A novel functional role for the classic CNS neurotransmitters, GABA, glycine and glutamate, in the kidney: potent and opposing regulators of the renal vasculature

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Running head: Functional role of GABA, glycine and glutamate in the kidney

Abstract

The presence of a renal GABA/glutamate system has previously been described; however, its functional significance in the kidney remains undefined. We hypothesized given its extensive presence in the kidney that activation of this GABA/glutamate system would elicit a vasoactive response from the renal microvessels. Functional data here demonstrate for the first time that activation of endogenous GABA and glutamate receptors in the kidney significantly alters microvessel diameter with important implications for influencing renal blood flow. Renal blood flow is regulated in both the renal cortical and medullary microcirculatory beds via diverse signaling pathways. GABA- and glutamate-mediated effects on renal capillaries are strikingly similar to those central to the regulation of CNS capillaries, that is, exposing renal tissue to physiological concentrations of GABA, glutamate and glycine led to alterations in the way contractile cells, pericytes and smooth muscle cells, regulate microvessel diameter in the kidney. Since dysregulated renal blood flow is linked to chronic renal disease, alterations in the renal GABA/glutamate system, possibly through prescription drugs, could significantly impact long-term kidney function.

Key words: GABA, glutamate, Glycine, microvascular function, pericytes.

New and Noteworthy: Functional data here offers novel insight into the vasoactive activity of the renal GABA/glutamate system. This data shows that activation of endogenous GABA and glutamate receptors in the kidney significantly alters microvessel diameter. Furthermore, it shows that these antiepileptic drugs are as potentially challenging to the kidney as NSAIDs.

Introduction

In the brain, the classical inhibitory and excitatory neurotransmitters, GABA/glycine and glutamate respectively, aid brain function by regulating cerebral blood flow. Understanding what initiates increases in CNS blood flow in response to neuronal activity remains contentious[20,32,47]. Regulation of renal blood flow is similarly complex and given the significance of the kidney in regulating systemic blood flow, is an important research area. Some CNS studies describe pericyte[23,54] cell-mediated regulation of capillary diameter as the primary mechanism for initiating increases in CNS blood flow[8], while others support smooth muscle cell (SMC)-mediated regulation of arteriole diameter[22,56]. Anatomically, pericyte-mediated regulation of the microcirculation seems more appropriate, since most neurons are in close apposition to CNS capillaries rather than arterioles[22].

In the kidney, there are distinct vascular beds in the cortex and medulla. The renal medulla is served solely by vasa recta capillaries and pericytes are spatially located along the vasa recta to regulate blood flow in this region[7,8,30,43,44,45] Conversely, glomeruli are located in the renal cortex and are served by afferent arterioles and efferent arterioles, bearing a full vascular smooth muscle coat, surrounded by SMCs that serve to regulate glomerular blood flow and glomerular capillary pressure. Regulation of blood flow in the renal cortex maintains GFR, and regulation in the medulla maintains urine concentration. These regional processes are highly metabolic, and as such need to be tightly regulated. Dysregulation of renal blood flow is linked to numerous pathologies, including hypertension, diabetic nephropathy, fibrosis and drug-induced nephrotoxicity[30,48,51] and interestingly, pericytes are intimately involved in almost all pathologies[47].

GABA, glycine and glutamate, their respective receptors, and enzymes involved in the synthesis and metabolism of GABA are all present in tubular and vascular compartments of the renal cortex and medulla[12,13,17,38,50,55,57]. Given the existence of renal GABA, glycine and glutamate and their established role in the regulation of CNS capillary diameter we

hypothesize that these neurotransmitters are similarly involved in the regulation of renal vascular function and hence renal hemodynamics.

Accordingly, we investigated the roles of GABA, glycine and glutamate in regulating cortical and medullary blood flow. We provide evidence as to the receptors and cell signaling pathways involved and show that agents traditionally considered neurotransmitters differentially regulate different vascular beds in the kidney. Lastly, data presented here show how prescription medication, used to target conditions of the CNS, can also act to dysregulate microvascular diameter, and thereby influence renal blood flow.

Results

GABA induces pericyte-mediated constriction of vasa recta capillaries in renal medulla

Superfusion of kidney slices with GABA (3 μM) evoked a significant and maximal decrease in subsurface (>50 μm) vasa recta capillary diameter at pericyte sites (12.4 ± 1.8%, $p < 0.05$, n = 10; Fig. 1a-b) but not at non-pericyte sites (0.9 ± 0.3%, Fig. 1a-b). Using Poiseuille's law, to estimate the effect of vasoconstriction at this magnitude (12.4%) on blood flow, this percentage decrease in vessel diameter would suggest a decrease in blood flow of ~41%. The GABA-evoked vasoconstriction was reversible, reproducible (Fig. 1a-b) and concentration dependent (Fig. 1c). The magnitude of pericyte-mediated vasoconstriction of vasa recta by GABA (3 μM) was similar to that previously reported for norepinephrine (NE), ATP, angiotensin-II (Ang-2) and endothelin-1 (ET-1)[3] (Fig. 1d). The GABA-evoked vasoconstriction of vasa recta by pericytes was coupled to increases in *in situ* pericyte and endothelial cell intracellular calcium (Fig 1e). Maximal increases in Fluo-4 fluorescence in pericytes spatiotemporally correlated with pericyte-mediated vasoconstriction of vasa recta (10.2 ± 2.7%, $p < 0.05$, n = 10; Fig. 1e, f). We were unable to adequately spatiotemporarily resolve whether calcium transients originated in endothelial or pericyte cells, nor the direction in which the signal propagated.

Superfusion of live kidney tissue with the $GABA_A$ receptor ($GABA_AR$) and $GABA_BR$ agonists muscimol (1 µM; Fig. 1g) and baclofen (200 nM[39]; Fig. 1h); respectively, caused a significantly greater vasoconstriction of vasa recta at pericyte sites (12.6 ± 1.1%, n = 6, and 13.1 ± 2.6%, respectively; $p < 0.05$, n = 3) than at non-pericyte sites (Fig. 1i), suggesting GABA-evoked vasoconstriction is mediated by $GABA_AR$ and $GABA_BRs$. Application of the $GABA_AR$ antagonist bicuculline (10 µM; Fig. 1j), or the $GABA_BR$ antagonist CGP (1 µM; Fig. 1k), evoked a significantly greater vasodilation of vasa recta at pericyte-sites (15.8 ± 2.7%; n = 10, and 13.1 ± 2.8%; n = 11, respectively, $p < 0.05$), than at non-pericyte sites (Fig. 1l), suggesting blockade of endogenous GABA binding to both $GABA_AR$ and $GABA_BR$ receptors. Co-application of both muscimol (500 nM) and baclofen (100 nM) resulted in a significantly greater vasoconstriction of vasa recta at pericyte sites (13.5 + 0.9%) than that measured in response to superfusion of tissue with agents alone ($p < 0.001$; Fig. 1m) and equated to the sum of the individual responses.

Co-application of GABA (3 µM) with bicuculline (10 µM), GABA (3 µM) with CGP (1 µM), or GABA (3 µM) with both bicuculline and CGP, resulted in a ~70% (n = 7), ~39% (n = 8) and ~57% (n = 7) reduction GABA-evoked vasoconstriction of vasa recta by pericytes, respectively; no significant change in vessel diameter was detected at non-pericyte sites (Fig. 1n). Combining bicuculline and CGP in the perfusate with GABA failed to elicit a significantly greater reduction in the GABA-mediated constriction than that mediated by either antagonist alone (Fig 1n). The effect of both bicuculline and CPG on vessel diameter was not due to GABA receptor desensitisation since exposure of kidney tissue to GABA (3 µM) alone for the same duration resulted in an irreversible pericyte-mediated constriction of vasa recta[7]. Collectively, data demonstrate that GABA (endogenous and exogenously superfused) acts at $GABA_AR$ and $GABA_BR$ to elicit pericyte-mediated vasoconstriction of vasa recta in the renal medulla.

Glutamate and glycine induce pericyte-mediated vasodilation in the renal medulla

Superfusion of live kidney slices with the GABA precursor glutamate (10 µM) caused a significantly greater dilation of vasa recta at pericyte sites (15.7 ± 3.9%) than at non-pericyte sites (1.6 ± 0.7%, $p < 0.01$; Fig. 2a). Glutamate-mediated dilation was reversible but not reproducible (Fig. 2b) and the magnitude of glutamate-mediated pericyte-evoked increases in vasa recta diameter was greater than that reported for adenosine, SNAP prostaglandin E_2 and bradykinin[3] (Fig. 2c). Glutamate at plasma concentrations (100 nM) also induced significant increases in vasa recta diameter at pericyte sites (7.0 ± 1.2%; Fig. 2d).

The NMDA receptor (NMDAR) agonist NMDA, (Fig. 2e and g), and kainite receptor agonist domoic acid, (Fig. 2f and g), both evoked pericyte-mediated vasodilation (14.9 ± 2.7%; n = 8 and 12.3 ± 2.8%; n = 6, respectively), of a similar magnitude to that that observed for glutamate (10 µM, Fig 2a). To attenuate binding of endogenous glutamate to NMDARs and kainate receptors, tissue was exposed to MK-801 (300 µM; Fig. 2h), and UBP-302 (25 µM; Fig. 2i) respectively. MK-801 and UBP-302 evoked a significant pericyte-mediated constriction of vasa recta (16.0 ± 1.9%; n = 6 and 13.1 ± 2.4%; n = 6, respectively, $p < 0.05$, n=6), with no change at non-pericyte sites (1.3 ± 0.4% and 1.1 ± 0.3%, respectively, Fig. 2j). Tissue was exposed to a several glutamate receptor antagonists (Fig 2K). Only MK-801 (NMDA receptor antagonist) significantly attenuated glutamate-evoked dilation of vasa recta by pericytes (71.5% n = 7; Fig. 2k). This effect was not due to receptor desensitisation since pericyte-mediated dilation of vasa recta is sustained while glutamate is present (Extended data Fig. 2).

