Neuroglial ATP release through innexin channels controls microglial cell movement to a nerve injury

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Microglia, the immune cells of the central nervous system, are attracted to sites of injury. The injury releases adenosine triphosphate (ATP) into the extracellular space, activating the microglia, but the full mechanism of release is not known. In glial cells, a family of physiologically regulated unpaired gap junction channels called innexons (invertebrates) or pannexons (vertebrates) located in the cell membrane is permeable to ATP. Innexons, but not pannexons, also pair to make gap junctions. Glial calcium waves, triggered by injury or mechanical stimulation, open pannexon/innexon channels and cause the release of ATP. It has been hypothesized that a glial calcium wave that triggers the release of ATP causes rapid microglial migration to distant lesions. In the present study in the leech, in which a single giant glial cell ensheathes each connective, hydrolysis of ATP with 10 U/ml apyrase or block of innexons with 10 µM carbenoxolone (CBX), which decreased injury-induced ATP release, reduced both movement of microglia and their accumulation at lesions. Directed movement and accumulation were restored in CBX by adding ATP, consistent with separate actions of ATP and nitric oxide, which is required for directed movement but does not activate glia. Injection of glia with innexin2 (*Hm*inx2) RNAi inhibited release of carboxyfluorescein dye and microglial migration, whereas injection of innexin1 (*Hm*inx1) RNAi did not when measured 2 days after injection, indicating that glial cells' ATP release through innexons was required for microglial migration after nerve injury. Focal stimulation either mechanically or with ATP generated a calcium wave in the glial cell; injury caused a large, persistent intracellular calcium response. Neither the calcium wave nor the persistent response required ATP or its release. Thus, in the leech, innexin membrane channels releasing ATP from glia are required for migration and accumulation of microglia after nerve injury.

INTRODUCTION

Microglia respond rapidly to central nervous system (CNS) injury by extending processes (Davalos et al., 2005; Nimmerjahn et al., 2005) and migrating to the lesion (Gehrmann et al., 1995). Extracellular ATP, a signaling molecule important to glial physiology and pathology (James and Butt, 2002), is crucial for microglial activation and migration both in vivo and in vitro (Honda et al., 2001; Inoue, 2002; Davalos et al., 2005). The mechanism of microglial movement by purinergic signaling is not completely understood, but there is recent evidence that ATP increases the activity of matrix metalloproteases (Choi et al., 2010). However, migration assays typically do not distinguish between extracellular ATP's activation of movement and its trophic attraction. Recent work on the leech suggests that activation is separate from the signal that directs migration, which in situ appears to be nitric oxide (NO) (Duan et al., 2009) and is produced both directly at the lesion (Kumar et al., 2001) and likely by the glial calcium wave (Li et al., 2003).

The sources of ATP that activate microglia remain elusive. Although injured neurons and glia undoubtedly release ATP pathologically through damaged or compromised membranes, there also appears to be a regulated, physiological release of ATP from damaged cells. Wang et al. (2004) have shown that ATP is released upon mechanical injury to the spinal cord of an anesthetized rat and persists in the extracellular space for several hours, despite the presence of ATPases, indicating that release is prolonged. They also demonstrated that the prolonged release of ATP does not derive from the lesion itself (which actually has reduced ATP release), but from the peri-traumatic region surrounding the lesion from metabolically active, activated cells. Based on what is known about astrocytic ATP release upon mechanical injury and stimulation, the authors hypothesize that astrocytes are responsible for the physiologically regulated release of ATP after injury. This theory is supported by more recent evidence demonstrating that microglia activation after spinal cord injury is not mediated by neuronal activity or plasticity, but is greatly enhanced by ATP application (Chen et al., 2010).

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Abbreviations used in this paper: 6-CF, 6-carboxyfluorescein; CBX, carbenoxolone; CNS, central nervous system; dsRNA, double-stranded RNA; eNOS, endothelial nitric oxide synthase; *Hm*inx, *Hirudo* innexin; IP3, inositol 1,4,5-trisphosphate; KGlu, potassium gluconate Ringer's solution; L-15, Leibowitz-15; NO, nitric oxide.

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Mammalian astrocytes can activate microglia both in vivo and in co-cultures in vitro (Verderio and Matteoli, 2001; Schipke et al., 2002; Bianco et al., 2005b). However, the exact mechanism of this glia–microglia interaction and how it functions in vivo under physiological conditions are still speculative. Astrocytic release of ATP in conjunction with intercellular calcium waves and extracellular ATP is widely accepted as the soluble signal responsible for the propagation of these waves, via its action on P2Y or P2X receptors, or both (Salter and Hicks, 1995; Guthrie et al., 1999; Scemes and Giaume, 2006; Suadicani et al., 2009).

Calcium waves occur in astrocytes and other glial cells (Newman, 2001; Metea and Newman, 2006; Scemes and Giaume, 2006) and perform crucial physiological functions. The release of ATP from glia is now recognized as the primary method by which calcium waves propagate between glial cells (Hassinger et al., 1996; Guthrie et al., 1999; Anderson et al., 2004), although the mechanism of ATP release, either through "hemichannels" (connexons), through pannexin1 channels (pannexons), or through exocytosis, is still controversial (Cotrina et al., 1998a,b, 2000; Guthrie et al., 1999; Lohr and Deitmer, 1999; Stout et al., 2002; Anderson et al., 2004; Suadicani et al., 2004, 2006; Dahl and Locovei, 2006; Bowser and Khakh, 2007). Among the possibilities, Pannexin1 (PANX1, protein; *panx1*, gene), a mammalian homologue of the invertebrate gap junction protein innexin, has emerged as a candidate to release ATP during the calcium wave (Bao et al., 2004; Dahl and Locovei, 2006; Locovei et al., 2006a,b; Scemes and Giaume, 2006; Scemes et al., 2007; Iglesias et al., 2009; Ransford et al., 2009). The PANX1 channel with its large pore (~500 pS) is calcium activated, ATP permeable, and found in vertebrate cells that produce calcium waves, including astrocytes (Bao et al., 2004; Locovei et al., 2006b). Furthermore, the immunoreactivity of PANX1 does not colocalize with gap junction plaques in cultured astrocytes, indicating that PANX1 plays a nonjunctioning role in these cells (Huang et al., 2007; MacVicar and Thompson, 2010). In pathological conditions, PANX1 has been implicated in the activation of the inflammasome (Pelegrin and Surprenant, 2006; Kanneganti et al., 2007; Pelegrin et al., 2008; Silverman et al., 2009) and the P2X7 "death" receptor complex (Locovei et al., 2007; Iglesias et al., 2008).

Mechanically induced calcium waves are a microcosm of what likely occurs during nerve trauma. Mechanical stimulation and trauma can induce calcium waves that propagate many hundreds of micrometers, and the waves are thought to be involved in the generation of glial scars and microglial activation (James and Butt, 2002; Mills et al., 2004; Ostrow and Sachs, 2005; Verkhratsky, 2006). Astrocyte calcium waves trigger responses in microglia (Schipke et al., 2002), most likely through the release of ATP (Verderio and Matteoli, 2001).

Unfortunately, the similar pharmacological profiles of pannexins and connexins, the mammalian family of gap junction proteins, have made it difficult to distinguish the two functionally to determine whether connexins might be involved in ATP release. This is not the case in invertebrates, including the leech, which have only pannexin-like proteins, the innexins, and not connexins. We previously found that *Hirudo* innexin (*Hm*inx)2 present in the leech glia (Dykes and Macagno, 2006) forms ATP-permeable membrane channels (innexons) when expressed in oocytes and is inhibited by carbenoxolone (CBX; ~50% inhibition at 1 μM) (Bao et al., 2007), which inhibits both pannexins and, at higher concentration, connexins (Bruzzone et al., 2005; Barbe et al., 2006), in addition to other actions (Dahl and Locovei, 2006). Moreover, at similar concentrations, CBX inhibits the leak of 6-carboxyfluorescein (6-CF) from leech glia, evidently through innexons. Dye is also retained in the glial cell under conditions of cellular acidification with CO_2, which closes *Hm*inx2 channels in oocytes (Samuels, 2009).

The CNS of the leech *Hirudo*, in which microglia were early described and characterized (del Rio Hortega, 1920), has been valuable for understanding basic mechanisms involved in the migration of microglia to lesions (del Rio Hortega, 1932; Morgese et al., 1983; McGlade-McCulloh et al., 1989; von Bernhardi and Muller, 1995; Babington et al., 2005; Duan et al., 2005; Ngu et al., 2007). Leech microglia are similar to mammalian microglia in their cytology, physiology, histochemical staining (e.g., with silver carbonate and *Griffonia* lectin), and phagocytic ability (Coggeshall and Fawcett, 1964; Morgese et al., 1983). The leech nerve cord consists of a chain of segmental ganglia containing neuronal cell bodies linked by a pair of axon tracts, each ensheathed by a single large glial cell forming the connectives (see schematic in Fig. 2 A). The nucleus of each connective glial cell is midway between ganglia. Although the two connective glia are each several millimeters long, they are structurally and functionally similar to mammalian astrocytes and oligodendrocytes, but do not form myelin (Gray and Guillery, 1963; Coggeshall and Fawcett, 1964; Kuffler and Nicholls, 1966; Deitmer et al., 1999). Just as for glia in other invertebrates (Oland et al., 2008) and glia and Schwann cells in vertebrates (Son et al., 1996; Griffin and Thompson, 2008), glia are important for nervous system development (Stent and Weisblat, 1985; Weisblat, 2007) and possibly regeneration (Babington et al., 2005) in the leech . The connectives contain thousands of microglia (Kai-Kai and Pentreath, 1981; Morgese et al., 1983). We hypothesized that the innexin family of proteins in glial cells is involved both in the release of ATP after an injury and in the subsequent activation of microglia in the medicinal leech. Additionally, ATP can initiate calcium signals in leech glial cells (Rose et al., 1995; Lohr and Deitmer, 1999; Müller et al., 2000), although calcium waves elicited upon injury or

mechanical stimulation have not previously been described in the leech. Because extracellular ATP mediates the propagation of calcium waves between separate glial cells in mammalian cells in culture, we hypothesized that ATP release may mediate glial calcium waves in general. Experiments were designed to determine whether neuroglial innexons are required for microglia migration and for generation and spread of the calcium wave after mechanical stimuli or injury. Although the rapid local response of microglial cell process extension toward small lesions or puffs of ATP and the translocation of cells migrating up to hundreds of micrometers to nerve lesions might be controlled by similar mechanisms, the focus of this study was on mechanisms controlling cell migration to lesions.

