may contribute to functional imaging signals and to CNS vascular disease.

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Neurotransmitters make cultured pericytes contract<sup>8</sup>, and raise  $[\text{Ca}^{2+}]_i$  and constrict pericytes on isolated retinal microvessels<sup>9–11</sup>. Since neocortical pericytes *in situ* show neuronally-evoked  $[\text{Ca}^{2+}]_i$  elevations<sup>12</sup>, pericytes might control blood flow downstream of arterioles (Fig. 1a). However, it is unknown whether pericytes regulate capillary diameter *in situ* in retina or brain.

In retina and cerebellum, an antibody to the NG2 proteoglycan<sup>13</sup> revealed two pericyte classes: those on straight parts of capillaries, separated by  $34.2 \pm 3.6\mu\text{m}$  ( $n=24$ ) in retina, and those at capillary junctions (Fig. 1a–c). Pericytes have processes along and around the capillary (Fig. 1d). Loading dye into retinal pericytes by whole-cell clamping revealed processes like claws around the capillary (Fig. 1e), ranging  $11.3 \pm 0.7\mu\text{m}$  ( $n=3$ ) from the soma, providing an anatomical substrate for generating the capillary constriction described below.

We stimulated retinal pericytes electrically, with a pipette pressed on their soma, aiming to raise  $[\text{Ca}^{2+}]_i$ , since in isolated vessels pericyte constriction correlates with a  $[\text{Ca}^{2+}]_i$  rise<sup>11</sup>. Stimulation constricted most (90% of 30) pericytes studied (Fig. 2a–d, Supplementary Movie 1), starting  $3.6 \pm 1.6$  sec, and reaching half-maximum  $12.6 \pm 3.6$  sec, after initiating stimulation. After stimulation, constriction relaxed 50% in  $60.3 \pm 12.4$  sec. For 17 pericytes on straight parts of capillaries (initial diameter  $8.6 \pm 0.5\mu\text{m}$ ) the mean vessel constriction was  $73 \pm 5\%$  (Fig. 2e). Stimulation constricted pericytes at branch points (initial diameter

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5.8±0.6µm) by 76±8% (n=11). Stimulating the capillary wall between pericytes did not constrict the capillary or adjacent pericytes (Fig. 2e, n=7): thus, endothelial cells and astrocyte endfeet do not constrict.

Removing extracellular calcium evoked a dilation of capillaries near pericytes (by 28% in 4 vessels, p=0.023) that slowly decayed, but no dilation at non-pericyte sites (p=0.22, Fig. 2f,g). Removing Ca<sup>2+</sup> abolished the stimulation-evoked constriction (Fig. 2f,h), consistent with a raised [Ca<sup>2+</sup>]<sub>i</sub> triggering constriction. When pericytes were stimulated to constrict, sometimes distant pericytes also constricted later (Fig. 3a, Supplementary Movie 1). However, the capillary between the pericytes did not constrict (Fig. 3b). The constriction of distant pericytes cannot be due to extracellular stimulus spread because stimulating between pericytes did not evoke a constriction (Fig. 2e). The speed of the constriction spread was 2.0±1.3µm/sec (n=4).

In brain, astrocyte [Ca<sup>2+</sup>]<sub>i</sub> elevations regulate arteriole diameter<sup>14–16</sup>. ATP is released when [Ca<sup>2+</sup>]<sub>i</sub> waves spread between retinal glial cells<sup>17</sup>, and constricts pericytes on isolated vessels by raising [Ca<sup>2+</sup>]<sub>i</sub> via P2X<sub>7</sub> and P2Y receptors<sup>9</sup>. In the intact retina, ATP and the P2Y receptor agonist UTP constricted capillaries near pericytes, leaving non-pericyte regions uncontracted (Fig. 3c–e, Supplementary Movie 2). This occurred in pericytes on straight parts of capillaries and at branch points, but not in all pericytes: 50–100µM UTP constricted vessels at 30% (12 of 40) of pericytes, while 0.5–1mM ATP constricted 25% (5 of 20 pericytes: insignificantly different, p=0.92). Peak constrictions evoked by 100µM UTP (56±9%, n=10, initial diameter 7.1±0.9µm) and 1mM ATP (35±6%, n=4, from 7.9±2.3µm) were not significantly different (p=0.19). The lack of response of some pericytes is similar to findings on isolated vessels (a P2X<sub>7</sub> agonist contracted 37% of pericytes and capillary constriction occurred in only 1/3 of these or 12% of the total<sup>9</sup>, and a muscarinic agonist contracted only 10% of pericytes<sup>11</sup>). UTP- and ATP-evoked constrictions showed desensitization (Fig. 3d) to 34±3% and 32±17% of their initial value. Glutamate (500µM) did not affect retinal capillary diameter (n=39), and noradrenaline (0.3–10µM) constricted pericytes on only 3 of 61 retinal capillaries despite always constricting arterioles (Supplementary Fig. 1).