Glycine (1 mM) elicited concentration dependent, reversible pericyte-mediated vasodilation of vasa recta (16.9 ± 2.7% and 10.2 ± 1.8%, $p < 0.05$, n = 10; Fig.3a-d) that was significantly greater than that at non-pericyte sites ($p < 0.01$, n = 6), (Fig. 3a-c). To determine the

receptors involved, tissue was superfused separately with glycine (1 mM) in combination with MK-801 and glycine in combination with the glycine receptor antagonist strychnine, respectively. Co-application of glycine and MK-801 (300 μM) resulted in pericyte-mediated constriction (10.8 ± 3.2%, n = 8; Fig. 3e) that reversed upon removal of MK-801 from the superfusate. Subsequent perfusion of tissue with glycine alone induced pericyte-mediated dilation of vasa recta (19.8 ± 4.7%; Fig. 3f). Strychnine failed to attenuate glycine-mediated dilation ($p > 0.05$; Fig. 3g, h), thus glycine-mediated dilation of vasa recta is likely mediated via NMDAR.

Signalling pathways involved in glutamate-evoked pericyte-mediated dilation of vasa recta

In the CNS, glutamate-evoked dilation of capillaries by pericytes is NO-dependent.[9] DAF-FM is an NO-sensitive fluorescent indicator and glutamate and glycine evoked an increase in pericyte DAF-FM (24 μM) fluorescence (235 ± 36.8%, 136.9 ± 9.8%, respectively, n= 7) that spatiotemporally matched pericyte-mediated vasodilation (8.0 ± 0.7% and 8.7 ± 3.6%, respectively; n-7, Fig. 4a-d). Thus glutamate- and glycine-mediated activation of NMDARs leads to NO production in pericytes (and endothelial cells; Fig 4B and D). To determine the source of NO, tissue was treated with glutamate and a selective inhibitor of nNOS, vinyl-L-NiO (1 μM), (data not shown); or a competitive inhibitor of the neuronal and endothelial isoform of NOS, L-NNA (100 μM; Fig. 4e). Only L-NNA significantly reduced the glutamate-induced increase in vasa recta diameter (62%, $p < 0.01$; n=6 Fig. 4f). Thus, eNOS plays a role in glutamate-evoked dilation of vasa recta by pericytes.

The guanylyl cyclase blocker, ODQ (10 μM) failed to alter the glutamate-evoked vasodilation of vasa recta ($p > 0.05$, n = 8; Fig. 4g, 4h) but the epoxyeicosatrienoic acid (EET) inhibitor PPOH (9 μM) significantly reduced the glutamate-evoked vasodilation at pericyte sites (13.1

± 1.9%) by 50.2% (to 6.5 ± 1.2%, $p < 0.01$, n = 9; Fig. 4i, j). Glutamate-mediated dilation in this preparation is therefore cGMP independent. In the CNS, prostaglandin E_2 mediates glutamate-evoked dilation of capillaries.[9] Here the EP_4 receptor antagonist, L-161,982 (1 μM), significantly attenuated the glutamate-mediated dilation by 77% ($p < 0.01$, n = 7; Fig. 4k, l). When applied to tissue alone, both L-161,982 and PPOH evoked pericyte-mediated vasoconstriction (extended data Fig 3), suggesting PGE_2 (or similar activators of EP_4 receptors) and EET derivatives of arachidonic acid (AA) are involved in the NO-dependent response following activation of NMDARs (see schematic in extended data Fig 1).

Signals that regulate afferent arteriole diameter

For comparison, we examined the effect of GABA, glutamate and glycine on juxtamedullary nephron afferent arterioles which provides the blood supply reaching the vasa recta. For consistency, reagents were added to the superfusate. Increasing concentrations of GABA (10 μM - 1 mM) led to incremental decreases in afferent arteriolar diameter (Fig. 5a). The GABA-mediated vasoconstriction was inhibited by bicuculline (10 μM), but bicuculline also produced a transient vasoconstriction when applied alone ($p < 0.05$; n = 6; Fig. 5b). Unexpectedly, glutamate similarly decreased afferent arteriole diameter (Fig. 5c), an effect that was significantly inhibited by blocking 20-HETE formation using HET0016 (1 μM; $p < 0.001$, n = 6; Fig. 5d). Glutamate seemingly governs the regulation of cortical and medullary blood flow via two discrete signalling pathways. The vasoconstrictor response to noradrenaline in the presence of HET0016 is retained, indicating glutamate-evoked vasoconstriction but not that evoked by noradrenaline (100 nm), is linked to HETE. Glycine caused a concentration dependent increase in afferent arteriole diameter (n = 6; Fig. 5e) that was inhibited by strychnine (1 μM; $p < 0.05$; n = 6; Fig. 5f). Strychnine alone induced pericyte-mediated constriction thus a vasodilatory role for strychnine-sensitive glycine receptors may exist in the cortex. Data show separate signalling pathways are involved in the regulation of microvascular function in the renal cortex and medulla.

Pericytes respond to GABA- and glutamate related drugs

Drugs used to target the CNS GABA/glutamate system are typically excreted unchanged by the kidney and thus may modulate vasa recta diameter via their direct action at pericytes. Concentrations chosen were based upon the therapeutic dose window of these drugs. Superfusion of the anticonvulsant gabapentin (58 μM[16]), a structural analogue of GABA, induced pericyte-mediated vasoconstriction (15.1 ± 0.7%; Fig. 6ai). The $GABA_AR$ modulators diazepam (70 nM[18,28,52]; Fig. 6aii) and topiramate (50 μM[14,49]; Fig. 6aiii), both evoked pericyte-mediated vasoconstriction (18.5 ± 1.8%, n = 8; and -16.1 ± 2.9%, n = 7, respectively). Sustained superfusion with either gabapentin or diazepam caused an irreversible decrease in vasa recta diameter at pericytes, which was sustained after agonist washout (Fig. 6bi-ii). The gabapentin and diazepam-mediated vasoconstrictions were significantly attenuated by bicuculline and the pericyte-mediated increase in vessel diameter elicited by bicuculline continued beyond the initial baseline (gabapentine + bicuculline ;13.4 ± 4.5%, $p < 0.05$; n = 8; Fig. 6bi-ii). Since the topiramate-induced constriction at pericyte sites is rapidly reversed, topiramate was co-applied with bicuculline without prior bicuculline incubation. When both topiramate and bicuculline were present in the superfusate no significant change in vasa recta diameter was recorded at pericyte sites (1.7 ± 0.3%; Fig. 6biii). Removal of topiramate from the superfusate resulted in a significant bicuculline-mediated vasoconstriction at pericyte sites (11.1 ± 1.5%, $p < 0.01$, n = 7). Diazepam-evoked and topiramate-evoked changes in vasa recta diameter were therefore the result of their direct modulatory action on $GABA_ARs$ expressed in the renal medulla.

The intravenous anaesthetic propofol has previously been reported to attenuate NMDA-induced dilation of cerebral parenchymal arterioles[18]. In live kidney tissue, propofol caused a slowly-reversible constriction of vasa recta at pericyte sites (1 μM[31]; 12.0 ± 0.9; n = 8; Fig. 6ci) and NMDA-evoked dilation at pericyte sites completely reversed when propofol was

added to the superfusate (vessel returned to 0.9 ± 2.1% of baseline diameter, $p < 0.01$; Fig. 6cii). Memantine is an uncompetitive NMDAR antagonist used to treat dementia. Superfusion of live tissue with memantine (1 μM[46]), caused an irreversible vasoconstriction of vasa recta at pericyte sites (13.6 ± 2.5%, n = 8; Fig. 6di). Co-application of memantine with NMDA reversed the NMDA-evoked dilation at pericyte sites (-9.2 ± 3.0%; Fig. 6dii) indicating memantine has inhibitory action on renal NMDARs. When NMDA was superfused alone, a prolonged increase in the vasa recta diameter at pericyte sites was observed even after the cessation of NMDA superfusion (Fig. 6cii, dii). Attenuation of NMDA-evoked dilation in the medulla was due to the pharmacological actions of either propofol or memantine, since extended perfusion of tissue with NMDA alone, elicited prolonged pericyte-mediated vasodilation.

Discussion

The data presented here demonstrate a novel functional role for GABA, glutamate and glycine in the regulation of renal microvascular function. Exposure of live kidney tissue to GABA, glutamate and glycine at concentrations similar to those in the urine[40] and plasma[26,35,42] resulted in pericyte-mediated changes in vasa recta diameter in the medulla, and smooth muscle cell-mediated changes in afferent arteriole diameter in the cortex. Since blood flow through vessels is intrinsically linked to vessel diameter these agents likely influence renal blood flow.

Grgic et al previously performed microarray experiments profiling pericytes separately from the surrounding medullary tissue[19]. Use of the GEO Profiles database[2] revealed that under basal conditions medullary pericytes significantly expressed 6-fold more of the gene for the GABA-A-α4 receptor subunit than other medullary cells ($p < 0.0001$, GEO accession GSE50439[19]), and that pericytes and medullary cells express genes for the other receptor subunits for GABA, NMDA and GlyR, though these showed no significant differences.