MATERIALS AND METHODS

Leeches and drugs

Adult leeches (*Hirudo sp.*; Leeches U.S.A. LTD.), 3–4 g, were maintained in artificial pond water (0.5 g/liter H_2O; Forty Fathoms; Marine Enterprises International) at 15°C. Their nerve cords were dissected and connectives pinned in 35-mm Petri dishes (BD) containing 1 ml of cured silicone rubber (Sylgard 184; Dow Corning). For dissections and experiments, nerve cords were kept in physiological saline (leech Ringer's solution; Kuffler and Potter, 1964). Nerve cords can be maintained for several days when incubated in Leibowitz-15 (L-15) culture medium supplemented with 2% fetal bovine serum, 1% glutamine, 0.06% glucose, and 0.0005% gentamicin. For the experiments, it was considered unimportant whether the piece of connective contained the glial nucleus (Elliot and Muller, 1981). Drugs were administered by bath application at their final concentrations. CBX and apyrase were dissolved in water. All drugs were purchased from Sigma-Aldrich.

Microglia accumulation studies

Nerve cords were prepared as described above. Connectives were crushed for 8–12 s with fine forceps (#5; Dumont) and incubated in leech Ringer's solution at room temperature for 4 h with or without drugs. The tissue was then fixed overnight at 4°C in PBS with 4% paraformaldehyde, pH 7.2, washed, and mounted on a microscope slide in 80% glycerol with dye (10 µg/ml; Hoechst 33258; Sigma-Aldrich) to stain nuclei. In the leech nerve cord, the only nuclei in the connectives are those of the microglia, which are dispersed among axons, and the cells of the sheath, which surround the connectives, in addition to the nucleus of the single glial cell associated with each connective (Coggeshall and Fawcett, 1964; Morgese et al., 1983). The two glial nuclei are distinctively larger and fainter when stained; all the neuronal nuclei are within the ganglia. Microglia nuclei were imaged with a fluorescence microscope (20× objective, NA 0.7; DM RZA2; Leica) using an appropriate UV filter set. A series of 10 optical sections was photographed at 2-µm intervals and a combined depth of 18 µm. An image of the connectives was also made with blue illumination and a fluorescein filter set to identify the location of the crush, which had less autofluorescence at fluorescein emission wavelengths (green); this was done to determine where to count nuclei without bias, as they were not visible with the fluorescein filter set. Each stack of images showing cells including microglia at the crush was combined and collapsed using the Metamorph program (MDS Analytical Technologies); the nuclei were counted in a 10,000-μm^2 region at the edge of each crush (see Fig. 2 A). Because some of the nuclei were those of sheath cells, which do not move (Morgese et al., 1983; McGlade-McCulloh et al., 1989), and the balance were microglial nuclei, the number of cells includes both types, although all the increases are in microglia. An accurate estimate of the number of microglial cells accumulating would be the difference between the crush count and "distal" count, where distal count measures the number of cells in a region of the connective at a distance of more than 1 mm from the crush. The counts were statistically analyzed with an ANOVA and post-hoc Fischer Test (Statistica; StatSoft).

Low light video microscopy

Dissected nerve cords were incubated for 30 min in Hoechst 33258 dye (0.001%), rinsed three times in leech Ringer's solution for 5 min, and kept overnight in L-15 culture medium with supplements (Ready and Nicholls, 1979) at 15°C to ensure that nuclei were stained and to lower the background. The tissue was then imaged on an epifluorescence microscope (40× water immersion objective, NA 0.75; WL; Carl Zeiss, Inc.) using a 12-V, 100-W tungsten halogen lamp operated at 8–10 V and UV filter set. For time-lapse studies, Metamorph imaging software controlled a shutter and acquired images from a CCD camera (XC-77; Hamamatsu Photonics), averaging 256 frames at video rates every 2.5 min for up to 4 h. Before photography, the connectives were crushed and drugs were added. Controls were uncrushed connectives and crushed connectives without drug treatment, depending on the specific experimental protocol. Microglia movement during a time-lapse series was measured by double-blind assistants (undergraduate students ignorant of the experimental aims and conditions) who tracked individual microglia nuclei or counted the number of nuclei that moved more than 30 or 50 µm, noting direction of movement. The counts were statistically analyzed by ANOVA and post-hoc Fischer Test using Statistica.

Measurement of extracellular ATP

Connectives cut from dissected nerve cords were incubated in L-15 overnight. They were then rinsed, crushed, and incubated in 150 µl Ringer's solution for a timed interval ranging from 15 to 120 min, and 100 µl of the supernatant was collected for ATP analysis. A 50-µl sample of the premixed luciferin/luciferase solution (Enlighten ATP Assay System; Promega) was added to each 100 µl of supernatant. Luminescence measurements were acquired using a multi-label counter (Victor 1420; PerkinElmer). Results were statistically analyzed with ANOVA and post-hoc Fischer test (Statistica). Standard concentrations of ATP were used to calibrate the assay and determine the concentration of ATP in the tissue. Although the luminescence measurements were consistently of the same order of magnitude, the sensitivity of the assay only allowed the reporting of a range of ATP concentrations. In the 150 µl of supernatant surrounding the crushed tissue, ATP concentrations were between 1 and 10 nM. Because the tissue volume of ~0.1 µl ($100 \times 100 \times 10{,}000$ μm^3) represented a small fraction of 150 µl, and the extracellular space a small fraction of that, ATP concentrations in the extracellular space in tissue were in the upper micromolar to low millimolar range.

mRNA-injected oocyte recordings

Solutions. Oocyte Ringer's solution (OR2) was composed of 82.5 mM NaCl, 2.5 mM KCl, 1.0 mM $MgCl_2$, 1.0 mM $CaCl_2$, 1.0 mM Na_2HPO_4, 5.0 mM HEPES, and antibiotics (10,000 U/ml penicillin and 10 mg/ml streptomycin), pH 7.5. The patch pipette solution (potassium gluconate Ringer's solution [KGlu]) was 140 mM potassium gluconate, 10 mM KCl, and 5.0 mM TES, pH 7.5.

mRNA-injected oocytes. *Xenopus laevis* oocytes were isolated by incubating small pieces of ovary in 2 mg ml^{-1} collagenase in calcium-free OR2 and stirring at one turn/second for 3 h at room

temperature. After being thoroughly washed with OR2 (with calcium), oocytes devoid of follicle cells and having uniform pigmentation were selected and stored in OR2 at 18°C. *Hm*inx2 and *Hm*inx3 had been cloned into the expression vector pCR-BluntII-TOPO. mRNA was transcribed by SP6 (*Hm*inx2) or T7 (*Hm*inx3) RNA polymerase from 10 µg XbaI- (*Hm*inx2) or SpeI- (*Hm*inx3) linearized plasmid using the mMessage mMachine kit (Applied Biosystems). mRNA was quantified by absorbance (260 nm), and the proportion of full-length transcripts was checked by agarose gel electrophoresis. 20 nL mRNA (50 ng/µl\e) was injected into oocytes. The injected oocytes were then transferred into fresh OR2 medium with elevated Ca^{2+} (5 mM) and incubated at 18°C for 24–48 h. For electrophysiological recordings, oocytes were transferred to regular OR2.

Voltage clamp. Voltage clamp recording was performed with two intracellular microelectrodes, one to pass current and the other to record voltage. The oocyte membrane potential was clamped at a given voltage from which it was depolarized with square 15-mV pulses of 5-s duration at 0.1 Hz, the amplitude depending on the experiment. Changes in current required for the voltage pulses indicated the conductance. Conductance of the pannexin/innexin channels was determined by comparison with control oocytes; in practice, most of the conductance was due to pannexons/innexons, as confirmed in later experiments in which CO_2 closed the channels.

RNAi constructs and glial injections

*Hm*inx1 and *Hm*inx2 cDNA constructs used for the RNA interference experiments (RNAi) were provided by E. Macagno and M. Baker (University of California, San Diego, La Jolla, CA), synthesized as described previously (Kennerdell and Carthew, 1998; Baker and Macagno, 2000), and the cDNA was sequenced to confirm their integrity. In brief, the templates for RNA synthesis were linearized plasmids, cut with xhoI and avrII, that contained the full-length sequence of either *Hm*inx1 or *Hm*inx2. Linearized DNA was confirmed by agarose gel electrophoresis with ethidium bromide to reveal bands. T7 promoters were inserted at the 5′ end of each strand so that double-stranded RNA (dsRNA) could be synthesized and annealed in one reaction. Concentration was determined by spectrophotometry at 260 nm as ultraviolet absorbance. Final concentrations of dsRNA were ~25–50 ng/µl.

The dsRNA for the RNAi experiments was delivered into the glial cells via pressure injection from a standard omega-dot borosilicate micropipette at 10–20 psi into a cut end of a connective that had been dissected the previous day and stored in supplemented L-15 overnight to permit the end to reseal. Nerve cords were dissected so as to position the connective glia nucleus, located in the middle of the connective near the cut end, to be injected. The dsRNA was mixed 1:1 with 4% Fast Green FCF (Thermo Fisher Scientific) in dilute KCl to see the delivery of the dsRNA solution into the neuroglial cell and record from it. The microelectrode tips were broken to a size of 0.5 µm to allow the dsRNA to pass. Standard negative resting potentials were recorded before beginning the injections. All connectives were then kept in supplemented L-15 for 2 d to allow for the dsRNA to be processed to RNAi to knock down expression of the protein before either the dye loss or accumulation experiments were performed. For the dye loss studies, one of the glial cells in the connective was injected with dsRNA *Hm*inx1 and the other with dsRNA *Hm*inx2 to be able to compare their effects on dye loss in one preparation. For accumulation studies, both glial cells in the same preparation were injected with the same dsRNA, for either innexin1 or innexin2, and comparisons were made between the different sets of connectives from the same animal, with image collection and data analysis performed as described above in Microglia accumulation studies.

In vivo dye release

A 2-mm piece of connective was dissected a day before injection, pressure injected with ds*Hm*inx1 RNA into one connective glial cell of the pair and with ds*Hm*inx2 RNA into the other glial cell, both at one end of the connectives, and incubated in L-15 culture medium for 2 d. 6-CF dye was then iontophoretically injected into both glial cells at the other end of the piece, and the dye was allowed to diffuse throughout the entire connective for 1.5–2 h. The tissue was pinned under slight tension. Micropipettes for injection were pulled from standard omega-dot borosilicate glass and filled with 0.1 M 6-CF (376 D; Invitrogen) via capillary action; the back end of the pipette was filled with 4 M potassium acetate or 2 M LiCl to provide electrical contact with the dye. A voltage shift of −40 to −60 mV was observed when the microelectrode penetrated the connective glial cell, and the resting potential became more negative as the membrane resealed. Dye was injected into the connective glial cells using a −2-nA current for 3–5 min. The glial cells were imaged with an epifluorescence microscope (40× water immersion objective, NA 0.75; WL; Carl Zeiss, Inc.) with a 12-V, 100-W tungsten halogen lamp operated at 8–10 V and fluorescein filter set. Metamorph imaging software controlled a shutter, and acquisition of time-lapse images from a CCD camera (XC-77; Hamamatsu Photonics) operated at video rates to collect and average 256 frames, opening the shutter for several seconds once every minute.