We tested whether ATP release from underlying glia might explain the stimulation-evoked pericyte constriction in Fig. 2, or the propagating constriction in Fig. 3A. Applying P2 receptor blockers (suramin, 100µM; PPADS, 100µM), which inhibit the spread of ATP-releasing Ca<sup>2+</sup> waves through retinal glia<sup>17</sup> and will reduce the actions of any released ATP on the P2Y<sub>2</sub>, P2Y<sub>4</sub> and P2X<sub>7</sub> receptors that trigger pericyte contraction<sup>9</sup>, did not affect the fraction of pericytes responding to electrical stimulation nor the constriction seen (p=0.96 and 0.63 respectively, n=10), eliminating the possibility that pericyte stimulation releases ATP from glia which constricts pericytes. Furthermore, although pericyte stimulation sometimes evoked a Ca<sup>2+</sup> wave (see Methods) in underlying glia, as did direct glial stimulation, the wave propagation speed (10.5±1.2µm/sec; n=8: 3 stimulating pericytes, 5 stimulating glia, no significant difference between pericytes and glia) was 5-fold faster (p=0.0014) than the propagation of the constriction described above. This suggests that the spread of constriction is not produced by a glial Ca<sup>2+</sup> wave but by a different mechanism, possibly an electrical signal in endothelial cells, or an effect of the pressure change generated by constriction of the stimulated pericyte. Pericyte-pericyte communication could allow detection of neural activity near capillaries to be propagated back to arterioles to amplify blood flow changes<sup>6</sup>, as in muscle<sup>5</sup>.

To assess if neuronal activity can regulate capillary diameter via pericytes, we stimulated electrically, or puffed solution containing GABA<sub>A</sub> and GABA<sub>C</sub> blockers (GABA<sub>A</sub>zine, 100µM; TPMPA, 100µM), within the retina near the inner plexiform layer. Stimulation

(Fig. 3f,g) evoked a pericyte constriction of  $39\pm 6\%$  (from  $4.6\pm 0.4\mu\text{m}$ ) in 13 of 43 capillaries tested, which was blocked by TTX ( $p=0.034$ : a second stimulus evoked no constriction in 6 of 6 cells in  $1\mu\text{M}$  TTX, but constricted 4 of 5 cells without TTX), while GABA blockers evoked a constriction of  $69\pm 12\%$  (from  $4.8\pm 0.4\mu\text{m}$ , latency  $34\pm 3$  sec, in 4 of 41 pericytes, Fig. 3h,i). The effect of GABA blockers suggests there is endogenously active neurotransmitter signalling to pericytes.