Interestingly, murine, rodent, and human smooth muscle cells consistently express the GABA-A-α4 subunit which mediates vasoactivity in these cells[4,59,60]. GABA-C-ρ1 also appears to be involved in the regulation of vascular tone[62,3] but we did not investigate the $GABA_C$ receptor subtypes in this study.

GABA acts at medullary pericytes to constrict vasa recta capillaries, whereas glutamate and glycine dilate vasa recta capillaries via pericytes. In the cortex, GABA and glutamate decreased afferent arteriole diameter whilst glycine increased vessel diameter. Interestingly, glycine, a co-agonist of glutamate for NMDARs, has previously been shown to increase renal blood flow[33,58]. Clearly these neurotransmitters have functionally disparate regulatory roles in the cortex and medulla, most likely due to differential expression of GABA/glutamate/glycine receptors in different kidney regions[5,6,8,25,35,55,57].The magnitude of constriction/dilation elicited by GABA, glutamate and glycine is akin to that evoked by renal vasoconstrictors (angiotensin-II and endothelin-1) and vasodilators (nitric oxide and bradykinin)[8], as such their impact on blood flow is likely significant.

GABA-evoked constriction of vasa recta by pericytes occurred via $GABA_A$ and $GABA_B$Rs and was associated with an increase in $[Ca^{2+}]_i$ in pericytes and nearby endothelial cells (data not shown). Thus GABA exerts depolarising characteristics in renal tissue as well as in the CNS[20]. Conversely, glutamate-mediated activation of NMDARs, resulted in pericyte-evoked dilation of vasa recta, that was linked to endogenous arachidonic acid derivatives PGE_2 and EETs via cGMP-independent endothelial derived NO. Glutamate-mediated signaling mechanisms observed in kidney thus closely resemble those reported in CNS capillaries[20,47]. Pericyte-mediated changes in capillary diameter in the renal medulla and CNS are likely governed by similar cell signaling pathways. The divergent glutamate-mediated responses in afferent arteriole and vasa recta here complements previous work highlighting regional variation in the expression of renal glutamate receptors[58].

Glycine-induced responses were similarly disparate across vascular beds in mechanism, though the magnitude of change in vessel diameter complements previous work showing increased blood flow in the cortex and medulla in response to glycine[1]. Glycine exposure evoked concentration-dependent vasodilation of juxtamedullary afferent arterioles through glycine-receptor activation. This is consistent with the observed increase in GFR reported by Johannesen et al[27], and we argue that this relaxation may reflect afferent arteriole responses throughout the renal cortex. In contrast, glycine-evoked pericyte relaxation along the vasa recta of the outer medulla involved activation of NMDAR indicating a separate regulatory mechanism in the renal medulla.

Whilst we did not probe what this GABA/Glutamatergic heamodynamic regulation may be responsible for, we postulate it relates to osmoregulation. Modulation of perfusion by vasa recta and the concomitant changes in pO_2 alters sodium reabsorption[57]. GABA, glycine and glutamate all enhance sodium excretion[1,53,57]. Glycine and glutamate have also been shown to cycle between loops of Henle, collecting ducts, and the ascending and descending vasa recta[10,11], and this amino acid cycling is suggested to counterbalance the high interstitial osmolality[34]. With both the vasoactivity and the cycling of these amino acids, collectively these systems could work together to regulate sodium and offer osmoprotection to renal structures in the interim.

Neuromodulator drugs that act at $GABA_A$Rs or NMDARs in the CNS to alter neurotransmission in epilepsy are excreted unchanged in the kidney. Renal pericytes are an interface between the tubular and vascular compartments and mediate tubulovascular cross talk in the kidney[7,8,9,41]. As such the presence of agents acting at either $GABA_A$Rs or NMDARs in blood, or tubular filtrate, could result in pericyte-mediated changes in vasa recta diameter and medullary blood flow (MBF). Propofol, gabapentin, topiramate and memantine all constricted vasa recta capillaries via pericytes. Patients with chronic kidney disease are

highly susceptible to gabapentin toxicity[61] and both gabapentin and propofol have been shown to negatively impact on renal function in certain patients[31,36,37]. For the first time, we provide vascular mechanisms by which this toxicity may occur. Drug-induced nephrotoxicity is a well-established phenomenon for many medicines; chronic NSAIDs exposure is known to result in decreased renal blood flow[29]. The pericyte-mediated constriction of vasa recta capillaries demonstrated for antiepileptic drugs tested here, is potentially as challenging to the kidney as NSAIDs. Careful consideration should therefore be given when prescribing anti-epileptics to patients that have compromised renal function or to patients taking other medications known to alter renal blood flow.

Methods

Preparation of kidney slices

Animal experiments were conducted in accordance with national and institutional ethical and welfare standards and in compliance with the United Kingdom Home Office Scientific Procedures Act (1986). Adult male Sprague-Dawley rats (250–300 g) were euthanized by cervical dislocation, after which both kidneys were removed. Kidney slices were prepared as previously described[7,8]. Previous experiments show that the majority of tubular and vascular cells within the kidney slices are 'live' for up to four hours[7], with a ratio of live to dead cells similar to that of reported previously for healthy rat kidneys, and thus confirmed that these tissue slices were viable for physiological experiments during this time frame.

Functional experiments

Live kidney slices were superfused with pharmacological agents as previously described[7,8]. Differential interference contrast images of pericytes on subsurface vasa recta capillaries were captured through a ×63 water-immersion objective. Pericytes were identified by their distinctive "bump on a log" morphology, as previously described[7,8,15] Real-time video images

of vasa recta were collected every 1 s by an attached Rolera XR camera and recorded using Image ProSoftware (Media Cybernetics, Maidenhead, UK). Images were analyzed using ImageJ software (http://rsb.info.nih.gov). For Ca^{2+}/NO imaging, slices were incubated with Fluo-4-AM/DAF-FM (60 min, 22 °C); fluorophores were excited at 488 nm and collected at 560 nm.

In vitro blood-perfused juxtamedullary nephron preparation

Kidneys were prepared for blood-perfused juxtamedullary nephron experiments as previously described[25]. Blood perfused kidneys were visualized under a light microscope (Nikon Optiphot2-UD; Nikon) and superfused with Tyrode's buffer containing 1% BSA at 37 °C. Perfusion pressure was monitored using a pressure cannula connected to a pressure transducer. After an equilibration period (10–15 min) with perfusion pressure held at 100 mmHg, experiments were initiated. All drugs were applied in the superfusate so the route of administration for the arteriole data is consistent with the vasa recta and pericyte experiments. Images of the afferent arteriole were recorded on DVD for later analysis. Diameters were measured every 12 s, mean arteriole diameter calculated and data expressed as means ± SEM.

Statistics

Values are mean ± s.e.m, *n* values represent numbers of pericytes (and accompanying non-pericyte site per kidney slice). Variations in data occurs between slices and not animals (Pearson's correlation)[7, 30]. All experiments were performed in at least three animals and post-hoc power calculation and Cohen's d tests performed. Statistically significant differences between pericyte and non-pericyte sites were determined using a Student's *t*-test; $P < 0.05$ was considered significant. When comparing more than two data sets statistical significance was calculated using one-way ANOVA and post hoc tests Tukey (when

comparing all groups) or Dunnett (when comparing against control group only); $P < 0.05$ was considered significant.

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514 **Figure Legends**

515 **Figure 1 | GABA evokes pericyte-mediated constriction of vasa recta capillaries.** Data was
516 taken from time series experiments in which naïve kidney slices were exposed to; GABA (3
517 µM; **a-f**), other vasoactive compounds (**g-l**), and in combination (**m-n**) for approximately
518 300s. a, representative trace of the repeatable GABA evoked constriction of vasa recta. b,
519 Vasa recta exposed to PSS (**bi**), GABA (**ii**), PSS (**iii**) and GABA (**iv**). Yellow circle = pericyte, red
520 lines = pericyte site and blue lines = non-pericyte sites. **c**, concentration dependent effect of
521 GABA. **d**, mean pericyte–mediated constriction of vasa recta evoked by vasoconstrictor
522 compounds. **e**, percentage change in vasa recta diameter (blue trace) and percentage
523 change of Flu-4 fluorescence (red trace). Images show Fluo-4-AM signal before (**fi**), during
524 (**ii**) and after (**iii**) superfusion of tissue with GABA, white lines denote a vessel, red circles =
525 pericyte, at which vessel diameter was measured (red brackets). **g**, **h**, Muscimol (1 µM) and
526 baclofen (200 nM) respectively evoked pericyte-mediated constriction, with the mean
527 vasoconstrictions shown in scatterplot (**i**). **j**, **k**, Bicuculline (10 µM) and CGP (1 µM), induced
528 pericyte-mediated dilation, with the mean dilations shown in scatterplot (**l**). **m**, Co-
529 application of muscimol and baclofen increases constriction of vasa recta at pericyte sites. **n**,
530 Bicuculline, CGP and both antagonists combined, all reduce the GABA-evoked constriction of
531 vasa recta at pericyte sites. Data shown from male Sprague-Dawley rats as mean ± s.e.m, n ≥
532 3 pericytes. Statistics were calculated in GraphPad PRISM (5.0). Statistical significance
533 between pericyte and non-pericyte sites were determined using a Student's t-test. A one-
534 way ANOVA and post hoc tests Tukey (when comparing all groups) or Dunnett (when
535 comparing against control group only) were used for multiple comparisons. ***$P < 0.001$;
536 **$P < 0.01$; *$P < 0.05$.

