Activity-dependent NFATc3 nuclear accumulation in pericytes from cortical parenchymal microvessels

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The brain microcirculation comprises multiple cell types, which include smooth muscle cells, endothelial cells, and pericytes. Pericytes are mesenchyme-derived pluripotent cells (7, 38) known to possess different physiological functions, depending on their tissue location (52). In the brain, the ratio of pericytes to endothelial cells is about 1:3 (42). These cells have been shown to significantly contribute to the integrity of the blood-brain barrier, participate in inflammation, regulate endothelial cell activity (37), and alter blood flow distribution by virtue of their ability to contract around capillaries and microvessels (22, 44, 51). In addition, pericytes play an important physiological role in blood vessel maturation, cell differentiation, and the formation of endothelial cell tight junctions (1). During angiogenesis, pericytes are recruited by nascent endothelial cell tubes, where they provide structural support and release growth factors that contribute to the differentiation and maturation of blood vessels (2, 32, 45), which highlights the functional importance of cell-to-cell communication in the formation and organization of the cerebral microcirculation. As opposed to vascular smooth muscle cells (VSMCs), not much is known about the factors that signal gene transcription activation in pericytes. Given the key role that pericytes have in vessel differentiation and maturation, we investigated whether synaptic-induced glutamate releases target pericytes at a time when the postnatal organization of the cerebral microvasculature is highly active. Moreover, to determine whether glutamate signaling was associated with a molecular step of transcriptional activation in pericytes, we evaluated whether glutamate could induce nuclear translocation of the nuclear factor of activated T cells (NFAT), which was previously shown to participate in vascular maturation and VSMC differentiation (16, 18).

NFAT is a calcium-regulated transcription factor that was first associated with gene expression changes in T cells (9, 25, 46). It is now clear that NFAT plays an important role in the differentiation of a diverse array of cell types outside the immune system (5). To date, five members of the NFAT transcription factor family have been identified, including NFATc1 (NFAT2/c), NFATc2 (NFAT1/p), NFATc3 (NFAT4/x), NFATc4 (NFAT3), and NFAT5. NFAT has been implicated in vascular development and vessel maturation (18, 19). NFATc3-/NFATc4-null mice die in utero at around the age of embryonic day 11 (E11), with defects in vascular pattern being attributed to endothelial cell dysfunction (18); however, single knockouts display no abnormalities. In the double-knockout mice, endothelial cells fail to respond to and give signals for vessel formation (5, 18). Along these lines, Hernandez et al. (23) showed that inhibition of the calcineurin/NFAT pathway prevents VEGF-mediated angiogenesis.

The subcellular localization of NFAT is an important regulatory event in the activation of this transcription factor. A rise in intracellular Ca2+ activates the Ca2+/calmodulin-dependent protein phosphatase, calcineurin, which dephosphorylates the NFAT molecule at specific NH2-terminal serine residues (reviewed in references 5, 24, and 46). NFAT dephosphorylation causes a conformational change in the molecule that exposes nuclear localization signals (41), which allows NFAT nuclear
import. NFAT nuclear accumulation is subject to further regulation by serine/threonine kinases, which promote the export of nuclear NFAT (3, 14).

We recently demonstrated activation of the NFATc3 isoform by Gq/11-coupled receptor agonists (uridine triphosphate, endothelin-1, and angiotensin II) and physiological intravascular pressure in VSMCs from extracerebral arteries (14, 15, 17, 53). Pressure-induced NFATc3 nuclear accumulation in VSMCs from cerebral arteries is dependent on Ca2+ influx through L-type voltage-dependent Ca2+ channels, endothelin-derived nitric oxide (NO), and cGMP-dependent protein kinase (PKG) (17). Both PKG and NFAT have been associated with the maintenance of the contractile phenotype of VSMCs, which suggests that NFAT may be a linker in PKG-dependent gene transcription (17, 39, 54).