In cerebellar slices, noradrenaline ( $1\text{--}2.5\mu\text{M}$ ) constricted 50% (10 of 20) of capillaries at spatially restricted locations near pericytes (Fig. 4a, Supplementary Movie 3). In constricting capillaries, noradrenaline reduced capillary diameter by  $63\pm 8\%$  (from  $7.2\pm 0.8\mu\text{m}$ ) at pericytes, but did not affect the diameter at non-pericyte locations (Fig. 4b,c). In all 7 constricting vessels tested, superimposing glutamate ( $100\text{--}500\mu\text{M}$ ) on noradrenaline decreased the noradrenaline-evoked constriction, i.e. dilated the vessel, by  $40\pm 8\%$  of the precontracted diameter at the pericyte, but had no effect elsewhere (Fig. 4b,d, Supplementary Movie 3;  $100\mu\text{M}$  and  $500\mu\text{M}$  glutamate produced a  $55\pm 10\%$ , and a  $34\pm 9\%$  dilation in 2 and 5 vessels, not significantly different,  $p=0.2$ ). In one capillary glutamate dilated the vessel beyond the initial diameter it had in the absence of noradrenaline (by 14%), and in 4 (of 20) vessels tested  $500\mu\text{M}$  glutamate alone (without noradrenaline) dilated capillaries (by  $22\pm 9\%$ ), showing that glutamate can produce a net dilation. Thus, pericytes can control capillary diameter in the brain as well as the retina.

After ischaemia, cerebral blood flow is initially increased, but later reduced due to defective vasodilatory pathways<sup>18–20</sup>. We simulated retinal ischaemia, which leads to a regenerative “anoxic depolarization”<sup>21</sup>, associated with increased opacity. This occurred after  $435\pm 24$  sec. Before the anoxic depolarization some pericytes (4/38) constricted capillaries (Fig. 4e,f), by  $53\pm 9\%$  (from  $6.8\pm 0.4\mu\text{m}$ ).

These data establish pericytes as likely regulators of blood flow at the capillary level. First, we demonstrate that neurotransmitters evoke pericyte-mediated capillary constriction *in situ*. Previously this was shown only in isolated retinal capillaries, and it was claimed that vasoactive agents do not affect capillary diameter *in situ*<sup>22</sup> (but see ref. 23). Second, we show that capillary diameter changes are generated by pericytes, not endothelial cells, because they do not occur in pericyte-free regions of capillaries. Third, although retinal vessels have more pericytes than brain capillaries<sup>24</sup>, the noradrenaline-evoked constriction of cerebellar capillaries indicates that pericytes can regulate blood flow throughout the brain. Fourth, we show that decreasing pericyte contractile tone dilates capillaries:  $\text{Ca}^{2+}$ -removal dilates retinal capillaries and glutamate dilates cerebellar capillaries via pericytes. Glutamate may release NO, which inhibits  $\text{Ca}^{2+}$  influx in retinal pericytes and decreases contraction of cultured pericytes<sup>25,26</sup>. This could contribute to the increase of blood flow evoked by neural activity. Finally, we show that pericytes may contribute to the vascular response to ischaemia.

The constriction that ATP and noradrenaline produce (35% in retina, 63% in cerebellum, respectively) would increase flow resistance 5.6-fold and 53-fold by Poiseuille’s law, and might prevent erythrocytes passing through capillaries. Thus, pericytes could significantly redirect blood flow at the capillary level. It is unclear whether ATP and noradrenaline act directly on pericytes<sup>9</sup>, or via astrocytes<sup>27,28</sup>.

The low fraction of pericytes responding to neurotransmitters (25% for ATP in retina, 50% for noradrenaline in cerebellum) contrasts with the reliability of electrical stimulation-evoked constriction (90%). This may reflect variable expression of transmitter receptors on pericytes at different vascular sites, or a loss of factors released by blood flow. The lack of blood flow and pressure in brain slices may also slow the capillary responses reported here:

arteriolar dilations produced by astrocyte  $[Ca^{2+}]_i$  rises are faster *in vivo* than in slices<sup>14,16</sup>. In addition, although some pericytes do not visibly constrict, the stiffness of their processes around the capillary may be increased by ATP or noradrenaline, opposing dilation by other agents or by the deformation necessary for erythrocytes to pass along small capillaries.

These data suggest that pericytes may contribute to regulating cerebral blood flow in health and disease. Spatially restricted constrictions of brain microvessels attributed to arteriole smooth muscle<sup>29,30</sup> may actually have been mediated by pericytes. These results challenge the idea that arterioles are solely responsible for the blood flow increase evoked by neural activity which underlies functional imaging techniques (see Supplementary Material).