Changes in fluorescence intensity with time were used as a measure of dye loss from the connective glial cell, particularly for comparison between tissues with and without injection with *Hm*inx2 RNAi. The fluorescence intensities of entire injected connectives were measured and normalized using Metamorph (MDS Analytical Technologies).

Calcium imaging

Leech nerve cords (with at least 3 mm of connective on each piece) were dissected, pinned in 35-mm Petri dishes containing 1 ml of cured silicone rubber (Sylgard 184; Dow Corning) and incubated in supplemented L-15 medium (Ready and Nicholls, 1979) overnight, or for an additional 2 d for experiments involving injection of RNAi. Calcium Green 1 or Oregon Green Bapta 2 (Invitrogen) was then iontophoretically injected (−5 nA for 10 min) into the cut end of the glial cell and opposite the end injected with RNAi. The dye was allowed to diffuse within the glial cell for 1 h before the experiments began.

The glial cells were imaged on an epifluorescence microscope (40× water immersion objective, NA 0.75; WL; Carl Zeiss, Inc.) with a 12-V, 100-W tungsten halogen lamp operated at 8–10 V and a fluorescein filter set. Metamorph imaging software controlled a shutter, and the acquisition of time-lapse images was from a CCD camera (XC-77; Hamamatsu Photonics), averaging 16 frames stored at 1-s intervals.

To evoke ATP-induced calcium waves, an electronic picospritzer (Picospritzer II; General Valve Corporation) was adjusted to deliver puffs of 3 mM ATP, pH 7.4, directly onto the connective through a micropipette (20 psi for 0.1 s). The concentration of ATP used was not damaging, as even nerve cords bathed in 3 mM ATP were not measurably harmed, and the bath concentration of ATP was negligible even after several experiments.

A sharp tungsten probe (Hubel, 1957) driven by a piezoelectric device was used to elicit reproducible, mechanically induced calcium waves in the glial cell. With the probe placed flush against the tissue, a 200-ms, 30–100-V pulse was applied to the piezoelectric device to cause a displacement of the probe and the tissue. The amplitude varied with the experiment and the sensitivity of the tissue. The calcium response was seen immediately upon stimulation.

Measurements of the calcium wave intensity and propagation were made using the Metamorph region analysis tool. Quantification of the calcium change was made by measuring the intensities of 5 × 5–μm^2 regions separated by 20 µm along the glial cell,

beginning adjacent to the probe and up to 100 µm distant from it (see Fig. 7 A). Cells were stimulated mechanically at 15 s after beginning the time-lapse recording. The first 10 images were averaged to provide a resting fluorescence, F, and then the change in fluorescence from that baseline, ΔF, was used to calculate a ratio ΔF/F for every region and time point. The maximum intensity of the calcium response, or peak value, was determined to be the average of the five consecutive points in any region with the largest average ΔF/F measurement. The time to half peak at various distances from the initiation site was used to calculate the velocity of the calcium wave. ANOVA (Statistica) was used for statistical analysis of both maximum intensities and times to half peak.

Online supplemental material

A video showing all the frames used for Fig. 5, including the two in Fig. 5 A, is included the supplemental material. Graphs showing the spread of the ATP-induced calcium waves in the connective glial cell are presented in Fig. S1, in which puffs of 3 mM ATP were applied to the connective. The results show that leech glia have ATP-induced calcium waves that are similar to those found in mammalian glia. It is shown in Fig. S2 that 20 U/ml apyrase had no measureable effect on the speed or amplitude of mechanically induced calcium waves in the connective glia, consistent with evidence that extracellular ATP was not required for the usual propagation of the waves. The online supplemental material is available at http://www.jgp.org/cgi/content/full/jgp.201010476/DC1.

RESULTS

ATP release after nerve injury

Leech microglia can be activated by ATP. The activation apparently involves P2Y receptors, as P2Y receptor antagonists including Reactive Blue 2 can block microglia activation and accumulation at lesions (Duan et al., 2009). To confirm the release of ATP after a crush injury, a luciferin/luciferase ATP assay was used to measure extracellular ATP

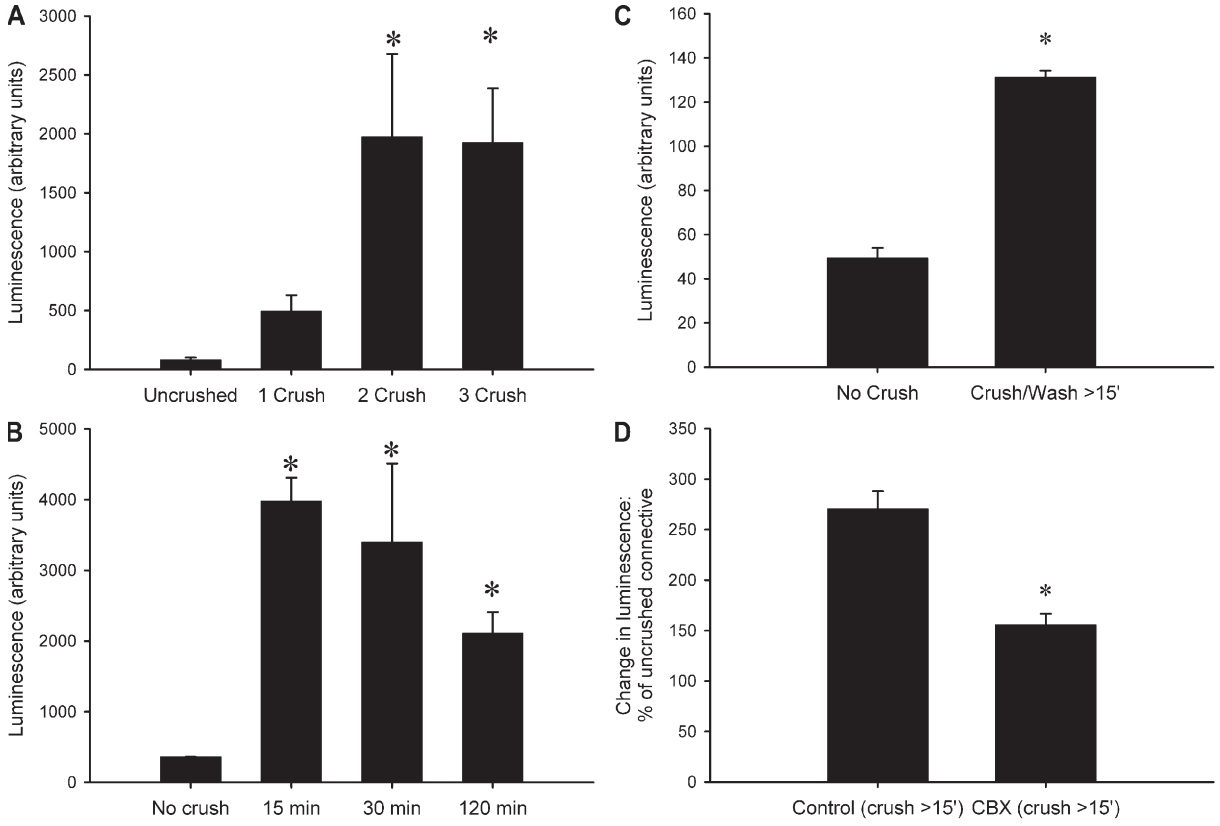

Figure 1. Measurements of extracellular ATP after nerve cord injury. (A) An ATP luminescence assay (luciferin/luciferase) was used to measure the increase in extracellular ATP after nerve cord crush. Connectives were dissected and kept overnight in supplemented L-15. No (uncrushed control), one, two, or three crushes were made to the connectives that were then incubated at 18°C for 30 min in 150 µl of leech Ringer's solution. 100 µl of the supernatant was analyzed for its ATP content. Connectives with two and three crushes showed statistically significant increases in extracellular ATP compared with the uncrushed control (*, $P < 0.05$; $n = 3$). (B) Extracellular ATP was elevated for at least 2 h after injury. Samples (100 µl) of supernatant of two connectives each crushed twice (total volume 150 µl) were collected at 15, 30, and 120 min after making the crushes, and samples were analyzed using a luciferin/luciferase luminescence ATP assay. Significant elevation of extracellular ATP remained for at least 2 h after injury (*, $P < 0.05$; $n = 3$). (C and D) Release of ATP >15 min after injury and the effect of 10 µM CBX. Connectives were crushed twice, incubated for 15 min at room temperature, washed three times with Ringer's solution, resubmerged in 150 µl of fresh Ringer's solution, and incubated at room temperature for 30 min. 100-µl aliquots of supernatant were analyzed with luciferin/luciferase luminescence assay to measure levels of extracellular ATP. In C, ATP was elevated 15 min after the injury ($P < 0.05$; $n = 3$). In D, in another experiment, the continued release of ATP was attenuated by treatment with 10 µM CBX. Control and CBX conditions are displayed as percentages of values for uncrushed tissues to control for the direct effect of CBX on the luciferase assay, for CBX may artificially increase the absolute luminescence values slightly ($P < 0.05$; $n = 3$).