In the central nervous system, a rise in intracellular Ca2+ has been associated with increased NFAT transcriptional activity in both neurons and astrocytes through a glutamate-mediated pathway (20, 25, 28). The activation of metabotropic glutamate receptors (mGluR) in cortical astrocytes increases intracellular Ca2+ concentration ([Ca2+]i) through an inositol 1,4,5-triphosphate-mediated pathway (11). The rise in astrocytic [Ca2+]i enhances the release of several vasoactive substances, some of which have been implicated in vessel maturation (i.e., prostanooids and NO) (33, 36, 40). Importantly, the activation of gene expression is necessary for the proper establishment of the cerebral microcirculation, and intercellular signaling is an essential step in this process. In the present study, we hypothesize that synaptically released glutamate signals pericytes, potentially via astrocytes, to increase NFATc3 nuclear accumulation. Nuclear translocation is a required step for NFATc3-mediated gene transcription and/or repression. Here we provide the first evidence that NFATc3 is expressed in pericytes from parenchymal microvessels. NFATc3 nuclear accumulation was induced by electrical field stimulation (EFS) and mGluR agonists, which suggests a mechanism by which activity-dependent glutamate release may regulate gene transcription in pluripotent vascular pericytes. Finally, we provide evidence that the flow of information for NFATc3 nuclear translocation in pericytes requires the participation of functional astrocytes.

**METHODS**

**Slice preparation.** Ten-day-old neonatal Sprague-Dawley rats were used. Coronal cortical slices were prepared following procedures approved by the Office of Animal Care Management at the University of Vermont. The cortex was immediately removed and placed in ice-cold artificial cerebrospinal fluid (aCSF) equilibrated with 95% O2-5% CO2 at 37°C for 10 min for stabilization. The stabilization period was followed by 10 min of preincubation with or without antagonists and subsequently by 30 min of incubation with the mGluR agonist (±)-1-aminoacyclopentane-trans-1,3-dicarboxylic acid (t-ACPD). The EFS protocol consisted of 3 min of stimulation (50 Hz, 0.3-ms pulses of 150 V). Brain slices were then fixed 15 min after the end of the stimulation to allow sufficient time for NFATc3 to translocate into the nucleus.

**NFATc3 and nuclei immunofluorescence colocalization.** At the end of the experimental protocol, cortical slices were immediately fixed in 4% formaldehyde/PBS overnight. After permeabilization and block of unspecific binding sites, primary antibody [rabbit anti-NFATc3 at 1:100 and goat anti-platelet-derived growth factor-β receptor (PDGFR-β) at 1:100 or goat anti-glial fibrillary acidic protein (GFAP) at 1:250 (Santa Cruz Biotechnology)] diluted in 0.2% gelatin-0.1% Triton-PBS was applied overnight at 4°C. Secondary antibodies (Cy3-anti-rabbit IgG and Cy3-anti-goat IgG at 1:500; Jackson ImmunoResearch) were applied for 2 h at room temperature. Nuclei were stained with the fluorescent nuclear acid dye Sytox (1:5,000; Molecular Probes). Individual cortical slices were then mounted (Aqua Polymount mounting medium; Polysciences) onto glass slides and were examined at ×60 magnification by using a Bio-Rad 1000 laser-scanning confocal microscope. NFATc3, PDGFR-β or GFAP, and nuclei were detected by sequentially monitoring the Cy5, Cy3, and Sytox fluorescence by using an excitation wavelength of 650 nm, 550 nm, and 488 nm and an emission wavelength of 670 nm, 570 nm, and 520 nm, respectively. Before the generation of an overlay image, a mask threshold was applied to each individual channel to exclude pixels that corresponded to nonspecific staining. Nonspecific staining was originally determined in images collected from brain slices incubated with secondary antibodies alone. To avoid differences in fluorescence intensity between preparations, brain slices from all experimental groups were processed simultaneously; images were acquired with the same settings, and care was taken to obtain a similar level of pixel intensity between channels. Nuclei were optically sectioned when the majority of the nuclear surface was in focus. For scoring of NFATc3-positive nuclei, multiple fields for each slice were imaged and counted by two independent observers under double-blind conditions by using Metamorph software (Universal Imaging). The software was programmed so that individual pixels within a given image would appear white if colocalization of the green nuclear acid stain and the Cy5-NFATc3 stain occurred. The criteria for considering positive nuclei for quantification were as follows: a cell was considered positive if colocalization (white pixels) was uniformly distributed within the nucleus and if >10% of the nucleus surface area included white pixels. Nuclei were considered negative if the surface areas of the nucleus included <10% white pixels and if the white pixels were distributed in the perinuclear borders. The percentage of white pixels (255) over the entire number of pixels comprising the nucleus was calculated by tracing each individual nuclei and then obtaining a histogram distribution for all the pixels (0–255) in the nucleus by using ImageJ software (version 1.33u; W. Rasband, National Institutes of Health, Bethesda, MD) (Fig. 1).