## Methods

### Preparations

Isolated rat retina (vitreous side up), or 200 $\mu$ m cerebellar slices, from P14–21 rats, were superfused with solution (33–35°C) containing (mM): NaCl 124, NaHCO<sub>3</sub> 26, NaH<sub>2</sub>PO<sub>4</sub> 1, KCl 2.5, CaCl<sub>2</sub> 1.8, MgCl<sub>2</sub> 2, D-glucose 10 (bubbled with 95% O<sub>2</sub>/5% CO<sub>2</sub>), pH 7.4. Kynurenic acid (1mM, blocks glutamate receptors) was included in the dissection and tissue storage solutions. Ischaemia was simulated by replacing 10mM glucose with 7mM sucrose, bubbling 95% N<sub>2</sub>/5% CO<sub>2</sub> and adding glycolysis and oxidative phosphorylation blockers (2mM Na-iodoacetate; 25 $\mu$ M antimycin).

### Imaging

Capillaries, lacking the continuous smooth muscle around arterioles (Supplementary Fig. 1) and <11 $\mu$ m diameter, were imaged every 2–10s. Capillaries, unlike arterioles (Supplementary Fig. 1), showed pericyte-mediated spatially-restricted constrictions in response to transmitters. For Ca<sup>2+</sup> imaging, retinæ were incubated with Fluo-4-AM (70 mins, room temperature); fluorescence was excited at 475nm and collected at 535nm; surface glia were stimulated electrically. Pericytes were labelled with NG2 antibody (Chemicon), which from P21 labels mainly pericyte somata<sup>13</sup>, using Alexa 555-conjugated goat anti-rabbit secondary antibody. Blood vessels were labelled with Alexa 488-isolectin B<sub>4</sub> (Invitrogen).

### Electrophysiology

Pericytes were whole-cell clamped with ~8M $\Omega$  electrodes containing Alexa 488 (1mg/ml) and (mM): K-gluconate 130, NaCl 10, MgCl<sub>2</sub> 1, CaCl<sub>2</sub> 0.1, HEPES 10, K<sub>2</sub>-EGTA 1.1, Na<sub>2</sub>-ATP 1, pH 7.0 with KOH, after pre-treating retinæ with collagenase (1mg/ml, 30 mins, 37°C) to remove connective tissue. Similar electrodes filled with extracellular solution were used to stimulate pericytes, by pressing the electrode against the cell and applying voltage pulses (40–90V, 0.02–0.2msec, 9Hz; 3 pulses evoked constriction), or to stimulate inner retinal neurons, by placing the electrode tip 22 $\mu$ m below the retinal surface and applying voltage pulses (60–90V, 0.02–0.06msec, 9–20 Hz for 20–30s: constriction cannot be due to extracellular current spread directly activating pericytes because no such response was seen when stimulation was applied to the capillary wall between pericytes).

### Statistics

Data are mean $\pm$ s.e.m. P values are from 2 tailed Student's t-tests or  $\chi^2$  tests.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

