Calcium Signaling of Glial Cells along Mammalian Axons

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Glial $[Ca^{2+}]_i$ signaling was examined in a mammalian white matter lacking neuronal cell bodies and synapses. Rat optic nerves (postnatal days 2 and 7) were stained with calcium indicator dyes and confocal images of $[Ca^{2+}]_i$ were recorded at $\sim 25^\circ C$ or $\sim 37^\circ C$. Glial cell bodies showed spiking or sustained $[Ca^{2+}]_i$ response to bath-applied glutamate (50–500 μM). The metabotropic glutamate agonist *trans*-ACPD elicited transient, sometimes spiking, $[Ca^{2+}]_i$ responses, whereas ionotropic agonists kainate and AMPA elicited a 6,7-dinitroquinoxaline-2,3-dione-sensitive, mostly sustained $[Ca^{2+}]_i$ response. Transient and spiking glial $[Ca^{2+}]_i$ responses also were elicited by adenosine and ATP (0.1–100 μM). Repetitive nerve stimulation (10–20 Hz) elicited $[Ca^{2+}]_i$ spiking in 15–25% of glial cells in postnatal day 7 nerves, with spiking typically occurring 15–60 sec after onset of nerve stimulation. At $37^\circ C$, the frequency of glial $[Ca^{2+}]_i$ spikes increased from ~ 0.06 Hz to ~ 0.11 Hz when axonal stimulation was increased from 10 to 20 Hz. This activity-dependent glial spiking was inhibited by TTX, could not be mimicked by increasing the bath K^+ by 20 mM, and occurred when nerves were stimulated in the absence of bath calcium. Activity-dependent and glutamate-induced glial spiking could be mimicked by altering ionic gradients known to favor release of glutamate via glutamate transporters, including elevation of intracellular Na^+ by veratridine concurrent with external K^+ elevation. We suggest that glial $[Ca^{2+}]_i$ spiking observed during electrical activity resulted from activation of glial receptors (e.g., metabotropic glutamate receptor, adenosine receptor) by substances (e.g., glutamate, adenosine) released from the optic nerve in a nonvesicular fashion, possibly through a reversal of sodium-coupled transporters when Na^+ and K^+ gradients are altered by prolonged nerve activity.

[Key words: metabotropic glutamate receptors, confocal imaging, optic nerve, white matter, axon–glial interactions, fluo-3]

When a neuron undergoes excitation, glutamate and other neuroactive substances are released. The immediate target of these substances is another neuron. However, the recent discovery that glial cells possess receptors for glutamate and other neuroactive ligands (for review, see Pearce and Murphy, 1988; Gallo

et al., 1990; Barres, 1991) raises the possibility of ligand-mediated, neuron–glial signaling. Different parts of the neuron can signal glia. One is the presynaptic terminal that is encapsulated by glial processes. The concentration of glutamate at the synaptic cleft can reach ~ 1 mM during synaptic transmissions, and even though the releasing neuronal membrane directly faces another neuron rather than glia, some glutamate can leak out and signal the glial membranes flanking the synapse (Dani et al., 1992).

Another part of the neuron that might signal glia is the axon. In the mammalian CNS, there are intimate contacts between axons and a variety of glia, including the myelinating oligodendrocytes, and O-2A glial progenitor cells and astrocytes that send processes to the axonal surface (Miller et al., 1989; Raff, 1989). Even though there are no known vesicular mechanisms for releasing neuroactive substances or excitatory amino acids in white matter, nerve fiber tracts in certain mammals and frogs have been shown to release glutamate (Wheeler et al., 1966; Weinreich and Hammerschlag, 1975) and adenosine (Kuperman et al., 1964; Maire et al., 1984) during electrical stimulation. To date, the best-documented case for axon–glia signaling is that which takes places between Schwann cells and the squid giant axon (Villegas, 1984; Lieberman et al., 1989; Lieberman, 1991). Collectively, these observations suggest that in the mammalian nervous system, ligand-mediated signaling between neurons and glia can occur in both the synaptic and nonsynaptic regions. The relative importance of synapse versus axons in signaling glia is unclear; for example, glutamate may be released at a higher density per unit membrane area at the synapse than along axonal membranes, but the total axonal area is much larger.

What are some of the detectable consequences of ligand-mediated activation in glia? Among the first to be measured were changes in membrane potential, as is the case for ligand-mediated signaling in squid Schwann cells and glial cells of amphibian optic nerve (Villegas, 1984; Tang and Orkand, 1986; Lieberman et al., 1989). Recently, calcium imaging in mammalian glia has revealed a dynamic intracellular signaling in glia, specifically in the case of glutamate. The first observations, made in neuron-free astrocyte cultures, showed that glutamate evoked calcium spikes (Enkvist et al., 1989; Cornell-Bell et al., 1990a; Glaum et al., 1990; Jensen and Chiu, 1990) and calcium waves in astrocytes network (Cornell-Bell et al., 1990a). Subsequently, Dani et al. (1992) showed that in organotypic brain slices, neuronal electrical activity triggered glial calcium waves and spiking. While the simplest explanation for the Dani et al. (1992) observation is that the glial calcium signals in brain slices were elicited by glutamate (or other neuroactive substances) released at the synapse, an interesting issue is whether some of the glial signaling may result from substances released from axons.

In this report, we examine whether axons alone can trigger

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glial calcium signaling by performing calcium imaging on neonatal rat optic nerves. By using the portion of the nerve between the eye and the optic chiasm, neuronal cell bodies and synapses are eliminated, leaving only glia and axons as the major cellular elements. Premyelinated nerves at postnatal day 7 (P7) were used to maximize the axonal surface available for releasing substances, which otherwise might not be possible in fully myelinated nerves where most of the axonal area has been sealed up. A method was developed to stain the axons and glial cells so that an intact nerve could be subjected to confocal calcium imaging as early as 30 min after nerve removal. We first bath-applied glutamate and other neuroactive ligands to see if optic nerve glia are capable of ligand-mediated calcium signaling. We then altered Na^+ and K^+ gradients in a fashion known to reverse the glutamate transporter to see if optic nerves are capable of releasing glutamate by this mechanism. We then stimulated the axons electrically to see if this triggered glial responses that mimicked the results obtained by direct ligand application, particularly with respect to glutamate.

Materials and Methods

Dye loading of neonatal rat optic nerves. Rat optic nerves (P2 and P7) were excised, stripped of the outermost dura matter, and stained with the calcium indicator fluo-3/acetomethoxyester (AM) (Kao et al., 1989) or calcium green/AM. In our earlier studies, we incubated the nerves in solutions containing the indicator, but found that after 2 hr only the outermost layers of cells were stained; apparently, the nerve was still wrapped by a residual pia layer that severely restricted dye penetration. We thus abandoned this loading method and adopted a different approach. In this method, a fine-tip glass pipette (2–5 M Ω), filled with 10–50 μM fluo-3/AM or calcium green/AM made in buffer or in a solution of 290 mM sucrose and 10 mM glucose, was gently inserted into the whole nerve by piercing the outermost pia sheath. The dye was injected into the extracellular space within the whole nerve by brief pulses of positive pressure delivered by a Picospritzer. This entire procedure was monitored closely under a microscope. During each pulse the nerve was seen to undergo slight expansion around the injection site; often a bolus of solution could be seen being propelled in one direction along the longitudinal axis of the nerve. The injection pressure was carefully adjusted to minimize the nerve expansion associated with each injection pulse, and the nerve was judged to have recovered from this slight distention before another injection was given. The volume of solution delivered in each injection was estimated to be about 0.1 μl , and typically 20–30 such injections were given over a period of 15–30 min. During injections, the bath containing the nerve was continuously perfused with oxygenated Krebs' solution at room temperature. Following injections, the nerve was washed for 10–30 min either at 25°C or at $\sim 37^\circ\text{C}$ before starting the imaging experiments.