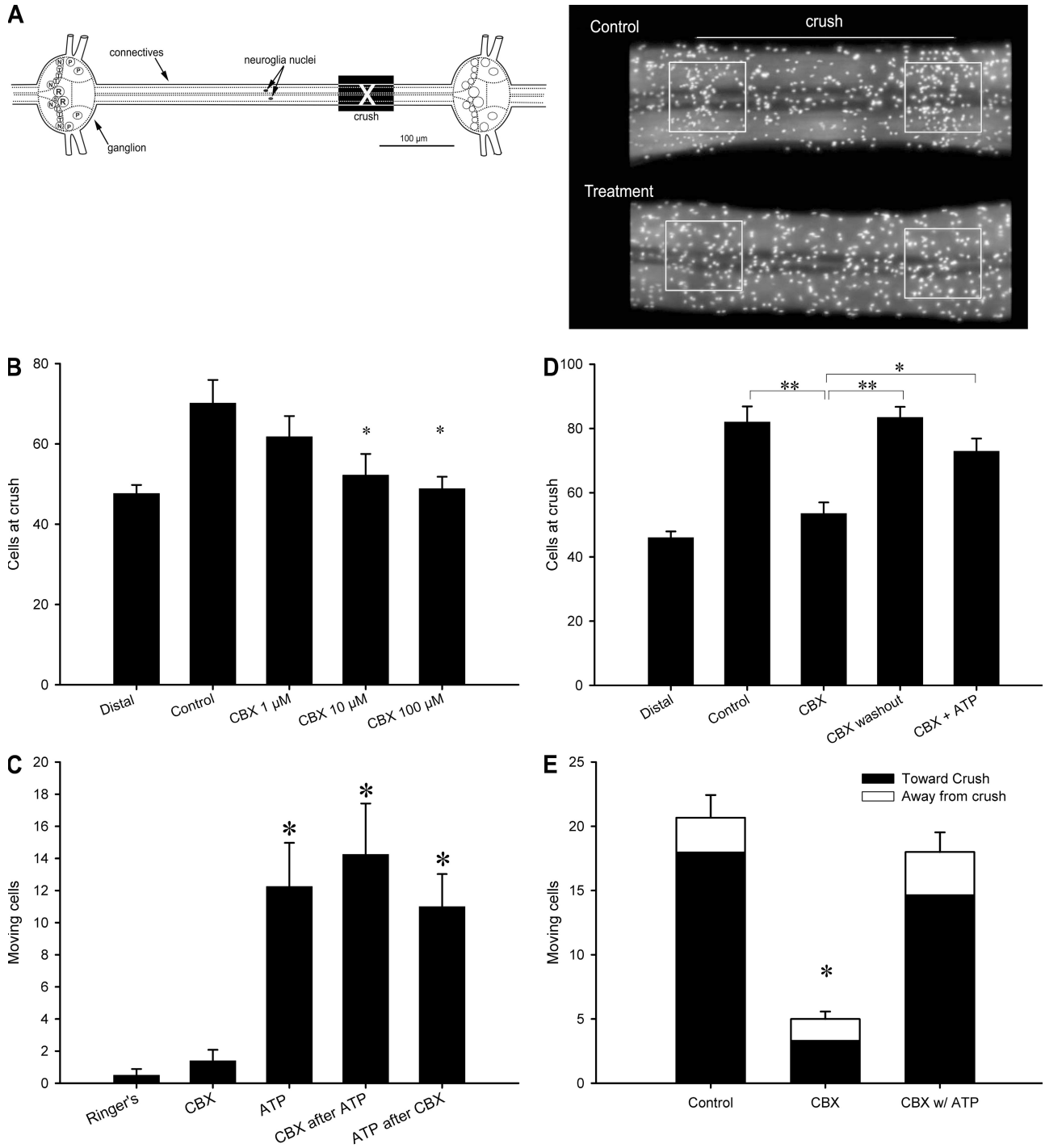

Figure 2. Effects of CBX and ATP on microglia migration and accumulation. (A) Schematic diagram of the leech nerve cord and, for the region depicted in the rectangle, micrographs of crushed connectives to show how cell accumulations are measured. Tissue was stained with Hoechst 33258 dye to show nuclei. The top and bottom micrographs are representative collapsed images (10 each through 18 µm) with and without drug treatment. The crushes are indicated, with squares (100 × 100 µm) outlining regions of measurement. Nuclei are mostly microglia but include some sheath cells. "Treatment" tissue in the micrograph was incubated in 10 µM CBX. (B) Cell accumulation at the site of a crush injury in CBX. Tissues were treated with CBX at 1-, 10-, and 100-µM concentrations at the time of crush, and the microglia were allowed to accumulate for 4 h. Tissues were then fixed and stained. Bars represent mean number of cells at the site of injury in a 100 × 100 × 18–µm^3 volume. "Distal" is a measure of the number of cells in an uncrushed region, ~1 mm from any injury, and is used as a baseline for the number of cells distributed randomly throughout the tissue. The difference between the "control" or other conditions and the distal is the true accumulation. Mean ± SEM are represented ($P < 0.001$; $n = 6$). (C) Microglia moving in 30 µM CBX and 100 µM ATP. Microglia nuclei were tracked using time-lapse recording, and those moving >30 µm were counted. CBX did not inhibit movement in ATP, although it did inhibit movement induced by crushing the connectives. Bars represent average number of

in the bathing solution for leech connectives in both crushed and noncrushed conditions. ATP release after two or three crushes rose to 10 times that of uncrushed controls, a significant rise (Fig. 1 A). In the 150 µl of supernatant surrounding the tissue, ATP concentrations were between 1 and 10 nM after a crush. Because the tissue volume was ~0.1 µl and the extracellular space ~5–10% of that (Nicholls and Wolfe, 1967), the extracellular ATP concentrations in the extracellular space were in the range of 0.5 to 5 mM. The measurements did not distinguish between ATP released directly through damaged membranes and that passing through innexons, nor did they distinguish ATP originating from glia or from other cells.

Microglia continue migrating to crushes for hours after the injury (McGlade-McCulloh et al., 1989; Masuda-Nakagawa et al., 1990; Ngu et al., 2007). To determine the time course of elevated ATP levels after nerve cord damage, ATP was measured at 15, 30, and 120 min from two pairs of connectives that were each crushed twice for a total of four nerve crushes (Fig. 1 B). The extracellular ATP concentration declined over time, but even 120 min after the crush was made, the ATP concentration remained significantly higher than uncrushed controls. There was no statistically significant difference in ATP concentrations between 30 and 120 min after the crush. Because ATP is hydrolyzed by extracellular ATPases, it is unlikely that such high concentrations would remain in the supernatant without continued release of ATP from the injured cells, particularly at a distance from the lesion. To distinguish between the ATP released immediately, possibly through damaged membranes, and extended release, the tissue was washed three times at 15 min after injury, and 30 min later the supernatant was removed and ATP was measured. Extracellular ATP remained elevated compared with uncrushed controls (Fig. 1 C). This continued release of ATP diminished when the tissue was treated with 10 µM CBX, implicating innexon channels in the extended release of ATP after an injury (Fig. 1 D).

Microglia migration and effect of CBX

Because the innexin inhibitor CBX reduced the continued release of ATP from the connectives after a crush injury, it was of interest to determine whether CBX could also inhibit the accumulation of microglia at the lesion. In the presence of 1, 10, and 100 µM CBX, connectives were crushed and the accumulation of cells were measured (Fig. 2, A and B). As discussed in Materials and methods, the nuclei accumulating after the nerve cord was crushed were all those of microglia. CBX prevented accumulation in a dose-dependent manner, with statistically significant reduction at 10 µM and greater. Concentrations of 10–30 µM CBX were therefore used in subsequent experiments.

To determine whether CBX might interfere directly with ATP activation of microglia rather than with release of ATP, a time-lapse study was performed with exogenous ATP, which activates microglia and causes them to move (Duan et al., 2009). Separate nerve cords were treated with 30 µM CBX either before or after the addition of 100 µM ATP (Fig. 2 C). ATP-induced microglial movement was not diminished by CBX.

If CBX prevents the release of ATP required for microglial movement, but the direction of movement is controlled by NO produced after the crush is made (Duan et al., 2009), the addition of ATP to the bath containing the crushed nerve cord treated with CBX might be expected to reverse the effect of CBX, enabling microglia to move to and accumulate at the lesion. To test this hypothesis, 10 µM CBX was added to the Ringer's bath 15 min before the nerve cord was crushed, which alone reduced accumulation, and 100 µM ATP was added to the bath. The result, shown in Fig. 2 D, indicates that the usual accumulation was restored to control levels with CBX and ATP together, and it was significantly greater than in 10 µM CBX, which was not significantly different from the distal, or basal, level of cells. The CBX treatment was reversible because in experiments in which the connectives were treated with 10 µM CBX for 4 h, washed three times in 15 min, and crushed, the number of microglia accumulating at the crush was elevated and not significantly different from positive controls that were crushed without drug treatment (Fig. 2 D). Experiments were next done to determine whether, if microglia moved little after a crush in CBX, the usual movement returned after the addition of 100 µM ATP (Fig. 2 E). Indeed, the number of cells moving in CBX was significantly reduced, but it was restored to control levels in CBX with ATP. The directional preference toward the crush was not changed. Thus, CBX inhibited the microglial response to the injury by blocking the release of ATP from the damaged tissue without affecting the ability of microglia to respond to

microglia moving in 30 min ± SEM ($P < 0.01$; $n = 5$). (D) Microglia accumulation in CBX and ATP. 10 µM CBX significantly reduced the accumulation of cells at the crush. In the CBX washout condition, the connectives were incubated in CBX for 4 h and washed for 1 h before crushing. "Distal" is a measure of cell nuclei in an uncrushed region. The reduction seen in CBX did not occur after CBX was washed from the tissue, showing that the effect of CBX was reversible. It was also eliminated in the simultaneous presence of 100 µM ATP, consistent with an effect of CBX to block release of ATP, but not the direction of movement ($n = 3$; *, $P < 0.5$; **, $P < 0.01$). (E) Microglial migration toward a crush and the effects of 10 µM CBX and 100 µM ATP. In the ~400-µm-long region adjacent to the crush, the number of microglia nuclei moving >50 µm in 2 h was determined from time-lapse images. Drugs were added 5 min before crushing. The CBX with ATP condition was similar to the control condition in both total movement and directional movement, but CBX alone decreased the movement significantly ($P < 0.05$; $n = 3$).