**mGluR immunofluorescence detection.** For immunodetection of mGluR1 and mGluR5 in pericytes in cortical brain slices, rabbit anti-mGluR1 (1:300) or rabbit anti-mGluR5 (1:300) (Upstate Biotechnology) and goat anti-PDGFR-β (1:100) antibodies were used. Immunofluorescence protocol was performed as described above.

**Calcium imaging.** Ca2+ imaging was performed with the use of a confocal spinning unit (Yokogawa CSU 10) by using the imaging system and a Zeiss microscope (AxioScope 2FS) equipped with infrared differential interference contrast optics, a water immersion objective (Zeiss ×63, numerical aperture 0.9), and an electron multiplying charge coupled device camera (Xon+885, Andor Tech). Cortical slices were incubated (1–3 h) at room temperature in aCSF containing 10 µM Fluo-4 AM (Invitrogen) and pluronic acid (2.5 mg/ml) in a custom chamber gassed with 95% O2-5% CO2. Using this loading protocol, we are able to visualize Ca2+ transients in both astrocytes and vascular cells. Fluo-4 was excited at 488 nm by using a diode-pumped solid-state laser (Melles Griot), and fluorescence emission was collected at >495 nm. Images were acquired at 3–16 frames/s. Fractional fluorescence ([F/F0]o) was determined by dividing the fluorescence intensity ([F]) within a region of interest (10 × 10 pixels, ∼2.5 × 2.5 µm) drawn on individual cells by a baseline fluorescence value ([F0]o) obtained from ≥50 images showing minimal
fluorescence change. Data were analyzed with the use of custom software created by A. D. Bonev (Univ. of Vermont).

Solutions and drugs. The composition of the aCSF (in mM) was 5 KCl, 124 NaCl, 1.3 MgSO4, 26 NaHCO3, 1.24 KH2PO4, 10 glucose, and 2.4 CaCl2, and it was equilibrated with 95% O2-5% CO2. The mGluR antagonist (RS)-1-aminoindan-1,5-dicarboxylic acid (Aida) and 2-methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP) were obtained from Tocris Cookson. FK-506 was kindly provided by Fujisawa. Cyclosporin A (CsA) was obtained from Calbiochem. All other drugs used were obtained from Sigma: mGluR agonist t-ACPD, tetrodotoxin, nifedipine, indomethacin (Indo), and Nω-nitro-L-arginine (L-NNA) and L-α-amino adipate (LaAA).

Data analysis. Results are expressed as means ± SE. Statistical significance was tested at the 95% (P < 0.05) confidence level by using one-way analysis of variance followed by Tukey’s multiple-comparison test, where applicable.

RESULTS

Methodological considerations. To determine the number of NFATc3-positive nuclei accumulated in pericytes, brain slices were immunostained for NFATc3, the pericyte marker PDGFR-β, and the nuclear stain Sytox (Fig. 1A). PDGFR-β was used as a pericyte marker since it is selectively expressed in these cells throughout development, and the lack of these receptors, as in null-knockout mice, results in the absence of pericytes and in the formation of weak capillary walls (32). Nuclei in focus corresponding to a PDGFR-β-positive membrane staining were only considered for the analysis. Colocalization of NFATc3 and Sytox is shown in white (Fig. 1B), the nucleus of each pericyte was manually traced, and the corresponding histogram was obtained. Figure 1C shows representative nuclei corresponding to a cortical arteriole; nucleus no. 1 is negative (<10% white pixels), nuclei nos. 2, 3, and 7 are positive (>10% white pixels), and nucleus no. 3 is negative because it lacks a PDGFR-β-positive membrane staining and the white pixels are located in the perinuclear zone. Thus, from the vessel shown in Fig. 1, five out of eight nuclei (62.5%) were considered positive (Fig. 1D).