537
538 **Figure 2 | Glutamate evokes pericyte-mediated dilation of vasa recta capillaries.** Data was
539 taken from time series experiments in which naïve kidney slices were exposed to glutamate

(glut; 10 μM) and other vasoactive compounds for approximately 300s. **a**, Representative trace of glutamate evoked vasodilation. Vasa recta exposed to PSS (**bi**), glut (**ii**), PSS (**iii**), and glut (**iv**). Yellow circle = pericyte, red lines = pericyte site and blue lines = non-pericyte sites, black scale bar = 10 μm. **c**, mean pericyte–mediated dilation of vasa recta evoked by vasodilator compounds glut (blue), SNAP (red), prostaglandin E_2 (PG; black), adenosine (AD; green), and bradykinin (BK; orange). **d**, Concentration-dependent effect of glutamate on vasa recta diameter. **e, f,** Both NMDA (100 μM) and domoic acid (10 μM) evoked dilation of vasa recta at pericyte sites, with the mean vasodilation shown in scatterplot (**g**)**. h, I,** MK-801 (300 μM) and UBP-302 (25 μM) evoked pericyte-mediated vasoconstriction, with the mean vasoconstriction shown in scatterplot (**j**)**. k**, Only MK-801 inhibits glutamate-evoked dilation of vasa recta by pericytes. Data shown from male Sprague-Dawley rats as mean ± s.e.m, n ≥ 3 pericytes. Statistics were calculated in GraphPad PRISM (5.0). Statistical significance between pericyte and non-pericyte sites were determined using: a Student's t-test for pericyte versus non-pericyte sites, ***P < 0.001; **P < 0.01, and A one-way ANOVA and post hoc Dunnett test for comparison of agonists against glut, *P < 0.05, $^{\#\#}$P < 0.01, $^{\#\#\#}$P < 0.001.

Figure 3 | Glycine evokes pericyte-mediated dilation of vasa recta capillaries. Data was taken from time series experiments in which naïve kidney slices were exposed to glycine (gly; 1 mM) **a**, Representative trace of glycine evoked vasodilation. Vasa recta exposed to PSS (**bi**), glycine (**ii**), PSS (**iii**) glycine (**iv**). Yellow circle = pericyte, red lines = pericyte site and blue lines = non-pericyte sites. **c**, mean repeatable pericyte–mediated dilation of vasa recta evoked by glycine. **d**, Concentration-dependent effect of glycine on vasa recta diameter. **e**, Representative trace showing that exposure of tissue to glycine in the presence of MK-801 (300 nM) resulted in pericyte-mediated constriction of vasa recta that was reversed when MK-801 was removed. **f**, mean data showing MK-801 inhibits glycine-evoked dilation of vasa recta resulting in constriction, when MK-801 is removed from the superfusate, glycine

566 evoked dilation of vasa recta at pericytes. **g**, **h**, Strychnine (1 µM failed to attenuate the
567 dilatory response of vasa recta to glycine. Data shown from male Sprague-Dawley rats as
568 mean ± s.e.m. Statistics were calculated in GraphPad PRISM (5.0). Statistical significance
569 between pericyte and non-pericyte sites were determined using: a Student's t-test for
570 pericyte versus non-pericyte sites, ***P < 0.001; **P < 0.01, and A one-way ANOVA and post
571 hoc Dunnett test for comparison of agonists against against gly 1 mM, *P < 0.05, $^{\#\#}$P < 0.01,
572 $^{\#\#\#}$P < 0.001 .
573
574 **Figure 4 | Glutamate signalling pathways.** Data was taken from time series experiments in
575 which naïve kidney slices were exposed to glutamate (glut) or glycine (gly) alone (**a-d**) or in
576 the presence of other comounds (**e-l**). **a**, **c**, Representative traces of percentage change in
577 vessel diameter (blue trace) and percentage change in DAF-FM fluorescence (red trace) in
578 response to exposure of vasa recta to glutamate (10 µM) and glycine (1 mM). DAF-AM signal
579 before (**bi** and **di**), during (**bii** and **dii**) and after (**biii** and **diii**) superfusion with glutamate or
580 glycine. White lines denote the vessel wall, yellow circle = pericyte and red brackets show
581 where vessel diameter was measured, white scale bar = 10 µm. e, Glutamate-evoked
582 dilation was significantly attenuated by L-NNA (100 µM). f, representative trace showing
583 percentage change in vessel diameter in response to exposure to glutamate and LNNA. ODQ
584 (10 µM) failed to significantly attenuate the glutamate-evoked dilation, (g, shows mean
585 data, h, shows the representative trace). Both PPOH (9 µM; **i**, mean data, **j**, representative
586 trace) and L-161,982 (1 µM; **k**, mean data, **l**, representative trace) significantly attenuated
587 the glutamate mediated dilation. Data shown from male Sprague Dawley rats as mean ±
588 s.e.m, n ≥ 3 pericytes. Statistics were calculated in GraphPad PRISM (5.0). Statistical
589 significance between pericyte and non-pericyte sites were determined using: a Student's t-
590 test for comparison between drugs. ***P < 0.001; **P < 0.01, * P < 0.05.
591

Figure 5 | Afferent arteriolar responses. Data was taken from afferent arterioles (AA) from juxtamedullary nephrons, in which AA were perfused with increasing levels of agonist (**a,c,e**) and then exposed to agonist in the presence of relevant antagonist (**b,d,f**) **a,** GABA causes a concentration-dependent constriction of afferent arterioles. **b**, Bicuculline (10 μM) attenuates the GABA-evoked constriction. **c**, Glutamate (glut) causes a concentration-dependent constriction of afferent arterioles. **d**, HET0016 (1 μM) inhibits the glutamate-evoked constriction, but not the noradrenaline (NA; 100 nM)-evoked constriction (control). **e**, **f**, Glycine (gly) causes a concentration-dependent dilation of afferent arteriole diameter, which in inhibited by strychnine. "Con" represents the control period, with "Rec" representing the recovery period. Data shown from male Sprague-Dawley rats as mean ± s.e.m, n = 6. Statistical significance was calculated using a one-way ANOVA with post hoc Dunnett's test against the control variable. * $P < 0.05$.

Figure 6 | Pericyte-mediated regulation of MBF in response to modulators of GABAARs and NMDARs. Data was taken from time series experiments in which naïve kidney slices were exposed a variety of different compounds. Scatter plots show mean data for gabapentin (58 nM; **ai**), diazepam (40 μM; **aii**), topiramate (10 μM; **aiii**), propofol (1 μ; **ci**) and memantine (1 μM; **di**) induced pericyte-mediated constriction of vasa recta capillaries. **bi-iii** all show representative traces of gabapentin, diazepam and topiramate-evoked vasoconstriction of vasa recta (respectively; black lines), which is attenuated by bicuculline (10 μM) for all agents (red lines). **cii**, Representative trace showing the NMDA-evoked (100 μM) dilation of vasa recta by pericytes (black line) is attenuated by propofol (1 μM; red line). **dii**, Representative trace showing NMDA-evoked dilation of vasa recta by pericytes (black line) is attenuated by memantine (1 μM; red line). Data shown from male Sprague-Dawley rats as mean ± s.e.m, n ≥ 3 pericytes. Statistics were calculated in GraphPad PRISM (5.0). Statistical significance between pericyte and non-pericyte sites were determined using: a Student's t-test for pericyte versus non-pericyte sites, $^{**}P < 0.01$; $^{*}P < 0.05$.

Extended Data Figure 1 | Schematic showing potential mechanism involved in GABA-, glycine- and glutamate-mediated changes in vessel diameter. Diagrams show a pericyte, with claw like processes, situated on a blood vessel in close proximity to Loop of Henle (LOH). GABA, glutamate (Glut) and glycine (Glyc) are supplied from the blood, tubular cells, endothelial cells and urine. **a**, Activation of GABAAR on pericytes causes an increase in $[Ca2+]_i$ through L-type VOCC and release from $[Ca2+]_i$ stores. Likewise, activation of GABAARs likely stimulates the production of IP3, which binds to IP3R and induces calcium release from the endoplasmic reticulum stores. Elevation of $[Ca2+]_i$ leads to calcium and calmodulin (CaM)- dependent activation of myosin light chain kinase (MLCK) in pericytes. This leads to contraction by phosphorylation of MLC and promotes interaction of α-smooth muscle actin (SMA). **b**, Glutamate and Glycine simultaneously bind to and activate inotropic glutamate receptors, NMDA receptors (NMDAR), on endothelial and/or tubule epithelial cells. Activation of NMDARs causes an increase in $[Ca2+]_i$ leading to the synthesis of nitric oxide (NO). NO diffuses to pericytes, supressing the synthesis of vasoconstrictor 20-HETE and triggering PGE2, which stimulates pericyte mediated vasodilation of capillaries via EP4 receptors (EP4R).

Extended Data Figure 2 | Glutamate evokes pericyte-mediated dilation of vasa recta capillaries. Data was taken from time series experiments in which naïve kidney slices were exposed to glutamate (glut; 10 µM) for approximately 500s. (**a**) shows a typical field of view for **v**asa recta before (**ai**), during (**ii**) and after (**iii**) exposure to 10 µM of glutamate. Pericytes are denoted by yellow dotted circle, red dotted lines show the pericyte site and the blue dotted lines show the non-pericyte site. **b**, Vasa recta capillary response to 10 µM glutamate. Black line shows pericyte site and grey line shows non-pericyte site. **c**, Mean constriction measured at pericyte site and non-pericyte site in response to different

vasoconstrictors. Data shown from male Sprague-Dawley rats as mean ± s.e.m, n ≥ 3 pericytes. Statistics were calculated in GraphPad PRISM (5.0). Statistical significance between pericyte and non-pericyte sites were determined using: a Student's t-test for pericyte versus non-pericyte sites, *P < 0.05.