Confocal fluorescence imaging of intracellular calcium. The nerve was mounted on a chamber with a glass coverslip on the bottom. A nylon gauze was placed over the nerve to prevent it from moving during imaging experiments. Calcium images were viewed from below with a 20 $\times$, 40 $\times$, or 60 $\times$ (oil) objective lens using a Nikon Diaphot microscope. The intracellular dye was excited with a laser at 488 nm (Noran Odyssey, Madison, WI), and confocal fluorescence images were monitored at 500 nm. Images were either recorded onto a hard disk or an optical memory disk recorder (Panasonic TQ3031F). In most experiments, each image was the average of 16 frames taken at video rate, and one image was collected every 1–5 sec. In some experiments (electrical stimulation), fast changes in calcium signals were studied by collecting images at video rate (one image every 33 msec). Image acquisition and analysis were controlled by the IMAGE-1 software. Optical thin sections [2.6 μm full width at half-maximum (FWHM) for the 40 $\times$ objective used in most experiments] were made along the longitudinal axis of the nerve. In most cases, images were collected from a focal plane that was two to three cell layers deep into the nerve interior as measured from the outermost pia surface. In general cells were brightly stained at the dye injection site and became much less so away from it. Trypan blue staining suggested that cells at the injection site were damaged. Hence, all our images were collected from a region away from the injection site where the stain looked fairly uniform. Intracellular calcium

was reported as fluo-3 fluorescence without calibration for absolute values. In some figures, a $\Delta F/F_0$ value was calculated as described previously (Jensen and Chiu, 1991). Experiments were done either at $\sim 37^\circ\text{C}$ (using a stage-mounted, air-heated incubator enclosing the observation platform of the Nikon microscope) or at room temperature of $\sim 25^\circ\text{C}$.

Bath application of glutamate and other agents. The solution bathing the nerve was perfused first with a normal Krebs' solution for 30–60 sec to obtain a baseline, and then the solution was switched to one containing glutamate or other agents. The bath volume was about 0.5 ml, and the perfusion rate was about 5 ml/min.

Electrical stimulation of axons. A different chamber was used for electrical stimulation studies. The stimulation chamber has two pairs of platinum wire separated by a distance of about 0.5 cm. The nerve was gently laid across the two pairs of wires, and one end of the nerve was used for stimulation while the other end for recording of compound action potentials. A brief (0.1–0.4 msec), supramaximal stimulation was applied, and the frequency of stimulation was 5–20 Hz. Calcium images were collected from the nerve segment in between the two pairs of wires at a rate of one image every 1–3 sec. In some experiments, images were collected at video rate. Only the nerve segment between the electrodes was locally perfused with a pair of glass pipettes. Two pieces of moistened tissue paper were placed over the nerve segments in contact with the platinum wires to prevent the ends of the nerve from drying during an experiment. To prevent evaporation further, the entire chamber was covered with a thick, wetted piece of cotton pad. The tips of the glass pipettes were about 2 mm apart, and were placed along side the nerve segment. Oxygenated Krebs' solutions were continuously delivered via one pipette by gravity, and sucked out gently via the other pipette. Both pipette tips and the nerve segment were positioned underneath a small piece of nylon gauze. The nylon gauze provided a meniscus surface for the perfusing solution to dissipate over the entire nerve segment. Further, the suction gently pulled the nylon gauze down onto the nerve, which prevented nerve movement during imaging experiments. Electrical stimulation studies were performed either at $\sim 25^\circ\text{C}$ or $\sim 37^\circ\text{C}$. Compound action potentials could be elicited from nerves mounted in this chamber for up to 30 min even though most experiments were finished in 5–10 min. We did not notice any significant differences between compound action potentials from control and dye-loaded nerves.

Solutions. Optic nerves were normally bathed in a Krebs' solution that contained (mM) NaCl, 120; KCl, 5; KH_2PO_4 , 1.2; CaCl_2 , 2.4; MgSO_4 , 1.3; NaHCO_3 , 26; and glucose, 10; and was continuously oxygenated with 95% O_2 , 5% CO_2 (pH 7.4). In later experiments, the Krebs' solution was slightly modified to contain (mM) NaCl, 130; NaHCO_3 , 20; KCl, 3; KH_2PO_4 , 1.2; CaCl_2 , 2.4; MgSO_4 , 1.3; HEPES, 3; and glucose, 10 (pH 7.4). Calcium-free solutions were made by omitting calcium, and adding EGTA (50 μM or 2 mM); the pH was adjusted to 7.4 with NaOH as necessary. High-K solutions were made by equimolar substitution of KCl for NaCl.

Materials. Excitatory amino acids (L-glutamic acid, kainic acid) and purines (adenosine and ATP) were purchased from Sigma (St. Louis, MO). 6,7-Dinitroquinoxaline-2,3-dione (DNQX) was purchased from Cambridge Research Biochemicals. 8-(p-Sulphophenyl)theophylline was purchased from Research Biochemicals Inc. (Natick, MA). The acetomethoxyester form of the calcium indicator fluo-3 (fluo-3/AM), calcium green, and CFSE [5-(and-6)-carboxyfluorescein diacetate succinimidyl ester] were purchased from Molecular Probes (Junction City, OR).

Results

Glial cells in rat optic nerves are resolved by confocal imaging

P7 rat optic nerves, injected with fluo-3/AM or calcium green/AM, displayed well-stained cell bodies throughout the entire nerve. If extensive dye injections were used, and a large number of frames were averaged (128 or 256 frames), the morphology of the cells could be very well resolved, as illustrated by the calcium images in Figure 1. In most of the experiments, however, we worked with cells that were dimmer than those shown in Figure 1 by reducing the dye injection to a minimum. The reason was that even though extensive dye injections gave brightly stained cells with well-defined morphology, it was our impression that because of the trauma needed to achieve this staining,

Figure 1. Confocal calcium image of a P7 rat optic nerve. The optical section was taken along the longitudinal axis of the nerve with a Noran Odyssey system with a 40 $\times$ objective giving an optical section of approximately 2.6 μ m thick (see Materials and Methods). The image represents the average of 256 frames. The optical section was taken approximately several cell layers into the nerve as measured from the outermost pia surface. The nerve was stained by pressure injection of fluo-3/AM (see Materials and Methods) into the extracellular space.

the brightly stained cells might in fact be unhealthy or partially damaged and thus loaded with calcium. Further, in order to be able to resolve temporal changes in calcium, we only averaged 16 frames for each image. These adopted procedures, namely, the use of faintly stained cells to better the chances of obtaining a healthy cell and the use of minimum frame averaging to get better temporal resolution, meant that most of our analysis was based on images in which only the cell bodies, but not the cell processes, were resolved. An experiment could last up to 10–15 min without significant dye bleaching.

Since the nerves in this study were obtained between the eye and the optic chiasm, no neuronal cell bodies were present. The major cellular components of a P7 optic nerve are axons (~ 0.2 μ m diameter) and glial cells (a mixture of oligodendrocytes, astrocytes, and O-2A glial progenitor cells), with the remaining minor component coming from endothelial cells lining the blood vessels. Since injecting dye into the optic nerve might stain both glial cells and endothelial cells, we first attempted to stain selectively the endothelial cells with a vital dye to see if they had some recognizable morphological criteria that would allow us to exclude these cells from analysis. We found that the endo-

thelial cells could be selectively stained by introducing the vital dye CFSE into the systemic circulation via the heart before excising the optic nerve. Endothelial cells stained in this way were mostly thin and spindle-like and tended to form a long, thin tandem along blood capillaries. When calcium dye was injected into the optic nerves, the morphology of most of the stained cells did not look like endothelial cells; rather, they were randomly distributed, and even though some tended to form a row, their cell bodies were rounder and bigger than the endothelial cells and were not spindle-like in shape. This suggested that most of the stained cells used in calcium imaging were glia, consistent with the fact that endothelial cells are known to be relatively few in number compared to glia. In this study, we assumed that the stained cell bodies on which we performed calcium analysis were glial cells, even though we cannot completely rule out endothelial cell contamination. The glial cells we referred to in this study were in fact a mixture of oligodendrocytes, astrocytes, and O-2A progenitor cells that we cannot, at present, distinguish. Axons were also stained but their small diameters (~ 0.2 μ m) made it impossible to resolve individual axons.