EFS induces NFATc3 nuclear accumulation in pericytes from cortical parenchymal microvessels. Figure 2A shows representative confocal images of control and EFS-stimulated cortical brain slices immunostained for NFATc3 and the pericyte marker PDGFR-β, as well as the nuclear stain Sytox. Colocalization of NFATc3 and the nuclear stain is shown by the white pixels. NFATc3 nuclear accumulation was significantly increased by EFS from a basal percent level of 13.4 ± 3.8% to 60.6 ± 9.0%, P < 0.001 (n = 8). This response was prevented by preincubation with the sodium channel blocker tetrodotoxin (1 μM), which suggests that increased neuronal activity activates the signaling pathway that mediates NFATc3 nuclear accumulation in pericytes (Fig. 2B).

mGluR activation induces NFATc3 nuclear accumulation in pericytes. Glutamate, the major excitatory neurotransmitter released during increased neuronal activity in the cortex, modulates the activity of both neurons and astrocytes via the activation of both ionotropic and metabotropic glutamate receptors (43). To provide additional support for the idea that glutamate mediates NFATc3 nuclear accumulation in pericytes via mGluR activation, cortical brain slices were incubated with a selective mGluR agonist, t-ACPD. Consistent with the observation that glutamate induces NFATc3 nuclear accumulation in cultured astrocytes (28), t-ACPD
(50 μM) induced a significant increase in NFATc3 nuclear accumulation in cortical astrocytes from native tissue, from
35.0 ± 17.1% to 90.2 ± 6.1%, P < 0.02 (n = 4) (Fig. 3A).
In addition, a similar response was observed in pericytes from parenchymal microvessels. In cortical pericytes, the percentage of NFATc3-positive nuclei increased from 7.8 ± 2.2% to 53.5 ± 7.6% (n = 10); P < 0.001 (Fig. 3B). The increase in pericyte NFATc3 nuclear accumulation induced by t-ACPD was inhibited by the specific antagonists for group I mGluRs (50 μM MPEP and 300 μM Aida) (Fig. 3B). To verify that t-ACPD-induced NFATc3 pericyte nuclear accumulation is dependent on the activation of calcineurin, brain slices were pretreated with the immunosuppressant drugs CsA and FK-506, which inhibit calcineurin and thus NFATc3 nuclear translocation. In the presence of CsA (1 μM) and FK-506 (1 μM), t-ACPD failed to induce a significant increase in NFATc3 nuclear accumulation (Fig. 3B).
To verify whether the EFS-induced NFATc3 nuclear accumulation was primarily mediated by the activation of mGluR, EFS was applied in the presence of the specific mGluR blockers MPEP (50 μM) and Aida (300 μM) (35, 56). Figure 3C shows inhibition of the EFS response in the presence of mGluR blockers; a statistically significant difference was observed between the percentage of NFATc3-positive nuclei in the presence of EFS + mGluR blockers and EFS, and no significant differences from control were observed.