Extended Data Figure 3 | EET and EP_4 receptor antagonists evoke pericyte-mediated constriction of vasa recta capillaries. Data was taken from time series experiments in which naïve kidney slices were exposed to receptor antagonists for approximately 500s. **a & c**, Mean constriction measured at pericyte site and non-pericyte site in response to L-161,982 and PPOH, respectively. **b & d**, representative trace showing vasa recta capillary response to 1 µM L-161,982 and 9 µM PPOH, respectively. Black line shows pericyte site and grey line shows non-pericyte site. Data shown from male Sprague-Dawley rats as mean ± s.e.m, n ≥ 3 pericytes. Statistics were calculated in GraphPad PRISM (5.0). Statistical significance between pericyte and non-pericyte sites were determined using: a Student's t-test for pericyte versus non-pericyte sites. **P < 0.01, ***P < 0.001.

Supplemental data

https://doi.org/10.6084/m9.figshare.17081993

https://doi.org/10.6084/m9.figshare.17082005

https://doi.org/10.6084/m9.figshare.17082026

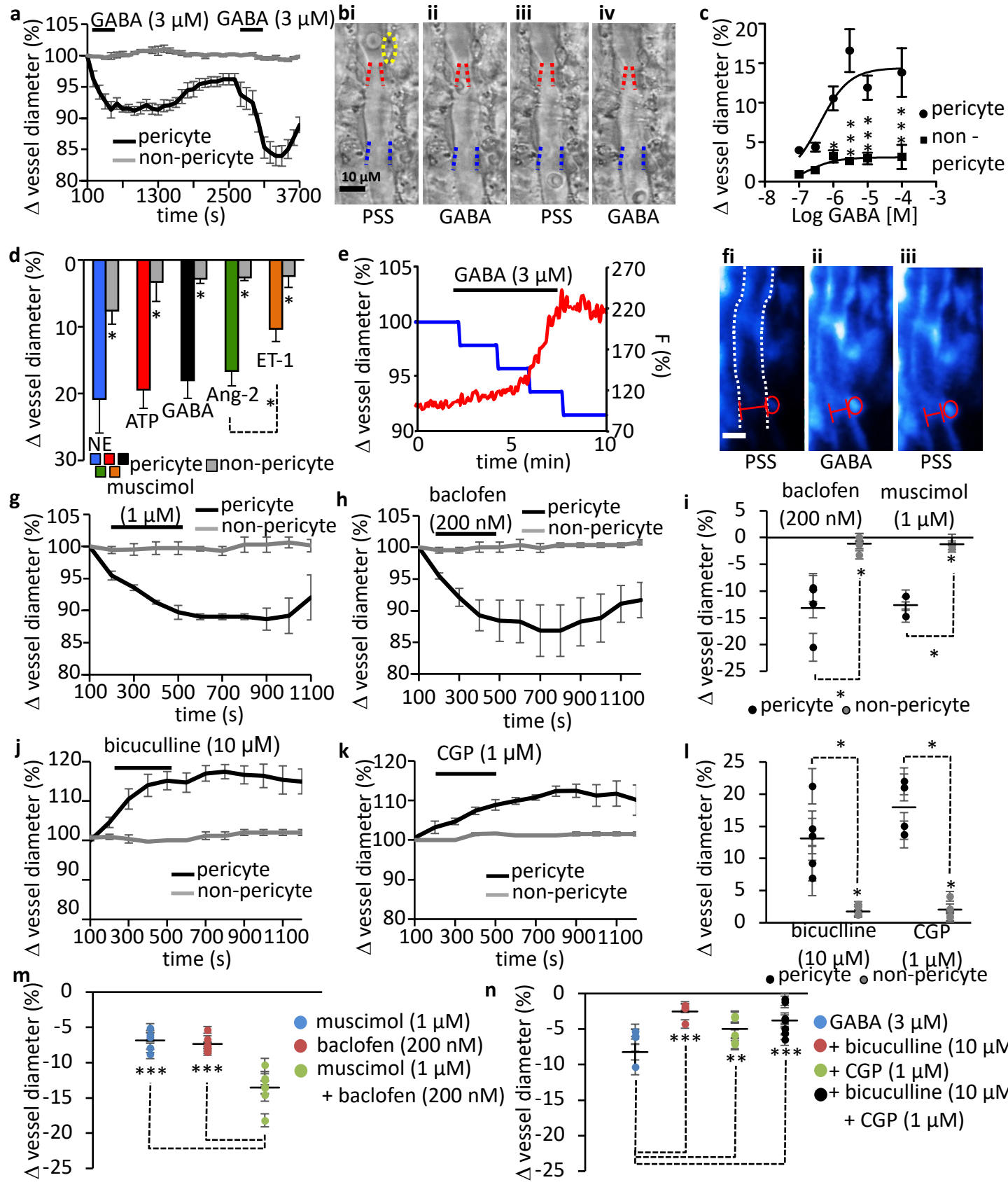


Figure 1 | GABA evokes pericyte-mediated constriction of vasa recta capillaries. Data was taken from time series experiments in which naïve kidney slices were exposed to; GABA (3 μM; **a-f**), other vasoactive compounds (**g-l**), and in combination (**m-n**) for approximately 300s. **a,** representative trace of the repeatable GABA evoked constriction of vasa recta. **b**, Vasa recta exposed to PSS (**bi**), GABA (**ii**), PSS (**iii**) and GABA (**iv**). Yellow circle = pericyte, red lines = pericyte site and blue lines = non-pericyte sites. **c**, concentration dependent effect of GABA. **d**, mean pericyte–mediated constriction of vasa recta evoked by vasoconstrictor compounds norepinephrine (NE; blue), Adenosine-5'-triphosphate (ATP; red), GABA (black), angiotensin-II (Ang-2; green), and endothelin-1 (ET01; orange). **e**, percentage change in vasa recta diameter (blue trace) and percentage change of Flu-4 fluorescence (red trace). Images show Fluo-4-AM signal before (**fi**), during (**ii**) and after (**iii**) superfusion of tissue with GABA, white lines denote a vessel, red circles = pericyte, at which vessel diameter was measured (red brackets). **g, h,** Muscimol (1 μM) and baclofen (200 nM) respectively evoked pericyte-mediated constriction, with the mean vasoconstrictions shown in scatterplot (**i**). **j, k,** Bicuculline (10 μM) and CGP (1 μM), induced pericyte-mediated dilation, with the mean dilations shown in scatterploy (**l**) . **m**, Co-application of muscimol and baclofen increases constriction of vasa recta at pericyte sites. **n**, Bicuculline, CGP and both antagonists combined, all reduce the GABA-evoked constriction of vasa recta at pericyte sites. Data shown from male Sprague-Dawley rats as mean ± s.e.m, n ≥ 3 pericytes. Statistics were calculated in GraphPad PRISM (5.0). Statistical significance between pericyte and non-pericyte sites were determined using a Student's t-test. A one-way ANOVA and post hoc tests Tukey (when comparing all groups) or Dunnett (when comparing against control group only) were used for multiple comparisons. ***P < 0.001; **P < 0.01; *P < 0.05.