Spontaneous changes in resting glial $[Ca^{2+}]_i$

In most studies, the fluorescence signal (intracellular calcium) at the cell bodies was monitored before and after various experimental manipulations. A meaningful interpretation of the stimulation data required that the baseline calcium level was steady, which was the case in many cells. However, some cells displayed spontaneous oscillations. These resting oscillations could occur with a fairly regular periodicity, or were irregular with long pauses between bursts of oscillation epochs. The resting $[Ca^{2+}]_i$ oscillations appeared to reflect internal release of calcium, since they could be observed when calcium was removed from the bath. Even though we have not systematically investigated this, preliminary observations suggested that the spontaneous glial oscillations occurred in the presence of TTX, suggesting that they may not be linked to spontaneous activity of cut axons. Interestingly, spontaneous changes in cytoplasmic calcium have been observed in cultured cortical astrocytes that are distinct from agonist-induced and membrane potential depolarization-induced changes (Fatatis and Russell, 1992). In subsequent experiments, cells with resting oscillations were excluded from analysis so as to distinguish them from stimulation-induced $[Ca^{2+}]_i$ responses. Two procedures were used: bath application of various agents and electrical stimulation of the optic nerves.

Bath application

Glutamate elicits spiking and sustained glial $[Ca^{2+}]_i$ responses

In previous studies of cultured cortical astrocytes, glutamate was found to induce oscillatory and sustained $[Ca^{2+}]_i$ responses in mixed cultures of type 1 and type 2 astrocytes (Jensen and Chiu, 1991). Here we examined whether similar patterns of glial responses can be elicited by glutamate *in situ*.

Individual glial cells exhibited two patterns of $[Ca^{2+}]_i$ response when glutamate was applied continuously to the bath in the presence of normal external calcium. The first pattern was calcium spiking, as shown by the single cell in Figure 2*A*. In this cell, continuous application of 100 μ M glutamate at 37°C elicited multiple sharp $[Ca^{2+}]_i$ spikes with an average interspike interval of ~5.5 sec. The second pattern was a sustained response, as illustrated by a different cell in Figure 2*B* at 37°C; there was an initial fast spike-like response that gave way to a plateau $[Ca^{2+}]_i$ elevation that showed a gradual decline in this cell. It should be noted that in other cells (data not shown), the plateau phase could be sustained for up to 2–4 min of glutamate application without signs of decline. In some glial cells, the response appeared to be a summation of these two patterns, with oscillations superimposing on a plateau $[Ca^{2+}]_i$ elevation. Spiking and sustained responses could be found in the same nerve. Both responses could be elicited at 25°C or 37°C. In a total of 13 nerves (P7), about 50% of the cells responded to glutamate. Of these responding cells, ~27% could be classified as roughly showing a sustained response, with the remaining ~73% showing some sort of spiking behavior.

Glutamate responses in calcium-free bath

Calcium spiking (Fig. 2*A*) suggests activation of metabotropic glutamate receptors. To examine the role of calcium release from internal stores in the glutamate-induced responses, optic nerves were bath-perfused with a calcium-free solution (0 Ca plus 50 μ M or 2 mM EGTA) for 10–15 min before adding glutamate to the calcium-free solution. Calcium spiking was observed in re-

sponse to glutamate under this condition (Fig. 2*C*). Figure 2*D* illustrates glial spiking under calcium-free conditions in another cell with a series of time-lapsed images. The images were ~20 sec apart, and started from left to right beginning with the top row. Following six images of steady baseline, glutamate (300 μ M) was applied continuously starting at the arrow. Subsequent images show that of the various cells in the field of view, glutamate induced one cell to spike.

The sustained calcium elevation elicited by glutamate (Fig. 2*B*) resembled an ionotropic response and the experiments performed in calcium-free bath solutions confirmed that this was due to influx. In the absence of external calcium, the cells that responded to glutamate responded only with transient single or multiple spikes (as in Fig. 2*C*) and sustained elevation of the type shown in Figure 2*B* were never observed ($N = 269$ cells).

Glutamate receptor agonists and antagonists

The spiking and sustained responses elicited by glutamate suggest the presence of metabotropic and ionotropic glutamate receptors in optic nerve glia. To strengthen the interpretation that these glial responses were receptor mediated, agonists specific for these two receptor classes were tested.

Figure 3*A* shows that AMPA (an ionotropic agonist) and *trans*-ACPD (a metabotropic agonist; Palmer et al., 1989) indeed elicited calcium responses from glia. The responses shown represented the average of 27 cells from the same nerve in the presence of normal external calcium. The cells were first exposed to *trans*-ACPD (200 μ M), and then washed and exposed to AMPA (200 μ M). The *trans*-ACPD response was transient, while that for AMPA was sustained, during continuous agonist application (Fig. 3*A*). Note that the 27 cells used in the average were chosen based on the fact they all responded to *trans*-ACPD. The AMPA response after the wash (Fig. 3*A*) was thus the response from the same group of cells that showed a prior response to *trans*-ACPD. In fact, virtually all cells that responded to *trans*-ACPD also responded to AMPA (72 cells in two experiments). In the absence of bath calcium (Fig. 3*B*), the AMPA response was abolished while the *trans*-ACPD response was present with only a slight change in shape. When examined at the single-cell level, the AMPA response never showed oscillations (data not shown); kainate (200 μ M), another ionotropic agonist, elicited similar responses to AMPA (data not shown). In contrast, the *trans*-ACPD response, when examined at the single-cell level, sometimes showed spiking that did not require the presence of bath calcium (Fig. 3*C*).

Figure 3*D* shows that the AMPA response was blocked by DNQX (10 μ M), a specific blocker of the ionotropic glutamate receptor (Honore et al., 1988). Interestingly, the kainate response was only partially inhibited by DNQX. Collectively, the results of Figure 3 show that AMPA elicited calcium influx while *trans*-ACPD induced internal calcium mobilization, consistent with the known ionotropic and metabotropic nature of these two glutamate receptor agonists. Figure 3*A* further suggests that both classes of glutamate receptors are coexpressed in the same cell.

Glial responses to other ligands

Besides glutamate receptors, glial cells express numerous receptors all capable of eliciting calcium responses, at least in culture (Salm et al., 1990; Helen et al., 1992). We tested five such substances, namely, norepinephrine, histamine, 5-HT, ATP, and adenosine, and found that all of them elicited a calcium response

Figure 2. Effects of glutamate on intracellular calcium fluorescence in optic nerve glia. Glutamate was bath-applied either in normal calcium (*A*, *B*) or in calcium-free solutions (*C*). *D* shows time-lapsed calcium images before and after glutamate (300 μM) was applied in calcium-free solutions. The images start from left to right, beginning at the top row, with ~ 20 sec in between images. Each image is the average of 16 frames. Calcium-free solutions in *C* and *D* were prepared with 2 mM EGTA and no calcium added. Temperature was 37°C (*A*, *B*) and 25°C (*C*, *D*). Optic nerves were from P7 rats.

in optic nerve glia in the concentration range of 0.1–100 μM . The responses to these substances were all similar to one another, but had several major differences with the glutamate response. First, when scored by the percentage of cells responding, most of these substances were more potent than glutamate

at the same concentration used. Adenosine appeared to be the most potent, in being able to elicit responses in about 90–95% of the cells at 100 μM , as compared to $\sim 50\%$ for glutamate at the same concentration. Further, cells responding to glutamate appeared not to be distributed randomly but tended to form

Figure 3. Effects of agonists and antagonist for metabotropic and ionotropic glutamate receptors on intracellular calcium in optic nerve glial. These agents were bath-applied. *A* shows the average response of 27 cells to *trans*-ACPD and AMPA with intervening wash. *B* shows a similar exposure to these agents done in calcium-free solutions ($N = 32$ cells). *C* shows a single cell's response to *trans*-ACPD in calcium-free solution. *D* shows the effect of DNQX on the AMPA response. Calcium-free solutions were made by adding 50 μ M EGTA with no calcium added. Temperature was 37°C.

groups located toward the pia edge of the nerve. In contrast, these five ligands appeared to trigger responses from cells that were more randomly distributed over the nerve, both along the edge and the nerve interior. Second, most of these ligands did not elicit the sharp and multiple spikes seen with glutamate. Rather, a typical response for these ligands was a single transient calcium elevation during continuous ligand exposure (e.g., Fig. 4*A*; see below).