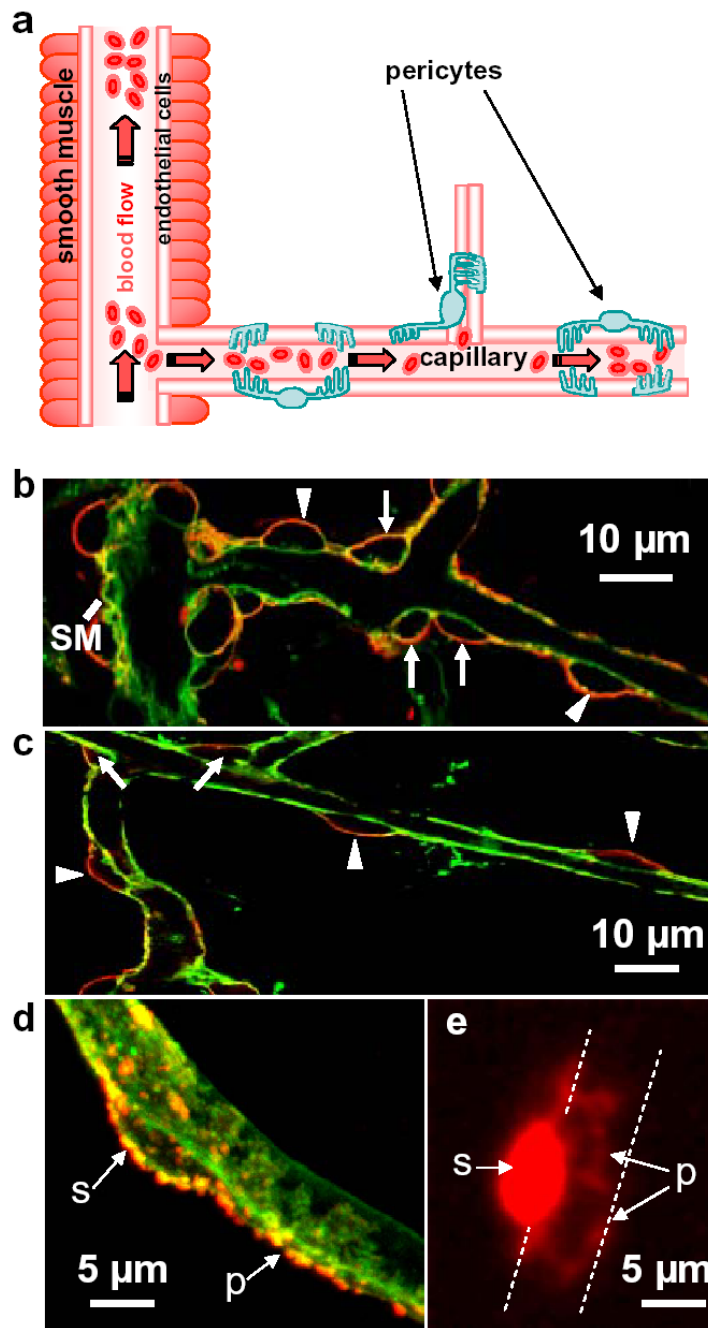
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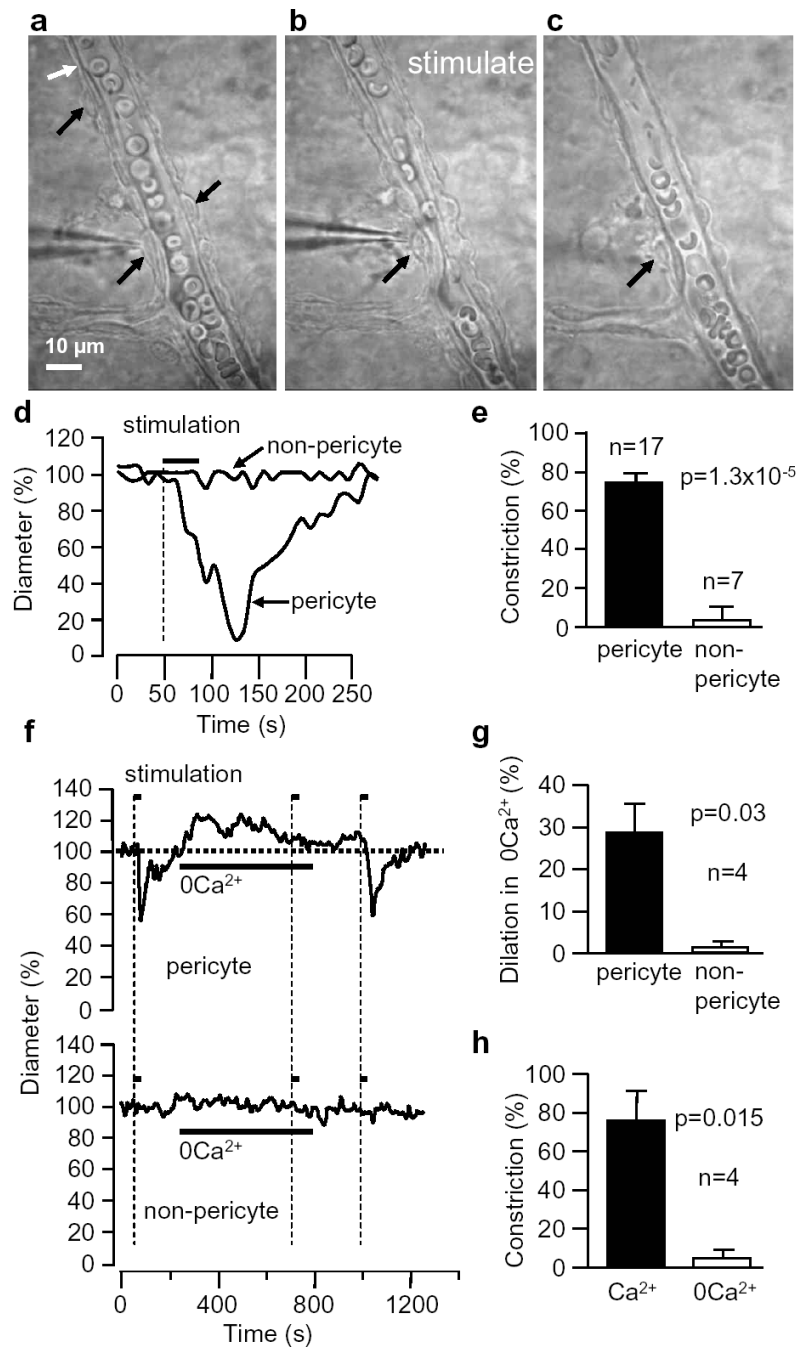