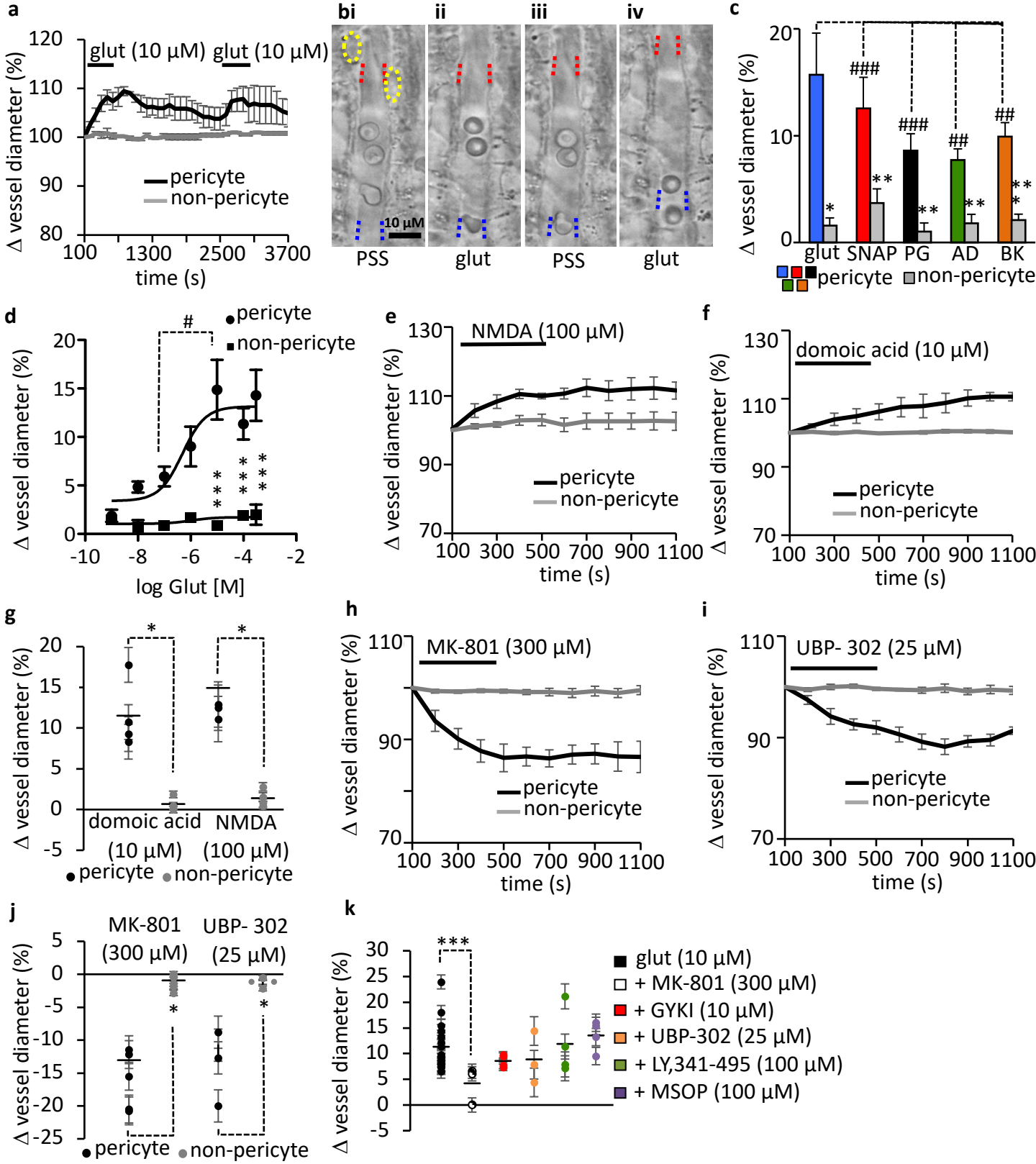


Figure 2 | Glutamate evokes pericyte-mediated dilation of vasa recta capillaries. Data was taken from time series experiments in which naïve kidney slices were exposed to glutamate (glut; 10 μM) and other vasoactive compounds for approximately 400s. **a**, Representative trace of glutamate evoked vasodilation. Vasa recta exposed to PSS (**bi**), glut (**ii**), PSS (**iii**), and glut (**iv**). Yellow circle = pericyte, red lines = pericyte site and blue lines = non-pericyte sites, black scale bar = 10 μm. **c**, mean pericyte–mediated dilation of vasa recta evoked by vasodilator compounds glut (blue), SNAP (red), prostaglandin E_2 (PG; black), adenosine (AD; green), and bradykinin (BK; orange). **d,** Concentration-dependent effect of glutamate on vasa recta diameter. **e, f,** Both NMDA (100 μM) and domoic acid (10 μM) evoked dilation of vasa recta at pericyte sites, with the mean vasodilation shown in scatterplot (**g**)**. h, I,** MK-801 (300 μM) and UBP-302 (25 μM) evoked pericyte-mediated vasoconstriction, with the mean vasoconstriction shown in scatterplot (**j**)**. k,** Only MK-801 inhibits glutamate-evoked dilation of vasa recta by pericytes. Data shown from male Sprague-Dawley rats as mean ± s.e.m, n ≥ 3 pericytes. Statistics were calculated in GraphPad PRISM (5.0). Statistical significance between pericyte and non-pericyte sites were determined using: a Student's t-test for pericyte versus non-pericyte sites, ***P < 0.001; **P < 0.01, and A one-way ANOVA and post hoc Dunnett test for comparison of agonists against glut, *P < 0.05, $^{\#\#}$P < 0.01, $^{\#\#\#}$P < 0.001

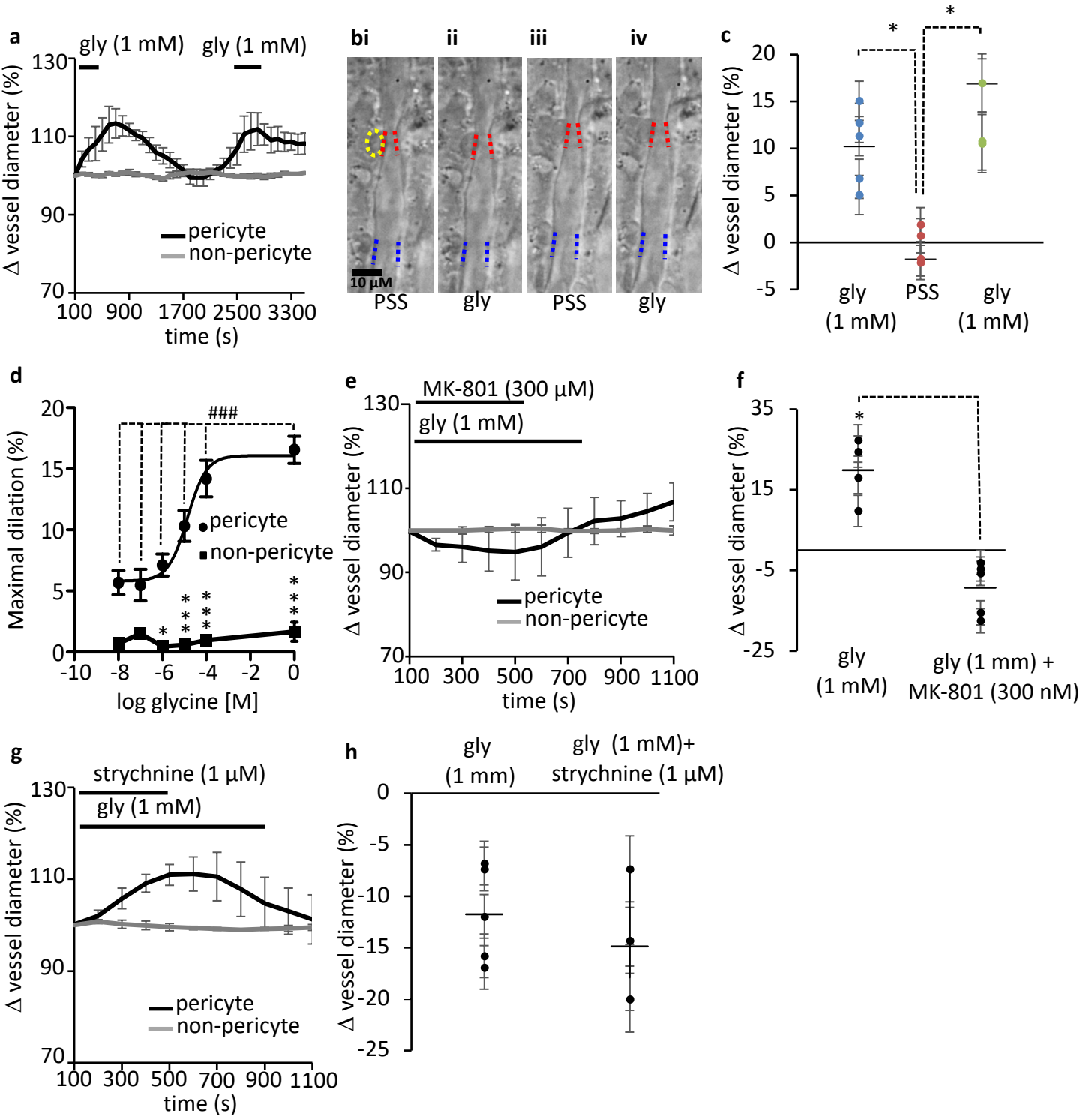


Figure 3 | Glycine evokes pericyte-mediated dilation of vasa recta capillaries. Data was taken from time series experiments in which naïve kidney slices were exposed to glycine (gly; 1 mM) **a**, Representative trace of glycine evoked vasodilation. Vasa recta exposed to PSS (**bi**), glycine (**ii**), PSS (**iii**) glycine (**iv**). Yellow circle = pericyte, red lines = pericyte site and blue lines = non-pericyte sites. **c**, mean repeatable pericyte–mediated dilation of vasa recta evoked by glycine. **d,** Concentration-dependent effect of glycine on vasa recta diameter. **e,** Representative trace showing that exposure of tissue to glycine in the presence of MK-801 (300 nM) resulted in pericyte-mediated constriction of vasa recta that was reversed when MK-801 was removed. **f,** mean data showing MK-801 inhibits glycine-evoked dilation of vasa recta resulting in constriction, when MK-801 is removed from the superfusate, glycine evoked dilation of vasa recta at pericytes. **g, h,** Strychnine (1 µM failed to attenuate the dilatory response of vasa recta to glycine. Data shown from male Sprague-Dawley rats as mean ± s.e.m. Statistics were calculated in GraphPad PRISM (5.0). Statistical significance between pericyte and non-pericyte sites were determined using: a Student's t-test for pericyte versus non-pericyte sites, ***P < 0.001; **P < 0.01, and A one-way ANOVA and post hoc Dunnett test for comparison of agonists against against gly 1 mM, *P < 0.05, ##P < 0.01, ###P < 0.001