We will conclude this brief survey of other ligands by presenting the data for adenosine, a substance of particular interest since this metabolite is known to be released from fiber tracts (Maire et al., 1982, 1984). Figure 4*A* shows a single cell's response to 50 μ M adenosine applied to a P7 optic nerve in the presence of normal external calcium. This response was due to internal calcium mobilization since a similar response also could be obtained in the absence of bath calcium (data not shown). Figure 4*B* shows that the adenosine response was reversibly blocked by 100 μ M 8-(*p*-sulfophenyl)theophylline (Bruns et al., 1986), a nonspecific blocker for the A₁ and A₂ adenosine receptors. While adenosine rarely gave calcium spiking, a related substance, ATP, was found to trigger calcium spiking in some optic nerve glia. This is illustrated in Figure 4*C*, as well as by the sequence of images shown in Figure 4*D*. In these time-lapsed images, taken every ~ 7 sec (from left to right starting with the top row), addition of 1 μ M ATP starting at the solid arrow elicited spiking in one of the cells (indicated by a white arrow).

Ionic conditions that favor reversal of the glutamate transporter can elicit glial spiking

Besides ligand application, we found that alterations of Na⁺ and K⁺ transmembrane gradients in optic nerve could also elicit glial calcium spikes. We first elevated extracellular K⁺ by 20 mM above normal, which is approximately the level seen when neonatal optic nerves underwent high-frequency firing (Connors et al., 1982). Figure 5*A* shows that when [K⁺]_o in the bath was elevated from a normal level of 6 mM to 26 mM (by equal osmotic substitution with Na⁺), there was at most a slight in-

crease in the fluorescence signal at the cell body without signs of spiking; in other similar experiments, a larger increase in fluorescence signal was observed.

From this level of elevated K⁺ (26 mM), which by itself did not give glial spiking, two other additional manipulations of the ionic gradients were found to produce glial spikes. The first was a concurrent elevation of intracellular Na⁺, achieved by adding veratridine (50 μ M) to the already elevated K⁺ solution (Fig. 5*A*). After so doing, the calcium signal at the cell body underwent a large, plateau-like increase and calcium spikes could be seen superimposed on the plateau. The second manipulation was simply to elevate the external K⁺ further (note that this was at the expense of further decreasing the transmembrane Na gradient due to equal osmotic substitution). This is shown in Figure 5*B*. Upon raising the external K⁺ from 26 mM (which did not elicit glial spiking) to 69 mM, robust glial spiking was seen.

It is interesting that these ionic manipulations, namely, raising intracellular Na⁺ and extracellular K⁺, coupled with the ensuing membrane depolarizations that resulted, are conditions known to favor reverse release from the high-affinity sodium glutamate transporter (Nicholls and Attwell, 1990). Several possibilities could account for the observation in Figure 5. One possibility is that the glial spiking is not receptor mediated but reflects a direct effect of the alterations of ionic gradients on glia. Another possibility is that alterations of Na and K gradients caused glutamate to be released (either from axons or glia) via a glutamate transporter, which then activates metabotropic glutamate receptors on glia. Even though we cannot distinguish between these two possibilities, work in progress using blockers for glutamate receptors and high-affinity glutamate transporters gave some support for the latter possibility (see Discussion).

Nerve stimulation

Having demonstrated that glial cells were capable of generating single or multiple spikes in response to ligands (e.g., adenosine, glutamate), as well as to ionic alterations that favored glutamate

Figure 4. Effects of adenosine and ATP on intracellular calcium in optic nerve glia. *A* shows a single cell's response to adenosine. *B* shows the inhibitory effects of a blocker, 8-(*p*-sulfophenyl)theophylline (8-SPT), on the averaged adenosine response (45 cells). *C* shows a single cell's response to ATP. *D* shows time-lapsed calcium images before and after ATP application. Images are shown at ~ 7 sec intervals. The white arrows point to a spiking glia after ATP application.

release from the optic nerve, we next examined if electrical nerve stimulation could mimic some of these glial responses. Since both axons and glial cells should be stained by the calcium dye, images captured at the glial cell body actually were composed

of a glial signal in series with an axon signal; the axonal signal would appear as a composite signal because signals from individual axons, each having a diameter of $0.2 \mu\text{m}$, were unresolvable. Our first goal was to see if we could separate the axonal

Figure 5. Effects on glial calcium fluorescence of altering extracellular and intracellular concentrations of Na and K in optic nerves. The bath solutions have normal calcium. Bath concentrations of Na and K were changed by equal osmotic substitution of K for Na (*A*), and intracellular Na concentration was raised by adding veratridine (*A* and *B*). Responses were from single cells from P7 nerves. Temperature was 37°C.

signal from the glial signal. This was crucial for subsequently interpreting signals seen at the glial cell body.

Axonal $[Ca^{2+}]_i$ signals

To resolve the axonal signal with little contamination from glial signal, we imaged the areas between glial cell bodies. At P7, the nerves are not yet myelinated and essentially naked axons, each 0.2 μm in diameter, fill the space between glial cells. Figure 6 shows two images, each representing the average of 256 frames, taken before (*A*) and immediately after (*B*) stimulating the nerve at 20 Hz. Since it took ~ 8 sec of frame averaging to produce one image, the stimulated image should be considered as the response averaged over an interval of ~ 8 sec. The consistent observation was an increase in fluorescence in the entire field of view, both in regions occupied by cell bodies and in regions between cell bodies.

We interpreted the fluorescence increase seen in regions between cell bodies to be coming mostly from axons, for the following reasons. First, such increase could be elicited in electrically stimulated P2 nerves, where in comparison with P7 nerves (Fig. 6), the areas between glial cell bodies were even larger because of the paucity of glial cells and their associated processes in such an early age; these large areas between cell bodies must be occupied mostly by axons. Second, in a limited number of experiments, a similar but much fainter increase in fluorescence

was seen when the axons were stained selectively by injecting calcium dye into the eyes.