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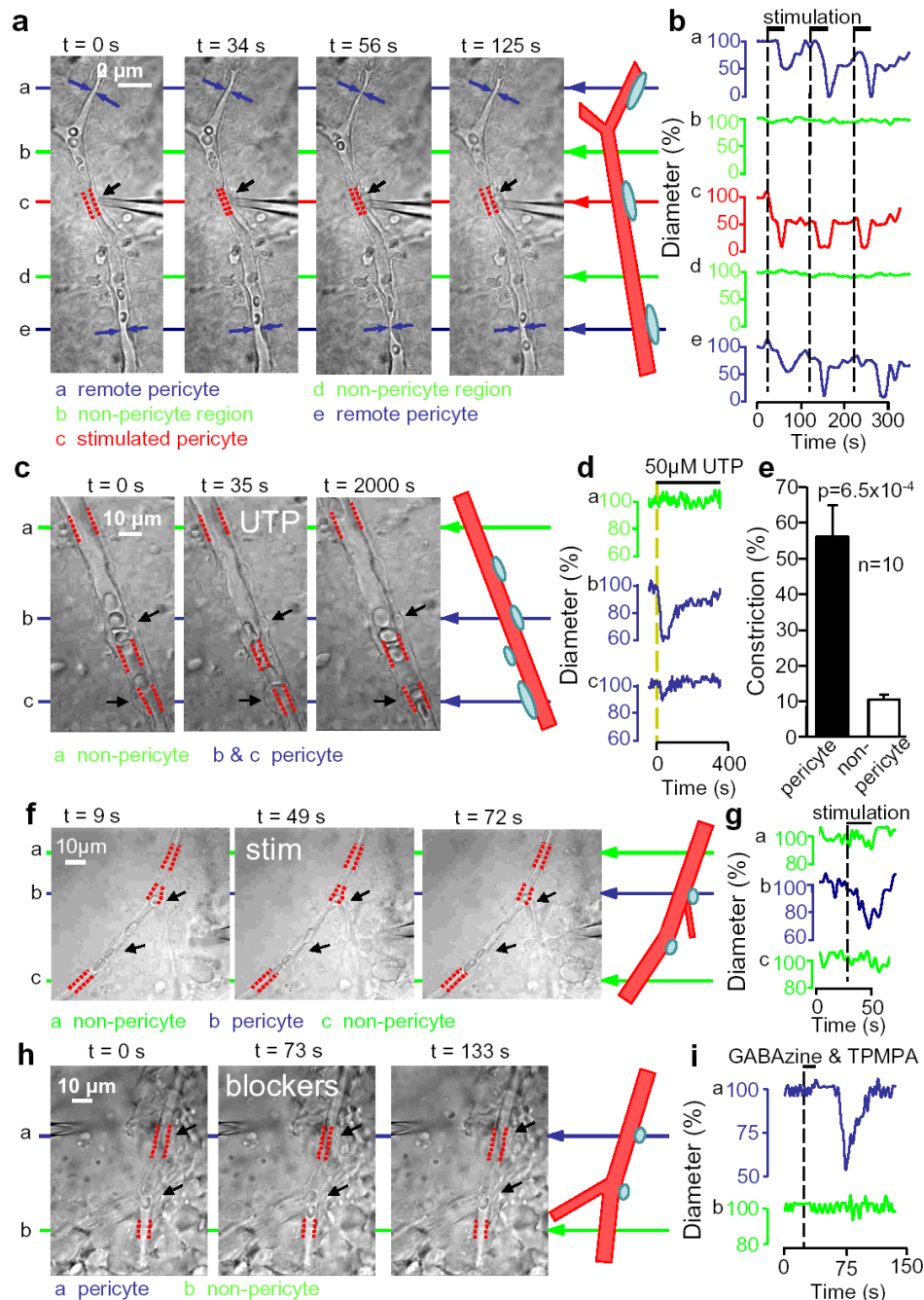
**Figure 1.**