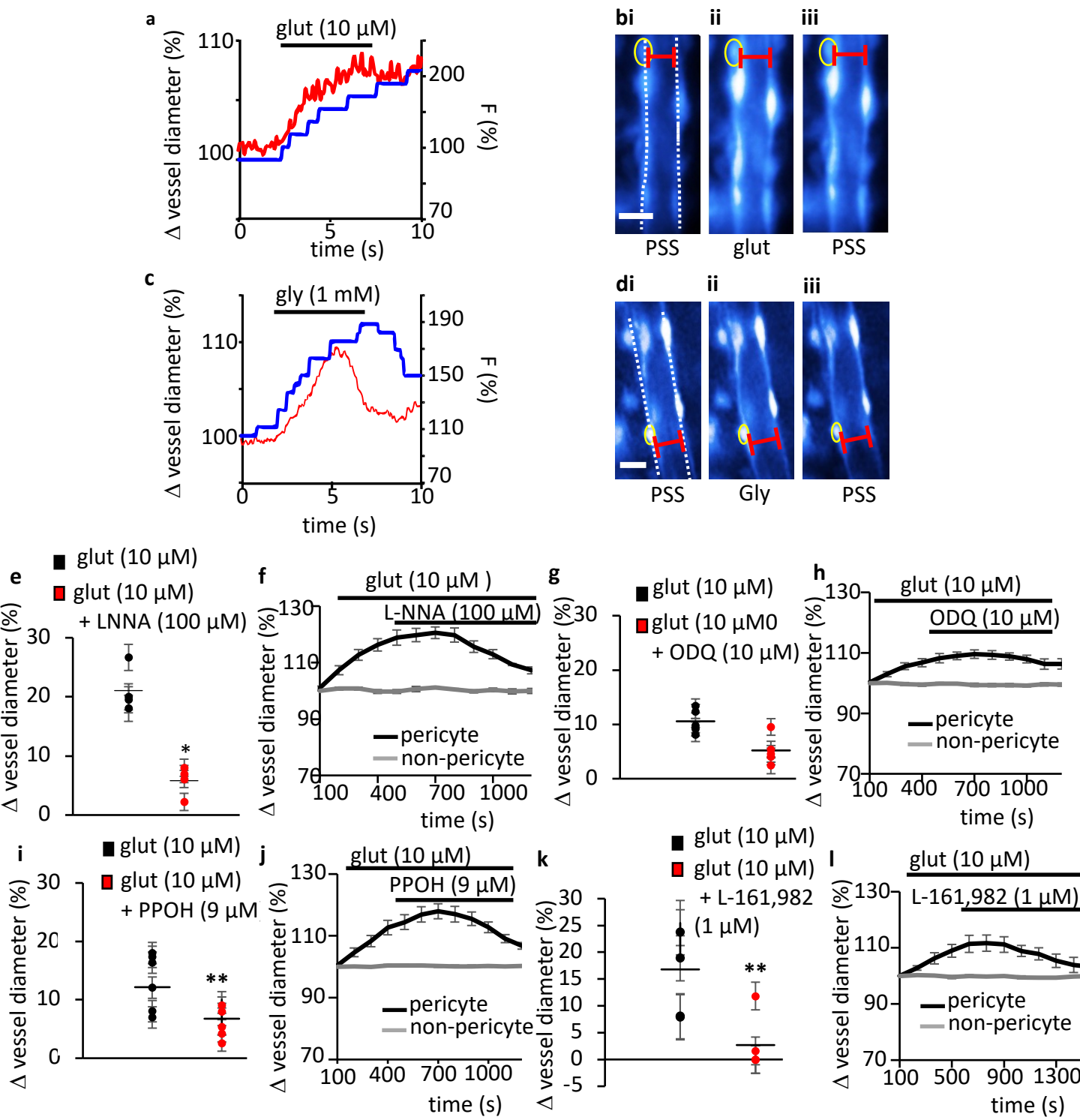


Figure 4 | Glutamate signalling pathways. Data was taken from time series experiments in which naïve kidney slices were exposed to glutamate (glut) or glycine (gly) alone (**a-d**) or in the presence of other comounds (**e-l**). **a, c,** Representative traces of percentage change in vessel diameter (blue trace) and percentage change in DAF-FM fluorescence (red trace) in response to exposure of vasa recta to glutamate (10 µM) and glycine (1 mM). DAF-AM signal before (**bi** and **di**), during (**bii** and **dii**) and after (**biii** and **diii**) superfusion with glutamate or glycine. White lines denote the vessel wall, yellow circle = pericyte and red brackets show where vessel diameter was measured, white scale bar = 10 µm . **e**, Glutamate-evoked dilation was significantly attenuated by L-NNA (100 µM). **f**, representative trace showing percentage change in vessel diameter in response to exposure to glutamate and LNNA. ODQ (10 µM) failed to significantly attenuate the glutamate-evoked dilation, (**g**, shows mean data, **h**, shows the representative trace). Both PPOH (9 µM; **I**, mean data, **j**, representative trace) and L-161,982 (1 µM; **k**, mean data, **l**, representative trace) significantly attenuated the glutamate mediated dilation. Data shown from male Sprague Dawley rats as mean ± s.e.m, n ≥ 3 pericytes. Statistics were calculated in GraphPad PRISM (5.0). Statistical significance between pericyte and non-pericyte sites were determined using: a Student's t-test for comparison between drugs. ***P < 0.001; **P < 0.01, * P < 0.05

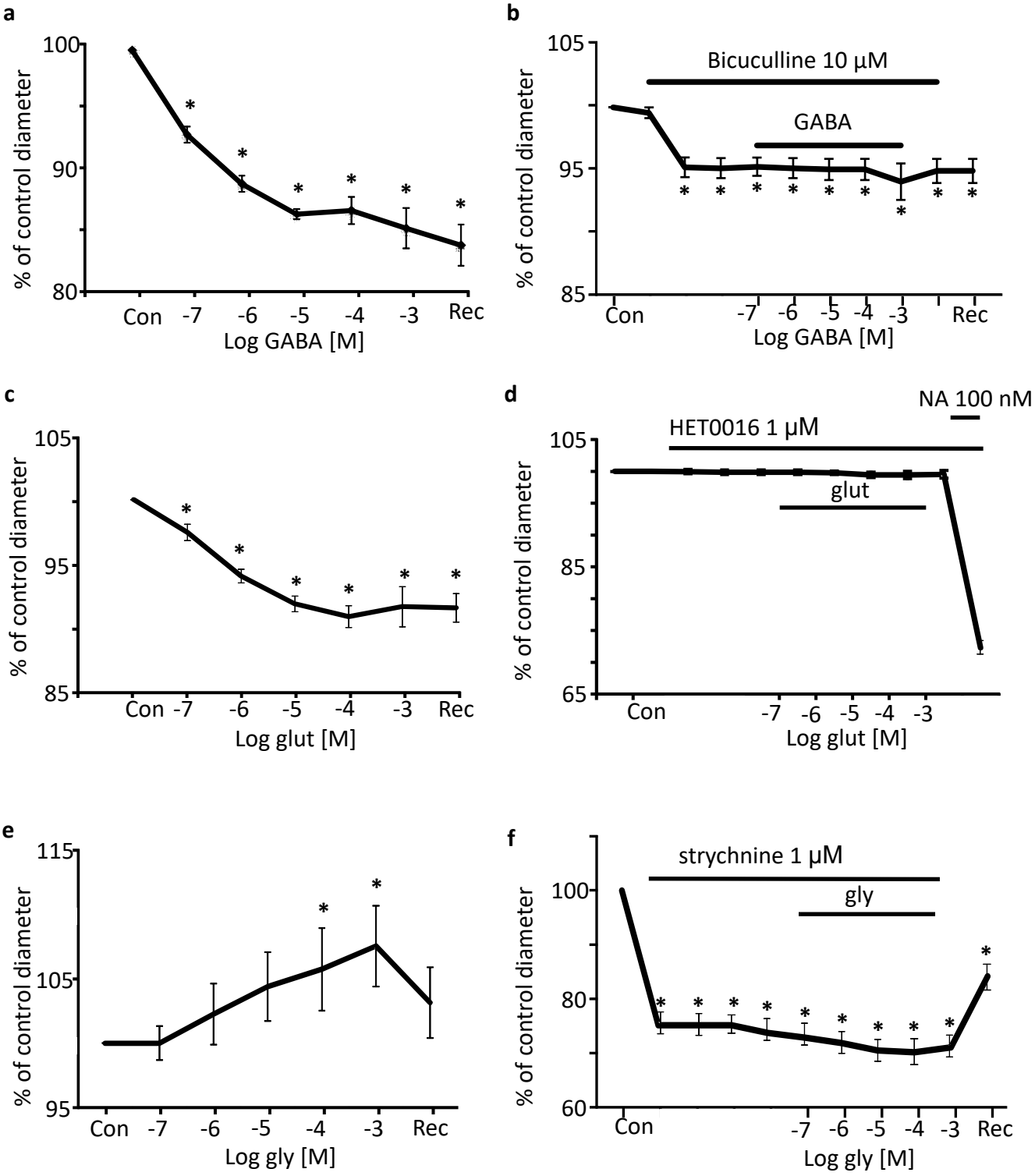


Figure 5 | Afferent arteriolar responses. Data was taken from afferent arterioles (AA) from juxtamedullary nephrons, in which AA were perfused with increasing levels of agonist (**a,c,e**) and then exposed to agonist in the presence of relevant antagonist (**b,d,f**) **a,** GABA causes a concentration-dependent constriction of afferent arterioles. **b**, Bicuculline (10 µM) attenuates the GABA-evoked constriction. **c**, Glutamate (glut) causes a concentration-dependent constriction of afferent arterioles. **d**, HET0016 (1 µM) inhibits the glutamate-evoked constriction, but not the noradrenaline (NA; 100 nM)-evoked constriction (control). **e**, **f**, Glycine (gly) causes a concentration-dependent dilation of afferent arteriole diameter, which in inhibited by strychnine. "Con" represents the control period, with "Rec" representing the recovery period. Data shown from male Sprague-Dawley rats as mean ± s.e.m, n = 6. Statistical significance was calculated using a one-way ANOVA with post hoc Dunnett's test against the control variable. * $P < 0.05$.