Third, one could argue that the signals seen between cell bodies were due to light scattered from responding cells either in or out of the focal planes. We considered this unlikely, since the images in Figure 6 were obtained with a very thin optical section of $\sim 1.5 \mu\text{m}$ (the thinnest offered by our confocal system) that should greatly reduce out-of-focus contaminations. Further, if the increase in signals between cell bodies was due to light scattered from a fluorescence increase that originated from the neighboring glial cell bodies in the same focal plane, then there should be a spatial gradient for the fluorescence increase, being highest at the cell body and becoming smaller at distance away from the cell body. In most cases this is not so, as illustrated in Figure 6*C*. Here, a profile for the fluorescence intensity is plotted along a line that crosses two cell bodies plus the area between them (the position of these two cell bodies and the line are shown in Fig. 6*B*). This line intensity scan is shown before (Fig. 6*C*, bottom trace) and after (top trace) electrical stimulation. The peaks of the intensity profile corresponded to the location of the cell bodies, while the valleys corresponded to the area between the cell bodies. It can be seen that electrical stimulation caused an increase in fluorescence (the vertical distance between the two intensity profiles) that is smaller at the peak (cell body) than at the valleys (regions between cell bodies). Figure 6*D* illustrates this observation by $\Delta F/F_0$, which simply expresses the fractional change in calcium fluorescence relative to the resting level. Figure 6, *C* and *D*, clearly shows that the fluorescence increase was smaller at the cell bodies and larger in the region between cells. This suggests that the fluorescence increase seen in the regions between cells cannot be completely explained by light scattering from cell bodies. In fact, the observation that the fluorescence increase is larger in regions between cell bodies is consistent with an axonal origin of the signal. The reason is that when an optical section of a given thickness is cut across a glial cell body, there will be less axons in a given volume than just outside the cell body.

Figure 7 shows video-rate recording of signals in regions between cell bodies during electrical stimulation. At a very slow rate of stimulation, each single stimulation triggered a clearly resolvable response that had a rising phase and a falling phase lasting a total of ~ 3 sec. This signal outlasted the duration of a compound action potential, which was about 2 sec. When the stimulation frequency was increased sequentially to 1, 10, and 20 Hz, successive individual responses fused together to form a plateau. $[Ca^{2+}]_i$ oscillations were never observed on the plateau response.

As expected from the signals being generated from axons, the signals were blocked by TTX (see Fig. 9*A* below). Following application of 4-aminopyridine (4-AP), a potassium channel blocker known to prolong action potentials in neonatal optic nerve, a single stimulation triggered a calcium response that was larger in amplitude and longer in duration. The prolongation of the calcium signal is shown in Figure 7*B* by normalizing the response before and after 4-AP application to the same peak value. The axonal $[Ca^{2+}]_i$ signals required the presence of external calcium. When nerves were perfused with a calcium-free solution (0 calcium and with 50 μM EGTA added) for 15 min, compound action potentials still could be elicited but the axonal signals were completely eliminated (see Fig. 9*C* below). This observation suggests that calcium influx accounted for the elevation of axonal calcium seen during nerve stimulation.

Figure 6. The effects of electrical stimulation on the fluorescence signal in P7 optic nerve before (*A*) and immediately following (*B*) the onset of continuous 20 Hz stimulation. The images were taken with a 60 $\times$ oil lens and an optical section of ~ 1.5 μ m thick (FWHM; see Materials and Methods). Each image is the average of 256 frames. *C* shows the absolute intensity profile along a line indicated in *B*, before (*C*, bottom trace) and during (*C*, top trace) stimulation. *D* shows a $(\Delta F/F_0)$ plot of the data in *C*. Temperature was $\sim 37^\circ\text{C}$. Scale bar, 20 μ m for *A* and *B*.

Glial $[\text{Ca}^{2+}]_i$ signals

Having characterized the composite axonal signal, we proceeded to examine in more detail the calcium images viewed at the glial cell bodies during nerve stimulation. During repetitive stimulation, the signal seen at most glial cell bodies rose to a plateau *similar in shape* to that of the composite axon signal (Fig. 7*A*). We therefore cannot resolve whether this was purely an axonal signal viewed in series or if there was a tonic glial component to it. Nevertheless, in about 15–25% of the glial cells, repetitive nerve stimulation elicited a response (spiking) that was so *different in shape* from the composite axonal signal that it can be unambiguously attributed to be glial in origin. In

the following data, the response recorded at glial cell bodies (see Figs. 9, 10) should be interpreted as being composed of a glial plus axonal component. For comparison, the signal from a region just outside of the cell body (the axonal signal) is also plotted to illustrate the dramatic difference between the axonal signal and the spiking signal viewed at the glial cell body.

Delayed multiple glial $[\text{Ca}^{2+}]_i$ spiking. When the nerves were subjected to a prolonged period of repetitive electrical stimulation, the most frequently observed pattern of glial response was multiple glial calcium spikes. Figure 8 shows a series of time-lapsed images, starting from left to right at the top row, at $\sim 37^\circ\text{C}$. Unlike Figure 6, each image was the average of 16 frames so as to obtain a better time resolution of calcium changes.

Figure 7. Video-rate recording of calcium fluorescence from regions between glial cell bodies during electrical nerve stimulation in P7 optic nerves. *A* represents the average of 64 regions from all over the field of view. The images were collected at video rate (one frame every 33 msec). *B* shows the effects of 4-AP on the fluorescence signal. The control and 4-AP responses have been normalized to the same peak value. Temperature was $\sim 37^{\circ}\text{C}$. Bath solutions contained normal calcium.

After collecting a stable baseline for 2 min, the last four control images are shown at ~ 7 sec interval before the nerve was stimulated continuously at 20 Hz starting at the arrow. The next image, taken ~ 7 sec after the onset of stimulation, shows the observation already described in Figure 6; namely, there was an overall increase in fluorescence, both at the cell bodies and in regions between them. As discussed above, this fluorescence increase probably reflects the composite axonal signal that encompassed the entire viewing area. For the next 55 sec, there was little change in the fluorescence signal (images not shown). Then, at 83 sec, a glial cell (indicated by an arrow) was noticed to give spikes. The spiking could be followed in the subsequent images. Note that the nerve was continuously stimulated during the glial spiking. Finally, turning off the stimulation reduced the fluorescence signal to a level close to baseline.

Figure 9*A* shows a plot of this type of glial spiking (top trace) from a different cell at 37°C , together with the composite axon signal (bottom trace) obtained from regions just outside the perimeter of the cell body. In response to a continuous stimulation at 20 Hz, both the axonal signal and the signal collected at the cell body initially underwent a parallel increase to a plateau level. However, at 55 sec after onset of the stimulation, the plateau response viewed at the cell body gave way to spikes.

In contrast, no spiking was observed in the axon signals in the cell's proximity (bottom trace). This, together with the images of Figure 8, allows us to attribute the spiking to glial cells. Figure 9*A* also shows that both the axonal signal and glial spiking were blocked by TTX ($5 \mu\text{M}$), suggesting that glial spiking was triggered by axonal activity; similar blocking effects were observed in 12 cells.

There are two interesting features to this multiple glial spiking. The first, already alluded to, was that spiking started with a delay following the onset of repetitive stimulation (Fig. 9*A*); at 20 Hz, this delay was about 15–60 sec at 37°C . The second feature was that the frequency of glial spiking was related to the frequency of axonal activities. We examined this using the stimulation paradigm shown in Figure 10. The axons were first stimulated for ~ 2 min at 10 Hz, and then at 20 Hz. We characterized 17 cells that showed spiking during the 20 Hz stimulation. For the cell in Figure 10, the first spike was initiated about 1 min after the onset of the 10 Hz stimulation, and four spikes with an average interspike interval of 12 sec were observed before the stimulation was increased stepwise to 20 Hz. Following this increase, glial spikes occurred more frequently, with the interspike interval reducing to 4.7 sec, until they were finally damped out. The composite axon signal from the cell's proximity (bottom trace) shows a stepwise increase without spiking (see also Fig. 7*A*). For the 17 cells tested at 37°C , the glial spiking frequency increased from 0.06 Hz to 0.11 Hz when the axonal stimulation was increased from 10 to 20 Hz in the same cell.

Even though we selected only cells with steady baseline in these stimulation experiments, we did find in a few cases that cells with spontaneous oscillations actually decreased their oscillation frequency when the nerves were stimulated repetitively.