Cellular targets of glutamate-induced signaling during mGluR stimulation. Because Gillard et al. (13) reported expression of mGluR on cerebral pericytes, we first determined whether, in our preparation, pericytes expressed these
receptors. Consistent with the report of Gillard et al. (13), we found that pericytes expressed both the mGluR1 and the mGluR5 (Fig. 4A). However, it is possible that mGluR could also be expressed in astrocytic processes that are in direct contact with the pericyte membrane and are thus an ideal upstream target from the pericytes. To test this hypothesis, astrocytic function was disrupted by the gliotoxin LαAA (2 mM). LαAA is a structural homolog of glutamate (21) and has been efficiently used as a gliotoxin in both in vivo and in vitro studies (29, 47, 48, 55). Following a 40-min incubation period, slices were exposed to the mGluR receptor agonist t-ACPD in the presence and absence of LαAA. In response to mGluR activation, the percentage of NFATc3-positive nuclei increased from 15.7 ± 5.3% (n = 10) to 68.6 ± 7.5% (n = 8). In contrast, t-ACPD failed to increase NFATc3 nuclei accumulation in the group preincubated with LαAA (28.35 ± 9; n = 8). In a control group, no significant differences were observed between control and slices incubated with the LαAA alone (% NFATc3-positive nuclei: 23.3 ± 12%; n = 4) (Fig. 4B). As a control experiment and to verify the specificity of the gliotoxin, we monitored [Ca2+]i changes in response to t-ACPD (astrocytic stimulation) and elevated K+ (neuronal and vascular stimulation) in slices preincubated in LαAA (>30 min). As shown in Fig. 4C, astrocytes failed to respond to t-ACPD in slices preincubated with the gliotoxin. On the other hand, both neurons and vascular cells responded with an increase in [Ca2+]i following exposure to elevated K+ (supplemental figure for this article is available online at the American Journal of Physiology-Cell Physiology website).

Possible signaling pathways mediating NFATc3 nuclear accumulation in pericytes on mGluR activation. In the brain, NO is a potent signal released by neurons, astrocytes, and endothelial cells (6, 34, 36, 40). A recent study from our laboratory showed that NFATc3 nuclear accumulation in extracerebral VSMCs depends on both Ca2+ influx and PKG activation through endothelium-derived NO (17). In our preparation, the NO synthase (NOS) inhibitor l-NNa (200 µM) completely abrogated t-ACPD-induced NFATc3 nuclear accumulation in pericytes (Fig. 5), which suggests that, in pericytes, like VSMCs, NFATc3 activation is NO dependent.
It is interesting that inhibition of glia function by the gliotoxin L-aminoadipate (L-AAA) prevented NFATc3 nuclear accumulation in pericytes in response to the mGluR agonist t-ACPD (Fig. 4B). Because NO has been shown to increase Ca\textsuperscript{2+} in astrocytes (31) and the rise in Ca\textsuperscript{2+} has been also linked to the release of prostaglandins implicated in NFAT regulation (27, 57), we addressed whether inhibition of cyclooxygenase would abolish NFATc3 nuclear accumulation in pericytes. Following incubation with Indo (10 \mu M), t-ACPD failed to induce NFATc3 nuclear accumulation in pericytes (21.83 \pm 6\%; n = 8), compared with the 53.46 \pm 7.6\% (n = 12) following t-ACPD alone. No statistically significant differences were observed between control (7.8 \pm 2\%; n = 12) and t-ACPD+Indo (21.83 \pm 6\%; n = 8) (Fig. 5).

**DISCUSSION**

This is the first study to report expression of the transcription factor NFATc3 in pericytes and astrocytes from cortical brain slices. We have found that EFS-induced increase in neuronal activity results in NFATc3 nuclear accumulation in pericytes. This response was dependent on the activation of astrocytic mGluR. Moreover, glutamate-induced NFATc3 nuclear accumulation in pericytes was coupled to NOS and cyclooxygenase
activity. In accordance with the reported effect of glutamate on NFATc3 transcripational activity in cultured astrocytes (28), our results showed that, in native astrocytes, t-ACPD, a mGluR agonist, significantly increased NFATc3 nuclear accumulation.

Glutamate release on neuronal depolarization mediates NFATc3 nuclear accumulation in pericytes from cortical parenchymal microvessels. An increase in neuronal activity results in the release of glutamate from neurons and astrocytes and subsequent activation of glutamate receptors in target cells. Neurons and astrocytes express ionotropic and metabotropic glutamate receptors (4). Recently, mGluR expression was also shown in pericytes and endothelial cells within the microvasculature of the cortex (13), which suggests a possible direct role for glutamate in the cerebral circulation. Consistent with this report, our results also suggest the expression of mGluR1 and mGluR5 (group I) in pericytes of parenchymal microvessels. Interestingly, however, the expression of group I mGluRs was observed only on the outer surface membrane, likely in contact with astrocytic processes (endfeet). Thus, it is also possible that the expression of these receptors was not specific to pericytes but rather to astrocytic membrane as well. We found that enhanced neuronal activity induced by EFS significantly increased NFATc3 nuclear accumulation in pericytes. This latter event depended on the activation of mGluRs, although other pathways are possible since EFS likely induces the release of multiple neuronal signals. However, a similar response was induced by the selective mGluR agonist, t-ACPD, in pericytes and cortical astrocytes. As expected, t-ACPD-induced NFATc3 pericyte nuclear accumulation was blocked by antagonists of the group I mGluRs. In addition, glutamate-induced NFATc3 nuclear accumulation is dependent on the activation of the Ca2+-calmodulin-dependent phosphatase calcineurin, which is consistent with the requirement of NFAT dephosphorylation as part of its activation mechanism (reviewed in Refs. 5, 24, 26, and 46).