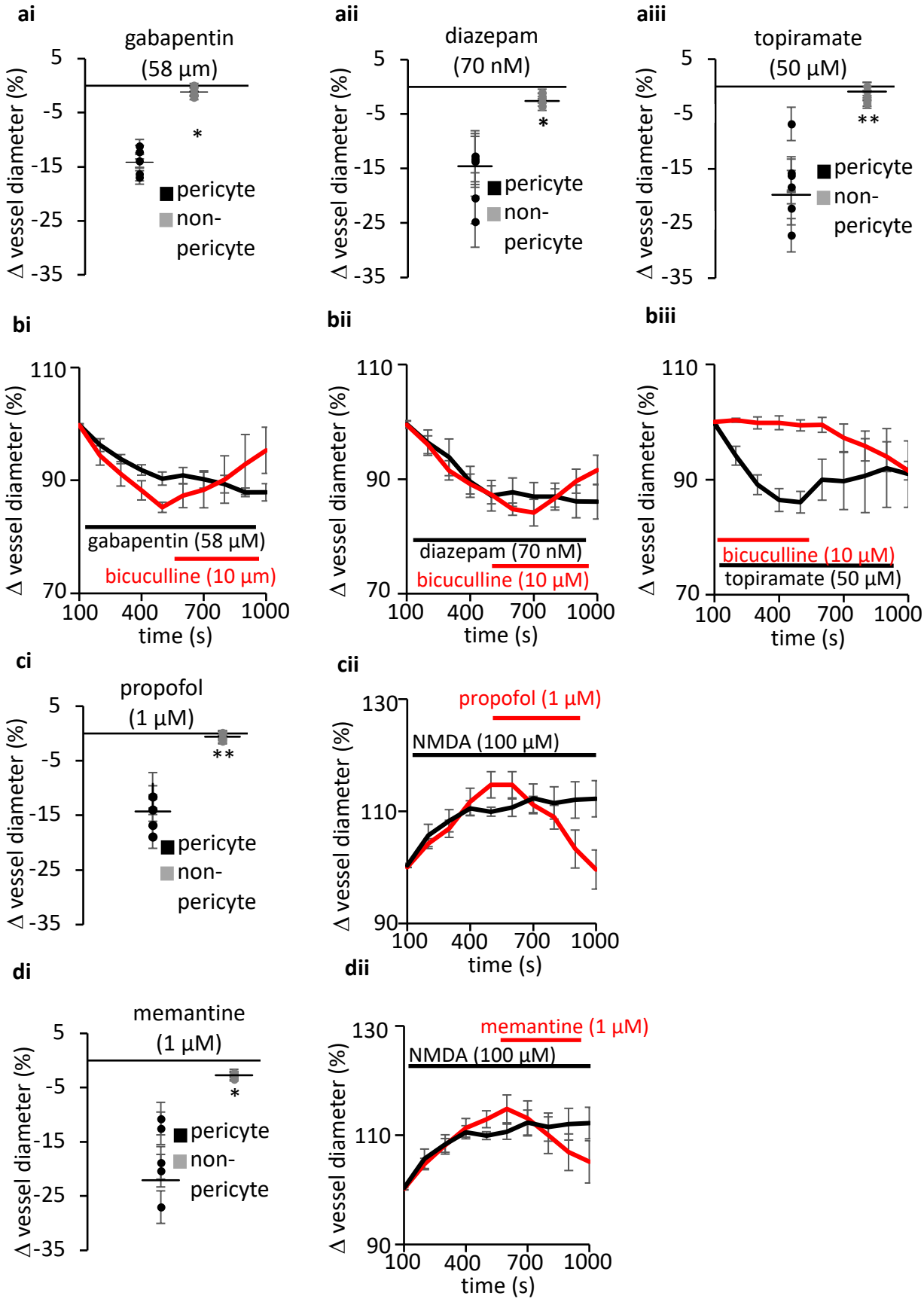
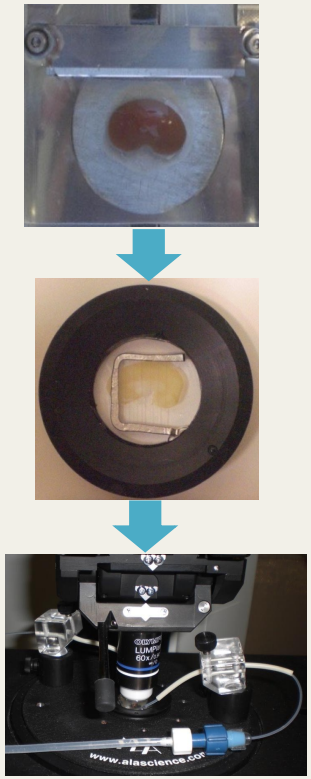


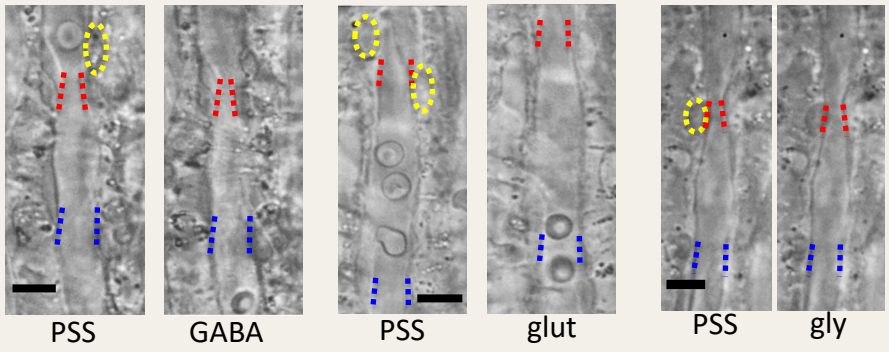
Figure 6 | Pericyte-mediated regulation of MBF in response to modulators of $GABA_A$Rs and NMDARs. Data was taken from time series experiments in which naïve kidney slices were exposed a variety of different compounds. Scatter plots show mean data for gabapentin (58 nM; **ai**), diazepam (70 μM; **aii**), topiramate (10 μM; **aiii**), propofol (1 μ; **ci**) and memantine (1 μM; **di**) induced pericyte-mediated constriction of vasa recta capillaries. **bi-iii** all show representative traces of gabapentin, diazepam and topiramate-evoked vasoconstriction of vasa recta (respectively; black lines), which is attenuated by bicuculline (10 μM) for all agents (red lines). **cii,** Representative trace showing the NMDA-evoked (100 μM) dilation of vasa recta by pericytes (black line) is attenuated by propofol (1 μM; red line). **dii,** Representative trace showing NMDA-evoked dilation of vasa recta by pericytes (black line) is attenuated by memantine (1 μM; red line). Data shown from male Sprague-Dawley rats as mean ± s.e.m, n ≥ 3 pericytes. Statistics were calculated in GraphPad PRISM (5.0). Statistical significance between pericyte and non-pericyte sites were determined using: a Student's t-test for pericyte versus non-pericyte sites, $^{**}P < 0.01$; $^{*}P < 0.05$

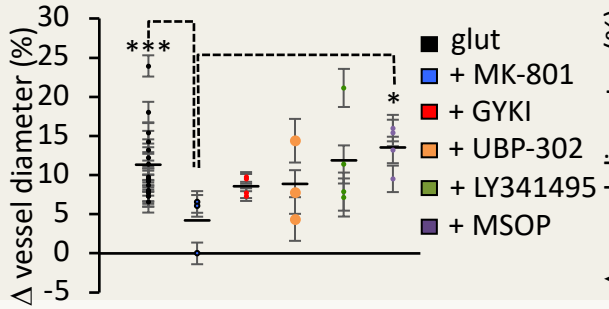
A novel functional role for the CNS neurotransmitters, GABA, glycine and glutamate, in the kidney: potent and opposing regulators of the renal vasculature

Live Kidney Slice Model

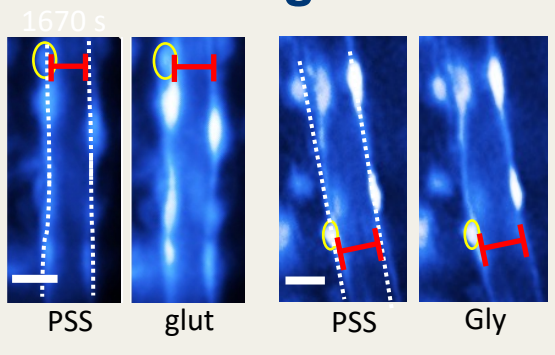


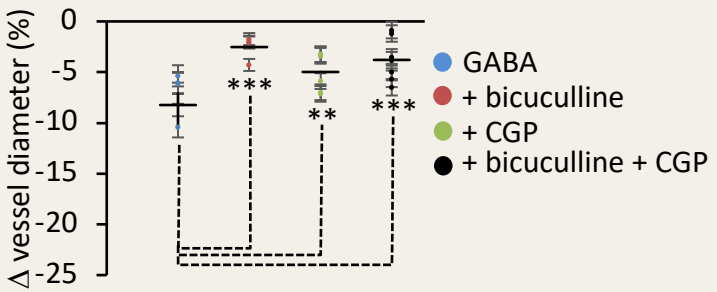
Vessel Diameter





DAF-AM signal





CONCLUSION Exposing renal tissue to physiological concentrations of GABA, glutamate and glycine alters the way contractile cells, pericytes and smooth muscle cells, regulate vessel diameter in the kidney.