Prompt and transient glial response. Another type of glial response that was observed, but to a lesser degree than the multiple glial spiking, was a prompt and transient response that started as soon as repetitive stimulation began. Figure 9*B* shows an example of this type of response. Following a rest period for baseline, the optic nerve was given a train of continuous stimulation at 20 Hz. Multiple glial spikes, similar to those already described in Figure 9*A*, began after a delay of ~ 70 sec. However, in this cell, a single calcium spike also was triggered at the onset of the stimulation. The composite axonal signal in the proximity of this glial cell body showed no spikes, either near the onset of or during the stimulation. This immediate response, when present, always occurred as a single spike. Further, these prompt responses could be seen in glial cells without subsequent delayed multiple spikes. Collectively, these observations suggested that the glial response triggered at the onset of stimulation was via a different mechanism than that which triggered the delayed, multiple spiking.

Glial spiking in the absence of external calcium. The delayed, multiple glial spiking elicited by continuous axonal stimulation was also observed in the absence of bath calcium. Figure 9*C* shows an experiment in which the optic nerve was first perfused for 15 min with a calcium-free solution (0 calcium added plus $50 \mu\text{M}$ EGTA) before the nerves were stimulated. Nerves still could give compound action potentials in calcium-free solutions. Only the last 2 min of the calcium-free incubation is shown. Application of 20 Hz stimulation ultimately elicited multiple glial spiking, following a delay of ~ 22 sec. Note that in the absence of external calcium, there no longer was an increase in the axonal signal obtained from a region next to the spiking cell body. This was important, since it directly confirmed

Figure 8. Time-lapsed calcium images of optic nerve in response to continuous 20 Hz electrical stimulation. Images were collected with a $40\times$ lens, and each image represents the average of 16 frames. The two vertical arrows indicate the beginning and end of stimulation. The arrow in the 83 sec image shows a spiking cell. Temperature was $\sim 37^{\circ}\text{C}$. P7 rat optic nerve.

that bath-applied calcium-free solutions had adequately achieved a calcium-free environment for the glial cell in question. These observations suggested that glial spiking triggered by axonal activity was due to internal release of calcium. However, we cannot rule out the possibility that calcium spiking is potentiated by calcium flux.

DNQX. One possible explanation for glial spiking is that during nerve stimulation, glutamate was released, activating glial glutamate receptors. In several experiments, glial calcium spikes were still seen with DNQX ($10\ \mu\text{M}$) present in the bath, suggesting that the ionotropic glutamate receptor may not be involved. Work is now in progress with more specific blockers for the metabotropic blockers to see if calcium spiking results from activation of metabotropic glutamate receptors.

Discussion

The key finding of this article is that glial cells along axons can generate intracellular calcium signals in a spike-like fashion, in response both to direct application of ligands like glutamate and ATP, as well as to axonal electrical stimulation. While an activity-dependent axonal-to-glial signaling mechanism mediated

through neuroactive substances has previously been demonstrated in the invertebrate PNS (Evans et al., 1991; Lieberman, 1991), this is the first evidence for a potentially similar process in the CNS of mammals. The present study on white matter, together with a previous study on gray matter (Dani et al., 1992), suggests that both the synaptic and nonsynaptic regions of a neuron can trigger dynamic glial calcium signaling in the mammalian nervous system.

In vivo presence of glutamate receptors in optic nerve glia

Previous studies have already provided evidence for the *in vivo* existence of glutamate receptors in white matter. These include demonstration of the ionotropic receptors in patch-clamp analysis of ionic currents in freshly isolated O-2A progenitor cells from rat optic nerves (Barres et al., 1990) and in glial cells of mouse corpus callosum slices (Berger et al., 1992), visualization of DNQX-sensitive cobalt uptake by O-2A cells (Fulton et al., 1992), and polymerase chain reaction (PCR) analysis of mRNA for ionotropic and metabotropic glutamate receptors in rat optic nerves (Jensen and Chiu, 1993). This report corroborates these findings by demonstrating that calcium signals appropriate for

Figure 9. Glial calcium spiking in response to repetitive electrical nerve stimulation. Responses are all from single cells in P7 optic nerves. In *A–C*, the *top* trace represents the signal collected from the cell body, while the *bottom* trace represents the signal collected from regions just outside the cell body of interest. Temperature was $\sim 37^{\circ}\text{C}$ for *A* and *B*, and $\sim 25^{\circ}\text{C}$ for *C*. The calcium-free solution in *C* was prepared with 50 μM EGTA and no calcium added.

Figure 10. The effects of increasing the frequency of electrical nerve stimulation on glial calcium spiking. Bath solutions contained normal calcium. The response was obtained from a single cell. The *top* trace shows response collected from the cell body, and the *bottom* trace, from a region just outside the cell body. Temperature was $\sim 37^{\circ}\text{C}$.

these two receptor classes can be elicited from optic nerve glia upon application of appropriate agonists. The results of Figure 2, *C* and *D*, suggest that the $[\text{Ca}^{2+}]_i$ spikes were generated largely by internal mobilization, most likely via receptor activation of the inositol triphosphate (IP_3) cascade (Milani et al., 1989). It is interesting that the metabotropic and ionotropic calcium responses in this study were elicited from the same cells (Fig. 3*A*). In comparison, in mixed cultures of type 1 and type 2 astrocytes the ionotropic response was elicited primarily in type 2 astrocytes, whereas the spiking, metabotropic response was observed only in type 1 astrocytes (Jensen and Chiu, 1991). We have not determined the glial cell type underlying the calcium responses. A strong candidate is type 1 astrocytes since these cells express metabotropic glutamate receptors *in vitro* (e.g., see Cornell-Bell et al., 1990a; Jensen and Chiu, 1991). Other candidates include the O-2A glial progenitor cells and the oligodendrocytes but the issue of whether these cells express metabotropic receptors, in culture or *in situ*, has not been addressed.

Axonal calcium signals induced by electrical stimulation

Activity-dependent calcium signals in optic nerves were first demonstrated by Lev-Ram and Grinvald (1987) using a photomultiplier-based detection system. Even though both glial cells and axons were stained in their studies, they have been able to provide argument that a fast component in their calcium signal originates from axons. In this study, we spatially resolved this component in areas between glial cell bodies. These areas, at least in neonatal nerves, should be filled mostly with densely packed, tiny axons with few intermingling glial processes. It is striking that axonal signals for a single compound action potential can be resolved at video rate (Fig. 7). The axonal signal was due to calcium influx, since it disappeared when nerves were stimulated in the absence of bath calcium (Fig. 9*C*). Axonal calcium signals have been suggested to result from voltage-gated calcium channels (Lev-Ram and Grinvald, 1987).

Axon-induced glial spiking: coupling mechanism

Repetitive axonal activity triggered calcium spiking in about 15–25% of the glial cells. How might axonal activity be coupled to glia? The lack of electrical and dye coupling between axons

and glia, together with the fact that the two membranes are separated by a periaxonal space of ~ 200 Å, suggests that coupling must be indirect, perhaps via some sort of mechanical perturbation or diffusible substances released during nerve activity.

Mechanical perturbation. Rat optic nerves exhibit activity-dependent shrinkage of the extracellular space (Ransom et al., 1985). Astrocytes are capable of generating calcium response when mechanically stimulated (Charles et al., 1991). Glial cells also undergo volume changes under physiological conditions. The shrinkage of the extracellular space during nerve activity may deform the glial membrane, triggering calcium spiking. We consider this possibility unlikely for two reasons. First, activity-dependent shrinkage of the extracellular space in rat optic nerves occurs largely in adult, but not in the neonatal nerves (Ransom et al., 1985) on which most of our studies were performed. Second, elevation of external K to 26 mM, which is slightly higher than the level detected during neonatal nerve activity (Connors et al., 1982), did not elicit spiking even though this may produce some glial swelling. Elevation of potassium of this magnitude, however, was insufficient to induce shrinkage of the extracellular space in the Ransom et al. (1985) work.