The observed expression of mGluR in pericytes raises the possibility that these are the direct target cells for the released glutamate during neuronal activation. It is also possible that the immunostaining did not provide accurate localization of the receptors and that the actual targets were upstream of the pericytes, such as the astrocytes. To address this important question, astrocyte function was abolished by exposing brain slices to a gliotoxin, LaAA. At low concentrations, LaAA is toxic only to glial cells, possibly via oxidative cell damage (47). Following LaAA incubation, the mGluR agonist failed to induce NFATc3 nuclear accumulation in pericytes, which suggests that the target cell was indeed the astrocyte. These results suggest that signaling from the neurons to the astrocytes is an essential step leading to NFATc3 nuclear accumulation in pericytes.

Possible signaling pathways mediating NFATc3 nuclear accumulation in pericytes on mGluR activation. Different sources of NO have been demonstrated in the nervous system. Neurons, astrocytes, and endothelial cells have been shown to express constitutive NOS (neuronal and endothelial NOS isoforms) (6, 34, 36, 40, 49, 50). Activation of the group I mGluRs in astrocytes increases intracellular Ca2+, which, in turn, results in the release of Ca2+-dependent vasoactive substances such as prostaglandins and NO (56). The close proximity of pericytes to astrocytic endfeet provides a potential intercellular pathway by which astrocytes, in addition to other cellular sources such as endothelial cells, may communicate with pericytes through the release of NO and/or prostaglandins (30, 34, 36, 56). Previously, we showed that NFATc3 nuclear accumulation in VSMCs depends on the activation of PKG through endothelial NO (17). In these cells, PKG regulates NFATc3 nuclear export through the modulation of c-Jun kinase 2 activity (14, 17). As in VSMCs, we found that glutamate-induced NFATc3 nuclear accumulation in pericytes is also dependent on NO. The cellular sources of NO and the targets of NO (astrocytes vs. the pericytes) are still to be determined. For example, it is well known that, following neuronal N-methyl-D-aspartate receptor stimulation, NO is formed and released (8, 12) and that NO can then diffuse and signal neighboring cells. It has also been shown that signals that increase Ca2+ in astrocytes can induce the production of NO in these cells, and NO then acts on the astrocytes to stimulate Ca2+-influx pathways that contribute to the refilling of astrocytic Ca2+ stores (31). Furthermore, the rise in astrocytic Ca2+ has been linked to the release of prostaglandins (57), which further supports the intercellular communication between astrocytes and pericytes and our observations that blockade of NO signaling with L-NNA and inhibition of prostaglandin synthesis with Indo both inhibit NFATc3 nuclear accumulation in pericytes. Future studies on the precise cellular sources and targets of these proposed pathways will be needed.

In summary, this is the first report demonstrating NFATc3 expression in both native cortical astrocytes and pericytes from parenchymal microvessels. Increased neuronal activity or application of the mGluR agonist resulted in NFATc3 nuclear accumulation in both astrocytes and pericytes from native tissue. In pericytes, this response was mediated by group I mGluRs, required functional astrocytes, NOS, and cyclooxygenase activity. Our findings support the concept of a neuronal-glial-vascular communication mediated by glutamate (10, 56). Given the important physiological role that NFAT, including NFATc3, plays in the establishment of the vasculature, the present study provides an important initial step in our understanding of the upstream intercellu-
lar processes leading to NFATc3 nuclear accumulation in pericytes.

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