Pericyte anatomy confers flow regulating capability downstream of arterioles. **a** Potential blood flow control sites in cerebral vasculature: arteriolar smooth muscle, and pericytes on capillaries. **b** Cerebellar molecular layer arteriole (left), surrounded by smooth muscle (SM), giving off a capillary. Capillary labelled with isolectin B4 (green); pericytes labelled for NG2 (red) are on the straight part of capillaries (arrow heads) and at junctions (arrows). **c** Retinal capillaries. **d** Soma (s) of cerebellar pericyte gives off processes (p) running along around capillary. **e** Dye fill of retinal pericyte reveals processes running around capillary (dashed lines).



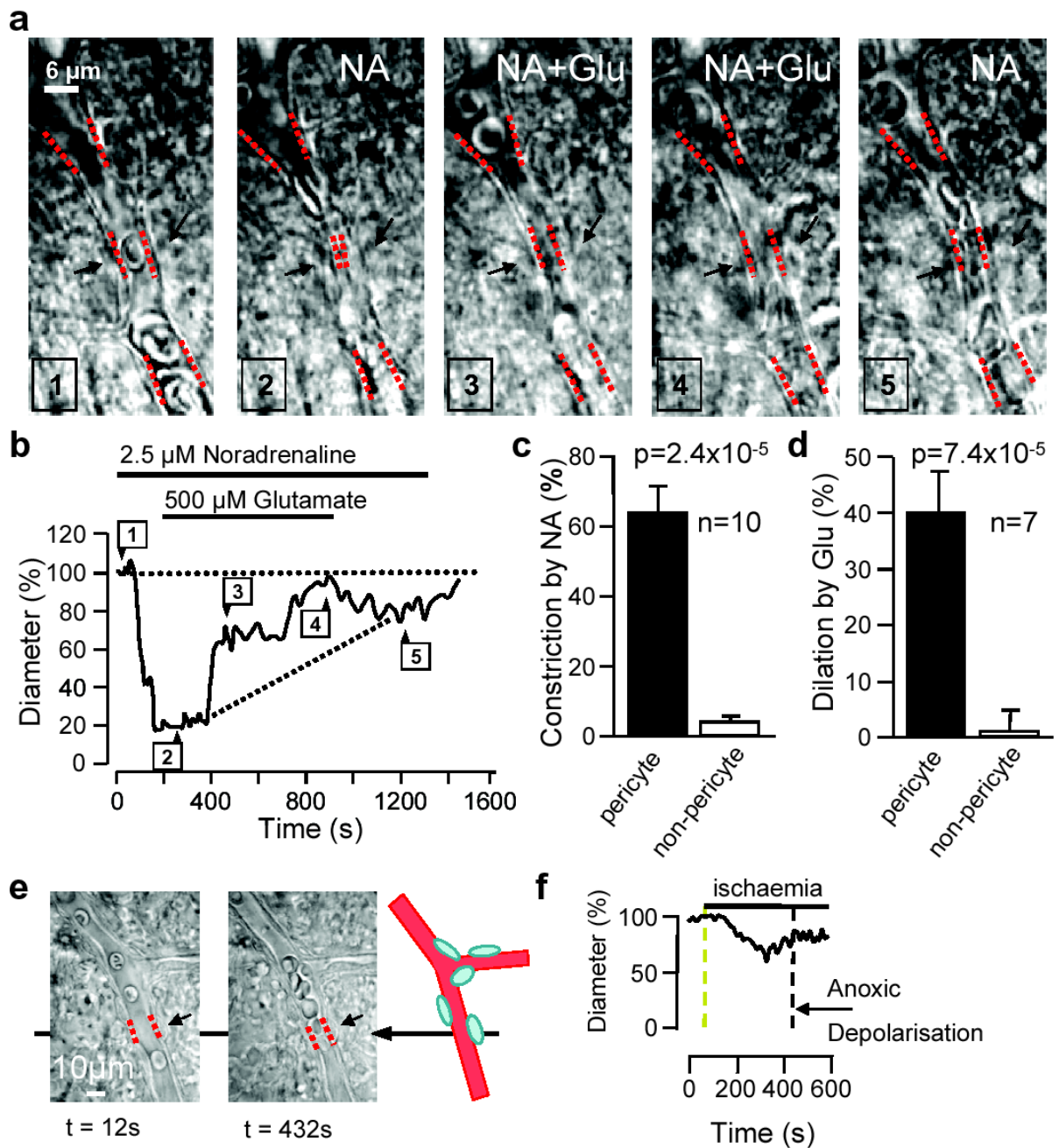
**Figure 2.** Electrical stimulation evokes a  $\text{Ca}^{2+}$ -dependent localized constriction of retinal pericytes. **a-c** Capillary with pericytes (black arrows) before (a), during (b) and after (c) stimulation. Erythrocytes are present within capillary; thin structures outside capillary are astrocyte endfeet. **d** Diameter of capillary in a-c at stimulated pericyte and a non-pericyte site (white arrow). **e** Mean ( $\pm$ s.e.m.) constriction when stimulating at pericyte or non-pericyte sites. **f** Effect of removing extracellular  $\text{Ca}^{2+}$  on resting diameter and response to pericyte stimulation, at pericyte and nearby non-pericyte sites. **g** Dilation produced by removing  $\text{Ca}^{2+}$ . **h** Effect of  $\text{Ca}^{2+}$ -removal on pericyte constriction, as in f.