Potassium. In neonatal nerves, repetitive nerve activity can elevate extracellular potassium by 15–20 mM (Connors et al., 1982), which could depolarize glia (Orkand et al., 1986), activating calcium channels and causing calcium influx. However, bath application of 26 mM K^+ failed to elicit glial spiking. Further, axonal activity can trigger glial spiking in the absence of bath calcium (Fig. 9C), suggesting that glial spiking did not result from calcium influx. However, we cannot rule out the possibility that influx via voltage-gated calcium channels might contribute to the overall glial response.

Glutamate. Glial $[Ca^{2+}]$ spiking during nerve stimulation may reflect activation of metabotropic glutamate receptors by glutamate released during nerve stimulation. Indeed, of all the ligands tested in this study, bath application of glutamate best mimicked activity-dependent, multiple glial spiking. Adult frog sciatic nerves and optic nerves, preloaded with radiolabeled glutamate, were found to release glutamate during repetitive electrical stimulation (Wheeler et al., 1966; Weinreich and Hammerschlag, 1975). Retinal ganglion cells are thought to use glutamate as a neurotransmitter (Ehinger et al., 1988; Marc et al., 1989).

Do neonatal optic nerves release enough glutamate during nerve activity to account for glial spiking? Glutamate release studies have not been carried out in neonatal nerves. However, data from adult fiber tracts allow a rough estimate of activity-dependent elevation in extracellular glutamate concentration (Wheeler et al., 1966). In myelinated bullfrog sciatic nerves preloaded with radiolabeled glutamate for 24 hr (Wheeler et al., 1966), the radiolabeled glutamate was found to be released at a rate of 0.1 pM per second collected outside a nerve trunk having a bulk tissue volume of 0.125 cc. The rate of release was approximately linear during the first 10 min of stimulation at 50 Hz. This translates into a rate of 0.016 pM of glutamate per cubic centimeter of nerve trunk volume per impulse. Since the nerves were artificially loaded with glutamate, this release rate may overestimate the actual release rate of the endogenous glutamate. Assume that the neonatal optic nerves used in this study also released glutamate at the same rate of 0.016 pM per cubic centimeter of nerve trunk volume per impulse. Of this 1 cc, about 0.22 cc was extracellular space, based on morphometric

analysis of a neonatal nonmyelinated optic nerve (see micrograph in Fig. 1 of Waxman et al., 1989). This corresponds to an elevation of glutamate concentration of $\sim 74 \times 10^{-6}$ μ M per nerve impulse (note this is a lower-limit estimate since it ignores glial uptake of the released glutamate). In a typical experiment in which the nerves were stimulated at 20 Hz for 1 min (1200 impulses; Fig. 9A), extracellular glutamate would rise to only ~ 0.1 μ M at this release rate. This argues that glutamate may not be released in sufficient amount to account for axon-induced glial spiking, which was mimicked by bath application of 50–500 μ M glutamate. However, that we did not systemically try lower bath concentrations of glutamate; in other glial preparations from invertebrate PNS like the squid, 10^{-9} M to 10^{-7} M glutamate is sufficient to give large glial electrophysiological responses that have been associated with Ca and IP_3 second messenger systems (Lieberman and Sanzenbacher, 1992). The fact that it takes 50–500 μ M glutamate in the optic nerve is more likely due to diffusion barriers and the fact that glia very actively take up glutamate, converting it to glutamine to inactivate its neuroactive and toxic properties. Another possibility is that activity-dependent glutamate release is much larger in neonatal than in adult optic nerves. For example, if the release is from axons, then the axonal membrane area undergoing excitation and hence capable of participating in release is several orders of magnitude larger before myelin insulation.

Adenosine. In addition to the synapses, purines (adenosine) are also known to be released in fiber tracts during nerve activity (Maire et al., 1982, 1984). In rabbit nonmyelinated nerve fibers, it has been calculated that 1800 impulses can elevate the extracellular concentration of purine-related substances by 138 μ M (Maire et al., 1982). Of these, adenosine amounts to $\sim 3\%$ (~ 4 μ M). We found that the optic nerve exhibits an exquisite sensitivity to bath-applied adenosine, with responses elicited with as low as 0.1 μ M. However, adenosine typically only generates one transient calcium elevation (Fig. 4A), which is in striking contrast to the sharp multiple spike patterns elicited by glutamate. ATP elicited a similar response as adenosine, but on several occasions caused calcium spiking; however, ATP has not been shown to be released in fiber tracts (see Maire et al., 1982, 1984). Release of adenosine from optic nerves might account for the single glial spikes sometimes seen at the start of nerve stimulation (Fig. 9B).

Release mechanism: reverse release of glutamate via glutamate transporters in optic nerve

If glutamate is indeed the major substance coupling axon activity to glial spiking, by what mechanism might glutamate be released? Whatever the mechanism, it is unlikely to be synapse-like since there are no vesicle-like structures in white matter, and since unlike synaptic release that is critically dependent on bath calcium, glial spiking can be induced by axonal activity in the absence of bath calcium.

A nonvesicular, calcium-independent release of glutamate may occur via reverse operation of the glutamate transporter. Recent studies have shown that this transporter is electrogenic and is coupled to Na and K ions. Under normal conditions, this transporter transports glutamate into cells. However, when the ionic gradients are suitably perturbed, the transporter can be driven to release glutamate from cells. Glutamate transporters are present in glia (see review by Nicholls and Attwell, 1990) as well as in some nonmammalian axons like those in squid and crab (Baker and Potashner, 1971, 1973). Even though a direct dem-

onstration of glutamate transporters in optic nerve is lacking, we showed that artificial manipulation of the ionic gradient in a manner known to drive the transporter in reverse (high external K, high internal Na; Fig. 5) resulted in glial spikes similar to those seen in response to direct glutamate application and axonal stimulation. Work in progress has shown that the percentage of spiking cells in Figure 5 was reduced by various inhibitors, including a nonspecific glutamate receptor blocker kynurenic acid (3 mM; Jensen and Chiu, 1990), and a blocker for the high-affinity glutamate transporter dihydrokainic acid (0.5 mM; Fletcher and Johnston, 1991). Collectively, these observations provide some support that glutamate transporters, of the type coupled to Na and K ions, are present in optic nerve *in situ*, and that these transporters can be manipulated to release glutamate to activate glial metabotropic receptors. Note that even though there is evidence for glutamate release through the glutamate transporters (Fig. 5; S. Kriegler and S. Y. Chiu, unpublished observations), we cannot yet ascertain whether the release comes from glia or axons. However, as discussed below, axons are the likely candidates in physiological stimulations.

Small-diameter optic nerve axons have large surface-to-volume ratios that may make them particularly prone to large ionic shifts of the type needed for reverse glutamate release

To date, glutamate release has been shown to occur either in certain pathological conditions or in experimental manipulations of ionic gradients (Nicholls and Attwell, 1990; see also Fig. 5). It is unclear if glutamate release via reversal of the transporter occurs physiologically. Rat optic nerves, particularly in neonatal stages, have certain morphometric features that make these nerves physiologically prone to large ionic shifts of the kind that favors glutamate release.

For example, premyelinated neonatal nerves exhibit prominent activity-dependent potassium accumulation in the extracellular space, a condition needed for release via the transporter. However, potassium elevation, at least to the extent seen during nerve activity (~ 20 mM), is apparently alone not sufficient to trigger release (Fig. 5). Figure 5 suggests that a concurrent elevation of intracellular Na is critical. Small-diameter axons (~ 0.2 μ m), with a large surface-to-volume ratio, are unusually susceptible to Na loading. Prior to myelination, Na influx occurs along the entire axonal surface. A lower limit calculation shows that the passage of a single action potential along a 0.2 μ m axon can elevate intracellular Na by 0.2 mM (influx of 6.25×10^{11} Na ions/cm² to charge an action potential 100 mV in size). Thus, repetitive stimulation can easily load axons with Na to a level that, together with the concurrent K elevation outside and the repetitive depolarizations of the axonal membrane, works in conjunction to favor glutamate release from axons. The delay in glial spiking onset (Fig. 9A) presumably reflects the time for activity-dependent axonal Na loading and extracellular K buildup. The gradual buildup of external K (and axonal Na loading) may also account for the decline of the axonal signal (action potentials are starting to be compromised) seen in some experiments (Fig. 9A).