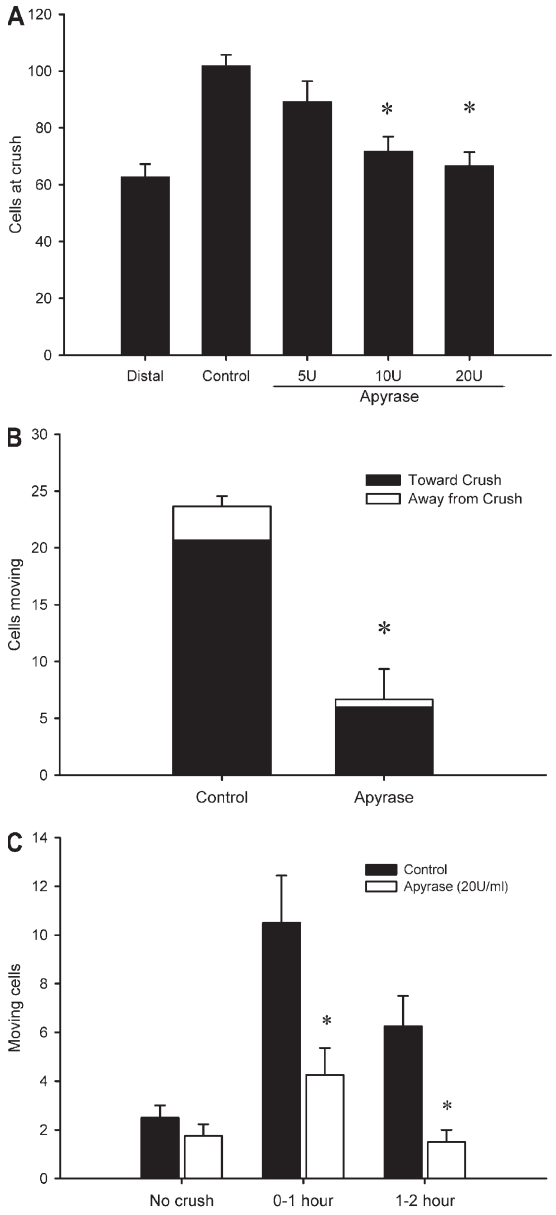

Figure 3. The effect of apyrase on microglia migration and accumulation. (A) Tissues were treated with apyrase at 5, 10, and 20 U/ml beginning at the time of crush, and after 4 h, the tissue was fixed, stained with Hoechst 33258 dye, and photographed. "Distal" was a measure of microglia in an uncrushed region, ~1 mm from any injury, and was used as a baseline for the number of cells distributed throughout the tissue before injury. The difference between the "control" and the distal is the measure of microglia accumulation, as sheath cells do not move. Bars represent mean number of cells at the site of injury in a 100 × 100 × 18–μm^3 volume ($P < 0.05$; $n = 3$). (B) Hydrolysis of ATP and microglial migration toward a crush. In the ~400-μm-long region adjacent to the crush, the number of microglia nuclei moving >50 μm in 2 h was determined from time-lapse images. The connectives treated with apyrase to hydrolyze ATP had approximately one fourth the number of microglia moving relative to the control; the fraction of those moving that moved toward the crush (i.e., the directional movement) was approximately the same as the control ($P < 0.05$; $n = 3$). (C) Diffusion of extracellular ATP released after a crush caused microglia to move in an adjacent tissue. Two sets of connectives were dissected, stained with Hoechst 33258 dye, and incubated in L-15 culture medium overnight. The next day, one set was imaged with time-lapse microscopy (every 2.5 min), whereas the other set was crushed and placed parallel to it, 40–50 μm away. The microglia moved in the intact connectives, which would not have occurred in isolation. This movement was blocked in 20 U/ml apyrase, indicating that extracellular ATP released from the crush was required to cause microglial movement. Movement was measured as translocation of microglial cell nuclei by >30 μm in 1 h ($P < 0.05$; $n = 3$).

ATP or influencing the signal that directs microglia to the lesion, which is considered to be NO.

Microglia migration and apyrase

Activation of purinergic receptors by extracellular ATP and possibly ADP has been shown to be a step in activating microglia, leading to their accumulation at lesions, and the data above showed that ATP was released by nerve injury. To test further the contribution of extracellular ATP for accumulation of microglia at a lesion, extracellular ATP and ADP were depleted by the addition of apyrase, an ecto-ATPase that hydrolyzes ATP into ADP and then AMP. Leech connectives were treated with apyrase at 5, 10, and 20 U/ml for 4 h beginning 10 min before the tissue was crushed. By definition, 10 U/ml apyrase hydrolyzes 10 mM ATP in the same volume in a minute at 30°C, but it is likely that little enzyme enters the connectives. Apyrase reduced the number of microglia found at the crushes in a concentration-dependent manner (Fig. 3 A). The accumulation levels were significantly lower than controls for 10 and 20 U/ml of apyrase. Time-lapse studies of crushed connectives confirmed that there were fewer microglia migrating to the injury when the tissue was treated with 20 U/ml apyrase compared with controls (Fig. 3 B). There was no effect of apyrase on the direction of movement, but apyrase decreased the amount of movement of microglia. This was consistent with earlier work indicating that NO rather than ATP affects the direction of movement of microglia, but that ATP is required for movement itself (Duan et al., 2009).

This experiment showed that extracellular ATP released after nerve crush was necessary for movement of the cells, but it did not rule out the possibility that an additional signal within the connectives, possibly even a mechanical one, was also required for simple movement. To determine more precisely the involvement of diffusible ATP released from the connective, an unharmed connective was placed in close proximity to an injured connective; the two connectives were pinned next to one another. Time-lapse microscopy of one pair of connectives was performed before and after its neighbors were crushed and showed that crushing one pair of connectives induced movement in an adjacent nerve cord (Fig. 3 C). To determine if extracellular ATP was a

crucial signal inducing movement, 20 U/ml apyrase was added before crushing. Apyrase inhibited the increased movement, supporting the idea that extracellular ATP released by nerve injury activates microglia without mechanical stress directly on the microglia. Although ATP may not be the only signaling molecule released by the connective after an injury, we conclude that its presence is necessary for the microglial response.

Innexon channels opened without injury and microglia movement

To understand the relationship between microglia movement and ATP release by innexon channel opening, such as occurs after injury, experiments were designed to open innexons without tissue damage. In oocytes, isotonic potassium gluconate saline opens leech *Hm*inx2 channels and releases ATP (Bao et al., 2007). This treatment opened innexon channels even when the cell's membrane was clamped to negative resting potentials, and CBX closed the channels opened by potassium gluconate (Fig. 4 A). Therefore, uninjured connectives were bathed in 120 mM of an isotonic KGlu, and the movement of microglia was measured from time-lapse photographs (Fig. 4 B). The microglia moved more than in control conditions, and the increased microglial movement did not occur in 10 µM CBX. This indicated that opening innexin channels in the leech CNS, which release ATP, was sufficient to cause microglial movement.

Effect of *Hm*inx RNAi in the connective glial cell on dye loss and microglia accumulation

Although the previous studies have supported the idea that ATP released through innexons is essential for the usual microglia migration and accumulation at lesions, they have not clearly identified the neuroglial cell ensheathing the axons and microglia of the connective as the source of the ATP and the location of critical innexon channels. To determine whether innexons in the connective glial cell were required for the microglial migration, dsRNA to *Hm*inx2, a glial innexin, and *Hm*inx1, a neuronal innexin used as a control, were injected into separate connective glial cells, and both dye loss and microglial accumulation were measured (refer to Materials and methods).

Loss of intracellularly injected dye is a sensitive assay for demonstrating the presence of functional innexon channels in leech glia in vivo, as dye loss is reliably and reversibly blocked by CBX (Bao et al., 2007) and CO_2 (Samuels, 2009). Segments of paired connectives including glial nuclei were isolated, and a day later glial cells were injected with *Hm*inx2 RNAi or *Hm*inx1 RNAi as a control, as described in Materials and methods. After an additional 2 d, the two glial cells were injected with similar amounts of 6-CF dye. Intracellular recordings confirmed cell viability, and dye loss from the *Hm*inx2 RNAi–injected cell was significantly less than its counterpart. A representative example of the dye loss seen during this experiment is shown in Fig. 5. This demonstrated that, at least functionally, the RNAi to *Hm*inx2 blocked the innexon channels responsible for dye loss from the connective neuroglial cell, presumably by knocking down expression of *Hm*inx2 in the cell.

Injection of paired glial cells with *Hm*inx2 RNAi blocked microglial accumulation at crushes compared with that for *Hm*inx1 RNAi–injected cells (Fig. 6). Thus, neuroglial cell innexons, presumably containing *Hm*inx2, that release ATP into the vicinity of microglia hundreds of micrometers from the crush were crucial for microglia migration and accumulation at the lesion.

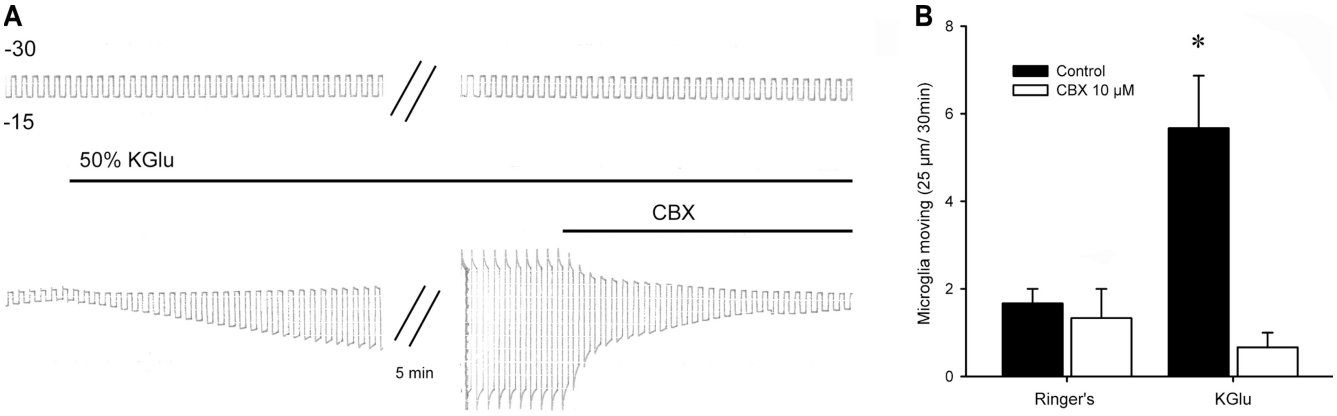

Figure 4. Microglia in connectives treated with isotonic potassium gluconate (KGlu) and the effect of 10 µM CBX. (A) A representative tracing of currents (bottom traces) from an oocyte injected with *Hm*inx2 and voltage clamped at negative potentials with shifts to positive potentials (top traces). The oocyte was treated with oocyte saline in which 50% NaCl was replaced with potassium gluconate (KGlu) to open the innexon channels and with 100 µM CBX to close them. A CBX-sensitive current increased severalfold in KGlu, reflecting increased membrane conductance due to innexon channel opening. (B) Cell nuclei stained with Hoechst 33258 dye were imaged using time-lapse microscopy, and microglia counted moving 25 µm for 30 min beginning at 30 min. The leech Ringer's solution bathing the tissue was then replaced with 140 mM KGlu for 30 min, and the nuclei moving were counted again for 30 min. Although CBX had no effect on the basal movement of the microglia, it blocked the KGlu-induced movement (t test, $n = 3$ and $P < 0.05$).