**Figure 3.**

Propagation and transmitter-evocation of retinal pericyte constriction. **a** Electrically stimulating a pericyte (black arrow) evokes local constriction (red dashes show vessel diameter) followed by constriction of distant pericytes (blue). **b** No constriction of intervening non-pericyte regions (green). **c,d** UTP constricts two pericytes (arrows) but not at non-pericyte region. **e** Mean ( $\pm$ s.e.m.) UTP-evoked constriction. **f,g** Pericyte constriction (top arrows; lower arrows show another pericyte) evoked by electrical stimulation (electrode, right) near inner plexiform layer. **h,i** Constriction of pericyte (top arrows; lower

arrows show another pericyte) evoked by puffing GABA receptor blockers (electrode, top left) near inner plexiform layer.



**Figure 4.** Effects of neurotransmitters on cerebellar molecular layer capillary, and of ischaemia on retinal capillary. **a** Localized noradrenaline-evoked constriction near pericytes (black arrows, clearest in panel 4), and dilation by superimposed glutamate. Noradrenaline-evoked constriction cannot reflect fluid movement caused by arterioles constricting, because this would make the capillary dilate. **b** Diameter at pericyte; numbers show image times above. **c** Mean ( $\pm$ s.e.m.) constriction by noradrenaline, at pericyte and non-pericyte sites. **d** Dilation (reduction of noradrenaline-evoked constriction) by glutamate (as percentage of pre-

noradrenaline diameter). **e** Retinal capillary before ischaemia and in ischaemia before anoxic depolarization. **f** Diameter at pericyte in **e**.