Glial cells in optic nerve are also candidates for glutamate release. However, the case for them may be less strong than for axons. Large membrane depolarizations (to beyond 0 mV), a condition favoring release, occur in axons but not glia during nerve stimulation. Glutamatergic optic nerve axons may have high levels of intracellular glutamate whereas glia *in situ* may metabolize glutamate to keep the intracellular glutamate con-

centration low. On the other hand, astrocytes in culture can generate a $[glutamate]_i/[glutamate]_o$ of 3000–10,000 and can release glutamate in response to high K and veratridine (see review by Martin, 1992). Glial intracellular Na has been observed to decrease during repetitive neuronal activity (Ballanyi et al., 1987), presumably reflecting K buffering via activation of the glial Na-K-ATPase that transports K in while exports Na out of glia. This fall in intracellular glial Na concentration may inhibit reverse glutamate release from glia.

It should be pointed out that besides glutamate, other transporter types present on the axons may also be so driven to operate in reverse mode, if they are coupled to the Na and/or K ions. For example, two types of adenosine transporters, one coupled to Na gradients and the other operating by Na-independent facilitated diffusion, have been demonstrated in certain cells (Javis, 1987).

Is frequency of stimulation physiological?

Glial spiking can be elicited in P7 nerves by stimulation rates of 10–20 Hz. These rates appear to be within the physiological range. Retinal ganglion cells of the developing retina are known to fire spontaneous action potentials at rates ranging from 10 to 30 Hz in bursts of 4–15 sec (Masland, 1977; Meister et al., 1991). It is possible that glutamate may be released from axons during even shorter stimulation periods than those used here, but that the release is small and only elicits glial responses restricted to the fine processes beyond our present resolution.

Is there a tonic glial calcium response to nerve stimulation?

Even though we have resolved a glial spiking response to electrical nerve stimulations, it is important to emphasize that we cannot rule out the presence of a tonic calcium response in glia. For example, the amplitude of the plateau response seen at the cell body of Figure 9B (top trace) during repetitive stimulation is larger than the amplitude of the axonal signal just outside the cell body (bottom trace). This might indicate a tonic glial component to the signal collected at the cell body since there are less axons per unit volume at the cell body. Resolving a tonic glial response might require injecting calcium dyes into single cells.

Are there receptors on axons?

We cannot completely rule out the intriguing possibility that some of the ligand-induced calcium signals in fact came from axons. For adenosine in particular, bath application gave an increase in calcium fluorescence at cell bodies and in regions between them. Even though in most cases the cell body response was highly localized, in some cases this localized increase at the cell body was not as clear. In some instances, we observed that the adenosine response was similar in the cell bodies and in regions between them. Adenosine receptors are known to be present on axons (Goodman et al., 1983; Ribeiro and Sebastiao, 1987), but whether they are linked to calcium elevation is not clear.

A model for neuron–glial calcium signaling

Figure 11 represents one model for neuron–glial calcium signaling. Neurons can signal glia via glutamate released both at the synapse (Dani et al., 1992) and along the axons (present results). The release from the synapse is vesicular and calcium dependent. Release from axons occurs through reverse operation of a glutamate transporter following activity-dependent al-

Figure 11. A model for glutamate-mediated neuron-glial calcium signaling. In this model, glutamate is released from a neuron both at the synapse via calcium-dependent vesicular mechanisms, and along axons via reverse of a glutamate transporter that is coupled to Na and K transmembrane gradients. The large surface-to-volume ratio of small axons (axonal diameter of $0.2 \mu\text{m}$ in neonatal optic nerves) allows significant changes in Na and K transmembrane gradients during repetitive nerve activity. The released glutamate activates glutamate receptors present on glia encapsulating the synapse (gray matter glia), as well as on glia distributed along the axons (white matter glia). This leads to intracellular calcium changes in glia, which can communicate between cells via gap junctions (Saez et al., 1989). Possible functional consequences of glial calcium changes are discussed in the Discussion.

terations of Na and K gradients across the axonal membrane. Other metabolites, like adenosine, may also be released via a similar mechanism along axons. The released glutamate activates metabotropic glutamate receptors on glia, leading to calcium spiking and calcium waves (Dani et al., 1992). It is interesting that in our studies on P7 nerves, we observed only single-cell spiking but never calcium waves, to either glutamate or axonal stimulation. This is in contrast to the work of Dani et al. (1992) in brain slices, where neuronal activity triggered calcium spiking in individual astrocytes as well as calcium waves that propagated throughout the glial network. One possibility is that astrocytes in neonatal optic nerves may not have developed gap junctions. In the visual cortex, coupling among glial cells was first detected at P11 and thereafter consistently observed in adult (Binmoller and Muller, 1992). Calcium imaging of adult optic nerves may resolve whether calcium waves exist in adult white matter and whether glutamate signaling occurs at the node of Ranvier.

What functions might be served by glial calcium signaling along axons? One is metabolic coupling. The metabolic status of axons can be reported to glia by glutamate released via a transporter that "senses" the changes in Na and K gradients incurred during repetitive activity. The glial metabotropic glutamate receptors translate this axonal message into frequency of calcium oscillations (Fig. 10). This glial calcium signal may drive various responses having metabolic consequences. Glutamate receptor activation in astrocytes can induce the release of other neurotransmitters (Gallo et al., 1991) that can modulate neuronal excitability. In the squid model of axon-Schwann signaling, glutamate released from axons causes an elevation of internal calcium in Schwann cells via an IP_3 second messenger system. This in turn triggers the release of another neurotransmitter, ACh, from Schwann cells (Lieberman and Sanzenbacher, 1992). Recent studies have suggested that glutamate induced the release of a substance from cultured astrocytes that may affect blood vessels (Murphy et al., 1990).

Another possible role for glutamate is development. Glutamate-mediated calcium signaling may regulate glial processes important during early stages of development. During the first week after birth, glial cells (O-2A) in the optic nerve migrate along naked axons from the chiasm to the eyes. In culture, perinatal O-2A progenitor cells migrate at $\sim 21\text{--}100 \mu\text{m/hr}$

(Wolswijk and Noble, 1989). Glutamate has been shown to induce rapid filopodia formation in astrocytes (Cornell-Bell et al., 1990b). Prior to eye opening, retinal ganglion cells may already be firing spontaneous action potentials down the axons. Glutamate released from spontaneously firing axons may cue migrating glia. Glial signaling may be developmentally regulated: glial calcium spiking occurs in response to spontaneously firing, growing axons, and ceases to do so when myelin insulates the axon surface. Metabotropic glutamate receptors could have a developmental role since they may be involved in modulating astrocyte proliferation (Nicoletti et al., 1990). Indeed, there is some hint from PCR analysis that mRNA for the metabotropic glutamate receptor in optic nerve may be downregulated as the nerve matures (see Fig. 7 of Jensen and Chiu, 1993). Finally, glutamate may influence early gene expression in cultured astrocytes (Arenander et al., 1989). Preliminary studies have shown that bath-applied glutamate and repetitive axonal stimulation induced the expression of early genes, like *c-fos*, in glial cells of neonatal optic nerve (Mack et al., 1992). Finally, proliferation of glial cells in neonatal optic nerves is dependent on electrical activity in axons (Barres and Raff, 1993) and it remains to be seen if glutamate-induced calcium signaling plays a role.

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