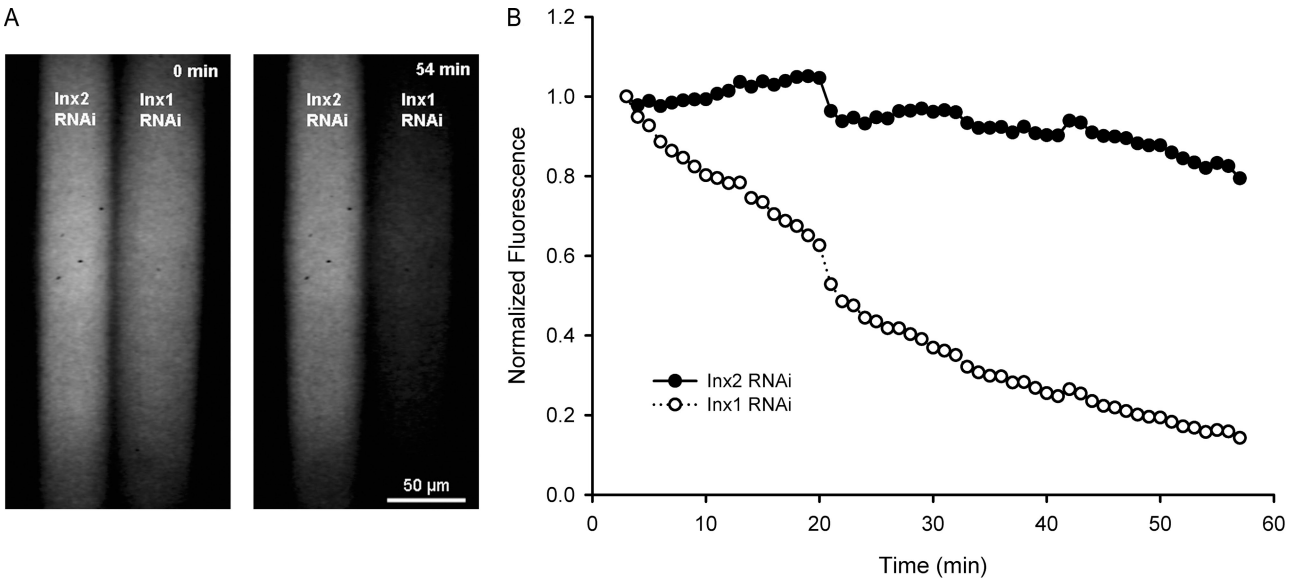

Figure 5. *Hm*inx2 RNAi, but not *Hm*inx1 RNAi, inhibited dye loss from the connective glial cell. (A) Photomicrographs of representative glial cells containing 6-CF dye. One pair of glial cells in the connectives was injected with *Hm*inx2 RNAi into the left cell and *Hm*inx1 RNAi into the right cell 2 d before the cells were injected with 6-CF dye, which was allowed to diffuse throughout the cells. The tissue was then slightly stretched to open the innexon channels. The left panel shows the cells at time 0, and the right panel shows them 54 min later. The glial cell injected with *Hm*inx2 RNAi retained substantially more dye than the control glial cell injected with the *Hm*inx1 RNAi. (B) Time course of dye loss from the experiment shown in A. The *Hm*inx2 RNAi–injected cell lost dye at a slower rate than its *Hm*inx1 RNAi–injected neighbor did. A movie of this experiment (Video 1) can be seen in the supplemental material.

Because of the technical difficulty in isolating RNA from the connective glial cells and the lack of antibody to *Hm*inx2, the knockdown of *Hm*inx2 expression has not been verified with RT-PCR or immunostaining. However, the functional knockdown demonstrated by dye retention showed that the effect was specific for neuroglial *Hm*inx2 innexons, demonstrating that glial innexons are necessary for the accumulation of microglia in response to a nerve injury.

Calcium gradient upon injury

The mechanism that opens the neuroglial innexon channels is hypothesized to be the calcium wave or calcium increase after cell damage. It was therefore important to determine whether calcium increased upon injury and for how long. Glial cells were injected with Calcium Green 1, and their fluorescence changes were monitored after the dye had distributed within the cell. Fig. 7 shows the calcium response after an injury at various distances from the site of the crush. The crush caused a substantial increase in intracellular calcium in the area adjacent to it. In the succeeding minutes, the area near the crush continued to increase its calcium response, whereas at greater distances the calcium response slowly decreased, increasing the spatial gradient. This gradient persisted beyond the time and distance shown in Fig. 7 for more than half an hour, extending more than 400 µm from the crush, which was the greatest distance examined. Because of the possibility that some dye leaked from the crush, the gradient might have been larger than indicated. The large calcium response and spatial gradient may be necessary not only for the movement, but also for the directed migration of microglia.

Mechanically induced calcium waves

To investigate the properties of the calcium response of the glial cells more precisely, calcium waves were elicited

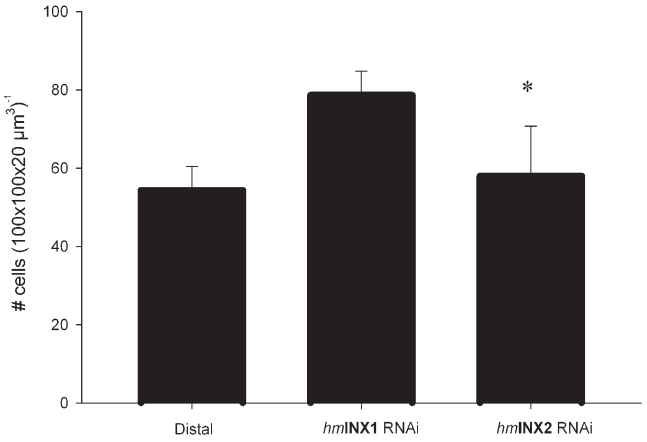

Figure 6. *Hm*inx2 RNAi injection into connective glial cells blocked microglial migration to nerve lesions. Connectives were injected with *Hm*inx2 RNAi or *Hm*inx1 RNAi 2 d before the experiments, as in Fig. 5 and Materials and methods, except that both connectives were injected with the same type of RNAi. Microglia accumulations after injury were performed according to normal laboratory methods. The *Hm*inx2 RNAi condition had significantly less accumulation than the *Hm*inx1 RNAi condition ($P < 0.05$; $n = 3$).

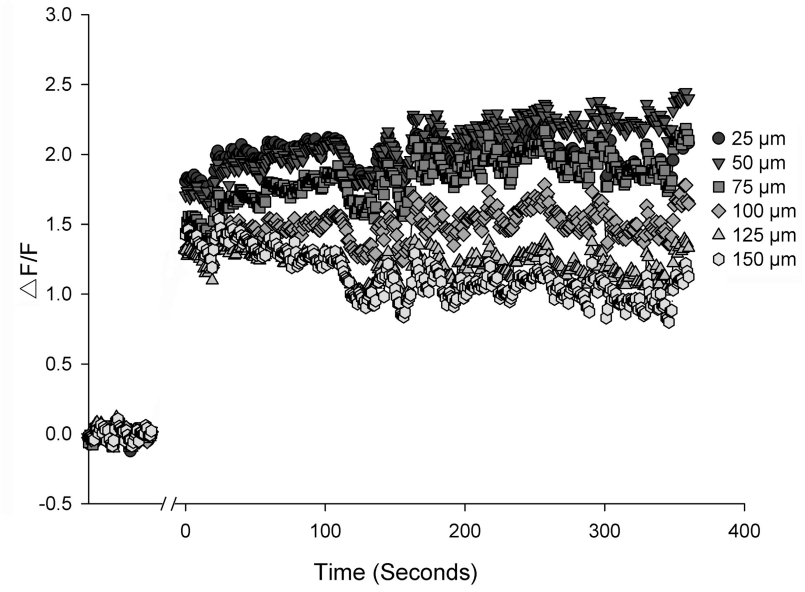

Figure 7. The post-injury calcium response. The fluorescence intensity of a glial cell injected with Calcium Green 1 was measured before and after the cell was injured by crushing the connectives. An interval of >1 min (at break in time axis) occurred before the jump in fluorescence at 0 s. The calcium response in the area adjacent to the crush and up to several hundred micrometers away developed a calcium gradient during the first few minutes after the injury. As indicated in the inset, the calcium responses in regions 100-µm^2 square were measured every 25 µm from near the crush to 150 µm distant. All regions measured had a large increase in fluorescence immediately after the crush was made; intensity in the regions nearer to the crush continued to rise, whereas the regions farther away began to decline, thereby creating a larger spatial calcium gradient.

by mechanical stimulation with a sharp tungsten probe attached to a piezoelectric device (Fig. 8, A and B). This is apparently a microcosm of what occurs during injury. It is noteworthy that innexons are stretch sensitive, opening with stretch. Although calcium responses have been described for leech glia in ganglia (Rose et al., 1995; Lohr and Deitmer, 2006), spreading waves of calcium had not previously been reported in leech glia. The linear geometry of the connective glia was particularly favorable for observing calcium waves, and their presence confirmed their evolutionary conservation. A calcium wave was also elicited using a short puff of ATP (Fig. S1), similar to what is known to occur in mammalian glial cells. The wave properties of the responses were analyzed as shown in Fig. 9, and the speed of propagation, determined from the time to half peak for responses in successive regions from the stimulus point, was 5.2 ± 0.4 µm/sec.

In mammalian astrocytes, calcium waves can propagate from cell to cell both via gap junctions by means of inositol 1,4,5-trisphosphate (IP3)-dependent, calcium-induced calcium release and via ATP-induced ATP release from the cells into extracellular space. In invertebrates, innexons would be required for either type of cell to cell propagation. Because a single glial cell ensheathes all the axons and microglia in each leech connective, one can imagine that innexons might not be required for the calcium signal. However, it also seemed plausible that the cell has an extracellular signaling component of its calcium wave that is mediated by ATP, and that the wave might be blocked or diminished by treating the tissue with CBX, in much the way CBX can block calcium waves

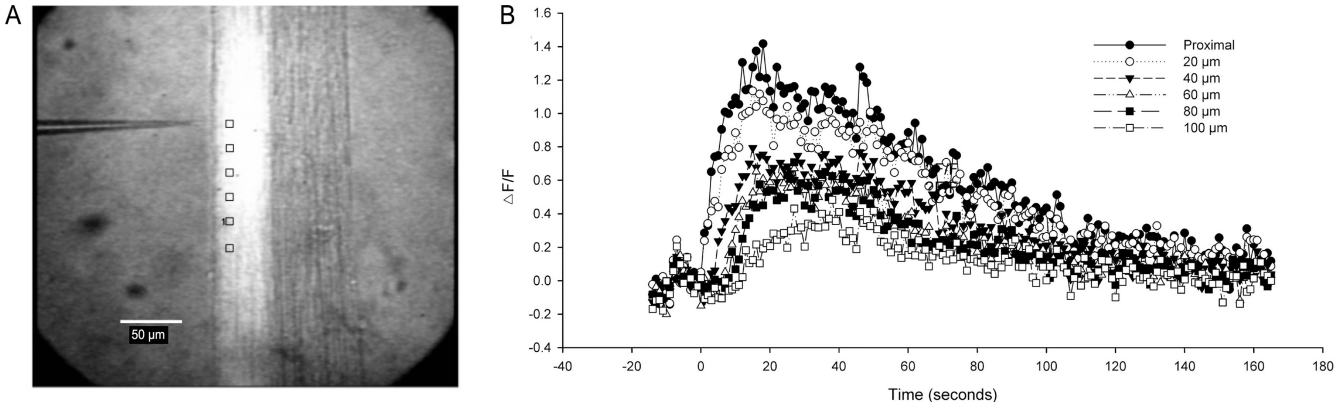

Figure 8. Mechanically induced calcium waves. (A) Measurements of calcium waves in connective glia. A probe coming from the left approaches a Calcium Green 1–injected glial cell. Blue squares are 25-µm^2 regions of measurement at 20-µm intervals beginning at the site of initiation. (B) An example of a mechanically induced calcium wave in a glial cell. Glial cells were filled with Calcium Green 1 and stimulated with a piezoelectric controlled tungsten probe (refer to Materials and methods). ΔF/F was determined for six 25-µm^2 regions located as shown with squares; the baseline F was fixed. The wave properties of the calcium response were evaluated by the increased delay of the calcium response with distance from the site of stimulation.

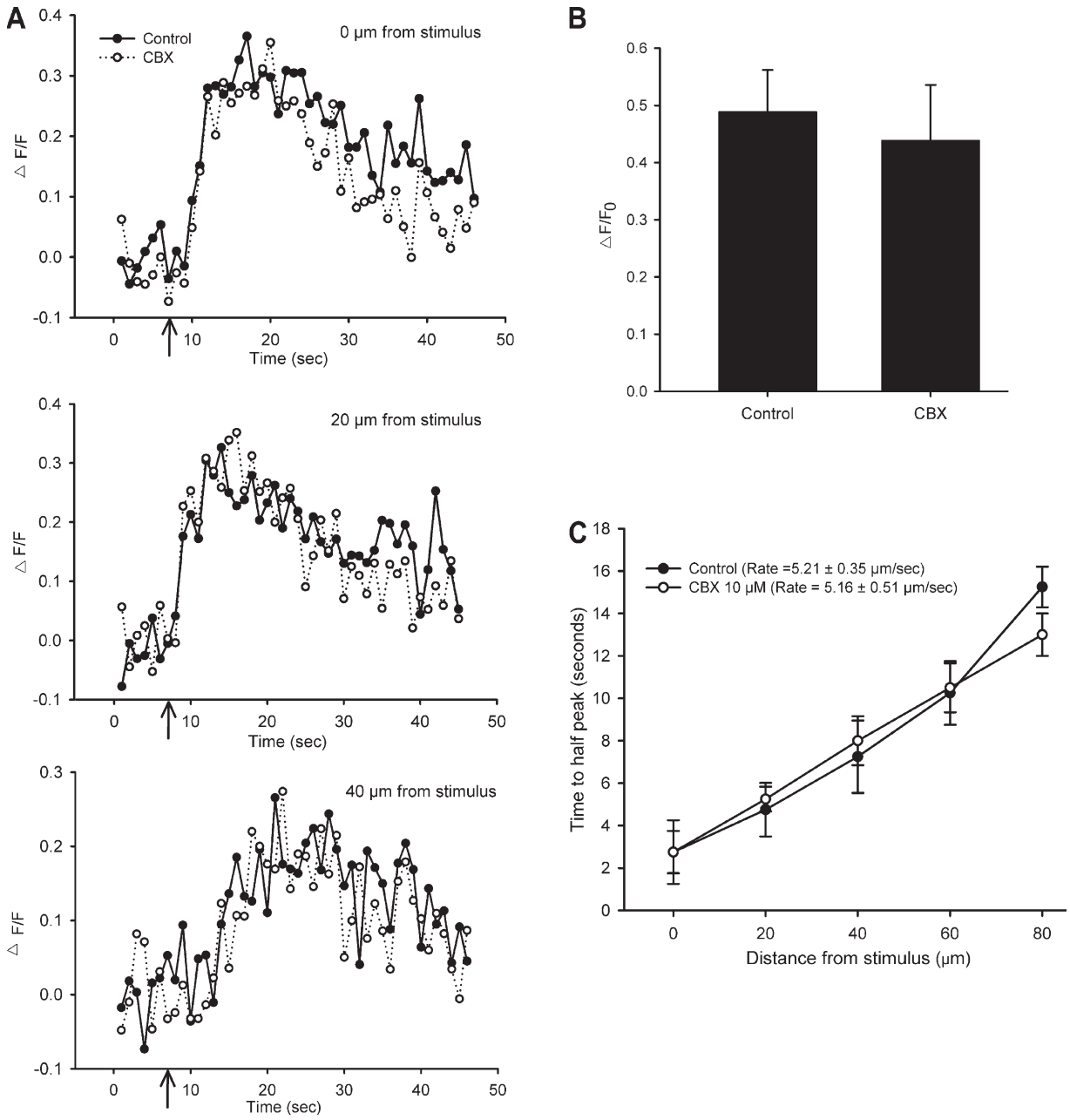

Figure 9. Mechanically induced calcium waves and the effect of CBX. (A) Mechanically induced calcium waves were induced with a sharp tungsten probe controlled by a piezoelectric device that briefly poked the connective, as shown in Fig. 8 A and described in Materials and methods. A 40-V piezoelectric touch was given after 7 s of recording to initiate the calcium wave in the Calcium Green 1–filled connective glial cell. Changes in calcium in three 25-µm^2 regions located at 20 µm intervals were measured and included in the graphs. The calcium response was calculated as the change in fluorescence divided by the resting fluorescence ($\Delta F/F$). The representative time courses of mechanically induced calcium responses in a connective glial cell with and without CBX were not distinguishable. Arrows indicate onset of the 200-msec stimulus. (B) The peak calcium responses during mechanically induced calcium waves in connective glial cells were not altered by CBX. Bars represent the average maximum fluorescence intensities in a 25-µm^2 region at the initiation site of the calcium wave induced by a mechanical stimulus and are statistically indistinguishable. Mechanically induced calcium waves were induced with a sharp tungsten probe controlled by a piezoelectric device. The stimulus was a 40-V pulse, which produced an ~50-µm displacement of the probe ($n = 4$). (C) Propagation speed of the calcium wave did not change in 10 µM CBX. These measurements were made for the same calcium waves as used in B. Five 25-µm^2 regions located at 20-µm intervals from the stimulus ("poke") site were used. At each region, the time to reach the half-maximum response after the stimulus, averaged for five responses, was determined. The distance of each region to the site of stimulation divided by the times to half peak from several adjacent regions was averaged to give the speed of propagation, as shown in the inset. There was no significant difference between the control and CBX conditions for the speeds ($n = 4$).

in astrocytes in culture (Iglesias et al., 2009). However, the pannexon/innexon channel inhibitor did not block or reduce the mechanically induced calcium response (Fig. 9, A and B). Also, the propagation speeds of the calcium waves, determined by the time to half peak, were not different in CBX from controls (Fig. 9 C). These experiments indicate that innexons were not required for the initiation or propagation of mechanically induced calcium waves. In addition, 20 U/ml apyrase did not influence the amplitude or propagation speed of the calcium waves (Fig. S2). This same concentration of apyrase blocked microglial migration (Fig. 3), showing that it penetrated the sheath and hydrolyzed the ATP within the glial sheath around the microglia. These experiments indicate that extracellular ATP was not required for the propagation of the calcium wave. An IP3-dependent, calcium-induced calcium release mechanism, known to mediate intracellular calcium waves in astrocytes, may have been operating. Indeed, the calcium waves in the leech glial cell persisted in calcium-free Ringer's solution (not depicted).

DISCUSSION

The experiments reported here showed that the rapid onset of movement of microglia to a lesion hundreds of micrometers distant in the leech CNS depended on extracellular ATP released through innexons in the glial cell, apparently as a consequence of a calcium signal or wave. CBX reversibly inhibited the migration and accumulation of microglia to a crush injury. This was not a direct effect on the microglia; instead, CBX decreased the release of extracellular ATP that triggered microglia movement. Consistent with evidence that ATP causes microglia to move and, separately, that NO is required for their directed movement, microglia overcame CBX block and moved to lesions when ATP was applied diffusely. Fig. 10 presents a model of the separate signals that includes a gradient of NO that in other systems has been reported to be generated by a glial calcium wave (Li et al., 2003). Importantly, knockdown of the glial innexin *Hm*inx2 was sufficient to block microglial accumulation.

Extracellular ATP, its actions, and the role of neuroglia

Mammalian microglia can be activated by ATP released from neighboring cells such as astrocytes (Verderio and Matteoli, 2001) and are perhaps influenced by their own autocrine release of ATP, as has been described for other immune cells (Chen et al., 2006; Yip et al., 2009). Although autocrine release of ATP from leech microglia conceivably occurs, leech microglia are at least initially activated by extracellular ATP and possibly ADP from the surrounding glia because functional glial innexons that release ATP are required for microglia accumulation at lesions. In mammals, ATP can act on microglia via P2Y12 receptors (Haynes et al., 2006) and produce a locally directed response (Davalos et al., 2005), but the idea that ATP may be a chemoattractant is based primarily on in vitro assays using a Boyden chamber (Honda et al., 2001), which does not distinguish between true chemotaxis and simple activation without directional movement. ATP signals provided by astrocytes, both in culture and in situ, also cause the release of cytokines, such as interleukin 1β (Ferrari et al., 1997; Verderio and Matteoli, 2001; Schipke et al., 2002). Among the neighboring cells, both axons and neuroglia are present as possible sources of ATP for the microglia. In the leech, both of these cell types have been shown to have innexons that can release ATP and are blocked by CBX (Bao et al., 2007). However, in mammals, large glial cells are activators of microglia and are known to be involved in the immune response to injury through their release of ATP (Bianco et al., 2005a,b; Abbracchio and Verderio, 2006). Therefore, the most likely cells responsible for the release of ATP in both mammals and leeches after CNS injury are the glial cells. This was confirmed by our experiments in which intracellular injection of the glial cell itself with dsRNA was used to selectively knock down the expression of the innexons in the glial cell using RNA interference. The knockdown, which functionally eliminated innexons as judged by reduction of dye loss, blocked the usual accumulation of microglia at the lesion. However, the possibility remains that once the microglia are activated by external stimuli, they then release ATP and further activate themselves.

Pannexons/innexons, calcium waves, and nerve injury

Both innexons and pannexons, which release ATP from glia, open in response to increases in intracellular calcium (Locovei et al., 2006b; Bao et al., 2007). Glial calcium waves, which occur in response to mechanical stimulation in mammals and can spread several hundred micrometers, have now been shown to occur in the leech. After a crush injury, the calcium signals spread as far and can persist for at least an hour, as shown in the present study. The trauma-induced increase in cytoplasmic calcium may by itself open the innexon channels, as both pannexon and innexon channels open when intracellular calcium concentrations rise. Alternative mechanisms that could also open the channels upon injury to the glial cell include the increased extracellular potassium released through damaged or compromised cell membranes or by tissue stretching, which would be only transient, however (Bao et al., 2007). Co-cultures of mammalian microglia and astrocytes have demonstrated that microglia respond to astrocytic calcium waves via purinergic signaling (Verderio and Matteoli, 2001).

Intracellular calcium waves, such as those seen in individual astrocytes, are mediated via the IP3-dependent, calcium-induced calcium release mechanism (Fiacco and McCarthy, 2004), and it is assumed that a similar mechanism operates in leech glia (Lohr and Deitmer, 2006). In short, any stimulus that causes an increase in calcium, such as an injury, a ligand such as ATP binding

to its receptor, or even spontaneous calcium oscillations, causes an increase in calcium to spread across the cell in a process that is dependent on the presence of IP3 (Kim et al., 1994; Salter and Hicks, 1995; Shao and McCarthy, 1995; Floyd et al., 2001; Parri and Crunelli, 2003). Calcium waves in leech glial cells persisted in the presence of the pannexon channel blocker CBX and the ATP-hydrolyzing enzyme apyrase, indicating that neither innexons nor ATP was necessary for their initiation or propagation. The waves also persisted in the absence of extracellular calcium, indicating that calcium entered the cytoplasm from internal stores. Because the leech connective glial cell is a single, large cell, the intracellular calcium wave can evidently propagate without extracellular release of ATP, but that release is required for mobilization of microglia.

Pannexons/innexons and the immune response

Inflammasomes are membrane platforms that play a crucial role in detecting foreign pathogens; they initiate and maintain inflammation and the innate immune response by activation of caspase 1 and the release of interleukin 1β (Lamkanfi et al., 2007; Franchi et al., 2009). P2X7 purinergic receptors have been implicated in the activation of the inflammasome, as have pannexins (Pelegrin and Surprenant, 2006; Kanneganti et al., 2007; Coutinho-Silva et al., 2009; Silverman et al., 2009). Such results are consistent with the idea that pannexin channels themselves are the pore-forming component of the P2X7 channels (Locovei et al., 2007). It has recently been shown that pannexin channels on both neurons and glia open in response to injury, and PANX1 has recently been implicated in the activation of the inflammasome in astrocytes and neurons (Silverman et al., 2009). The present study has shown that, at least in the leech, glial pannexons/innexons are necessary for an additional element of the innate immune response, namely, the migration and activation of microglia to the site of injury. Whether there is a relationship between microglia and activation of the inflammasome is not known.

Signals controlling microglia migration to lesions

In the leech, NO has been identified as an essential signal for the directed migration of microglia to lesions (Chen et al., 2000; Duan et al., 2003), and it is separate from the complementary signal provided by ATP and ADP, which activates microglia and is required for microglia movement (Duan et al., 2009). NO is produced by glia, but not neurons, immediately after nerve injury and has been localized to the lesion (Shafer et al., 1998; Kumar et al., 2001). NO operates by activating guanylate cyclase, and cGMP increases at the lesion and in a gradient beyond it after the nerve cord is crushed (Duan et al., 2009). Other signaling molecules, such as endocannabinoids and opioids, have been shown to block microglial migration through the regulation of NO (Yahyavi-Firouz-Abadi et al., 2007; Lipitz, 2008). However, despite the strong evidence for the involvement of NO in microglial migration, no evidence has emerged supporting NO itself as a signal initiating microglial movement. Instead, it appears that NO may actually stop microglia that reach the lesion (Shafer et al., 1998; Chen et al., 2000) in addition to separately providing the directional cue for microglia at a distance from the lesion. At the same time, ATP released through compromised plasma membranes from all cells or released at a distance from the lesion through innexons drives movement itself by activating P2Y receptors, but does not provide the directional cue (Duan et al., 2009). Consistent with this hypothesis, apyrase and CBX blocked microglial movement to lesions, but in CBX, directed migration was fully restored when ATP was added diffusely to the bath. In mammalian microglial cell migration, it has not been determined whether NO is important for guidance or whether there is a relationship between an NO signal and the ATP signal. However, it is interesting to note that both endothelial NO synthase (eNOS) and pannexons/innexons are activated by increases in intracellular calcium (Fleming and Busse, 1999; Bao et al., 2007) and by injury.

Model of events after nerve cord injury

A schematic diagram of the sequence of events that occurs upon injury to the leech nerve cord is proposed in three panels in Fig. 10. Fig. 10 A shows the resting condition depicting key molecules and the usual scattered distribution of microglia. Resting microglia in the leech nerve cord are spindle shaped and extend along axons within the sheath of connective glial cell (Elliot and Muller, 1981; Morgese et al., 1983). In this state, the cell's innexons are generally thought to be closed, with ATP sequestered in the cytoplasm and calcium sequestered in the endoplasmic reticulum.

Fig. 10 B shows the response beginning immediately after injury (at left), with a large and sustained increase in intracellular calcium that forms a spatial gradient highest at the crush. This calcium gradient may open innexon channels up to several hundred micrometers from the crush. ATP is released both through the damaged membrane and up to hundreds of micrometers along the connective through the glial cell's newly opened innexons. This is the ATP signal that initiates microglial movement at a distance from the lesion (Duan et al., 2005). The speed with which the signal involving ATP travels from the lesion is too rapid to be explained by simple diffusion alone, and ecto-ATPases might in any case limit diffusion of ATP itself, although in the present study ATP released from one crushed nerve cord was able to activate microglia in another cord nearby. Instead, a type of propagation or facilitated diffusion involving ATP release would account for spread of the signal. Activated microglia change shape, retracting their cytoplasmic extensions and rounding before moving.

Fig. 10 B also shows NO, which at the lesion is generated by calcium activation of eNOS in various cells. There is also evidence that NO is generated by glial calcium waves, which would account for its distant action. Because NO itself is rapidly inactivated and would not diffuse far from the lesion, NO appearing at a distance from the lesion may originate in the glial cell as a result of the calcium wave. The sustained calcium gradient may generate an NO gradient because the eNOS present in the glial cell is calcium sensitive (Shafer et al., 1998). NO provides the directional cue for the microglia, and, in high concentrations at the lesion itself, is a stop cue at the site of injury (Chen et al., 2000). It is not known exactly how NO, by activating soluble guanylate cyclase, influences the directionality (Duan et al., 2009), but cGMP controls migration of cells in diverse species and cell types (Veltman and van Haastert, 2008; Bulotta et al., 2009; Teves et al., 2009), as well as regulating motility of smooth muscle (Brown et al., 2001) and growth cones (Tornieri and Rehder, 2007). A candidate for providing directionality in leeches and perhaps mammals is a spatial gradient of the NO that is higher near the lesion. NO is also produced in response to a variety of molecular signals in the leech, including cannabinoids and opioids (see above).

Fig. 10 C shows an accumulation of microglia that collect within hours after the injury and are arrested by higher levels of NO. As the tissue recovers from the injury, the signals produced by the injury, such as the calcium gradient, NO, and extracellular ATP, likely decrease. The microglia that have migrated are no longer resting and are involved in the phagocytosing of cellular debris and producing laminin (von Bernhardi and Muller, 1995). These steps precede axon sprouting and regeneration of connections (Duan et al., 2005; Ngu et al., 2007).

In summary, it is now apparent that experimental inhibition and down-regulation of the pannexon/innexon family of proteins can influence the cellular response to injury and interfere with ATP signals required for migration of microglia. Recently, Iglesias et al. (2009) found that pannexons form the ATP-permeable hemichannels in mammalian astrocytes, using connexin43-null mice

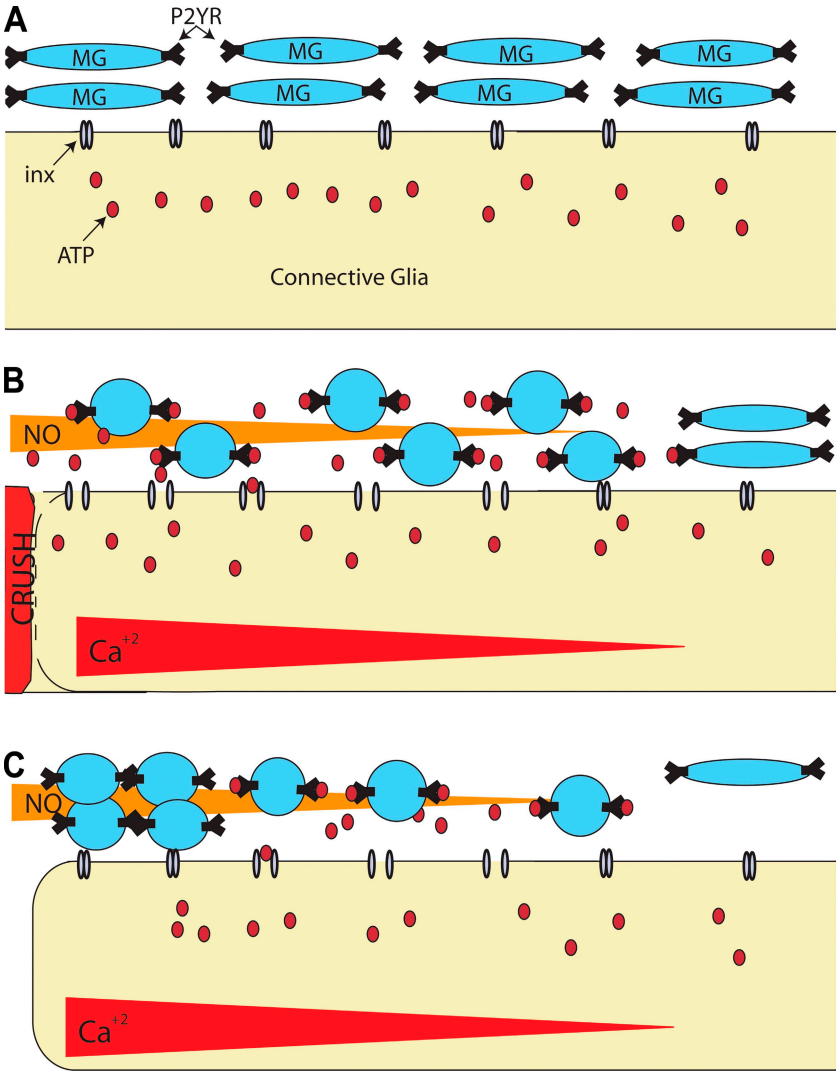

Figure 10. Diagram of signals postulated to control microglial cell migration to lesions. (A) The nerve cord before injury. (B) The cord from injury (on left) to several hours later. Note the presence of the NO and Ca^{2+} gradients, and the opening of the innexon channels. It is during this phase that the microglia become round and move toward the crush in response to ATP. (C) Recovery from injury after microglia have begun to accumulate at the site of injury. MG, microglia; inx, innexon; P2YR, purinergic receptor.

and panx1 knockdown in astrocytes to distinguish the function of connexins and pannexons. Pannexin/innexin membrane channels are evolutionarily conserved, so their release of ATP in response to cellular stresses and role in activating microglia in the leech support the idea that they may have a similar function in mammals.

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