FGF-1 induces ATP release from spinal astrocytes in culture and opens pannexin and connexin hemichannels

Juan M. Garré,a,b Mauricio A. Retamaletd, Patricia Cassinaa, Luis Barbeitoa,2, Feliksas F. Bukauskasb, Juan C. Sáezb,c,d, Michael V. L. Bennettb,1,2, and Verónica Abudaraa,1,2

(Received for review March 1, 2010) and lucifer yellow (LY) through electrical coupling of neurons (7), and HEK293 cells (8). Expression of Px1 can induce vertebrate cell types, including also called innexins. Px1 forms hemichannels (HCs), or pan-

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In addition to Cxs and Px1, spinal astrocytes express P2X7-Rs and P2YRs (10). During sustained application of ATP, P2X7-Rs may trigger permeation of relatively large molecules including ethidium” (Etd”) (11) and allow the release of ATP (12). This response was thought to result from channel dilation; however, single channel conductance and reversal potential do not change during ATP treatment (13), and a more likely mechanism is the opening of Px1 HCs induced by P2YRs or P2X-Rs when they bind ATP (8, 12) (however, see ref. 14). Proinflammatory molecules such as bacterial lipopolysaccharide and basic fibroblast growth factor 2 (FGF-2) enhance ATP release via Cx HCs in Cx43-expressing C6 glioma cells (15). Moreover, in cortical astrocytes the activity of at least Cx43 HCs is enhanced in two proin-

flammatory conditions, application of TNF-α and IL-1β (16) and oxygen/glucose deprivation (4). After injury or trauma, inflammatory mediators, such as ATP, are released by reactive microglia and astrocytes as well as dying neurons, and accumulated ATP may cause neuronal death via P2X-R activation (17). Conversely, sublethal ischemia (preconditioning) increases the number of surface Cx43 HCs and leads to the release of ATP from astrocytes and accumulation of its catabolite, adenosine, which may protect neurons from subsequent ischemic insults (18).

The acidic fibroblast growth factor, FGF-1, is also inflammatory in the adult spinal cord, and, as for ATP, its extracellular level is increased in response to cell injury (19, 20). FGF-1 activates spinal astrocytes, and activated astrocytes are implicated in neurodegenerative disorders such as amyotrophic lateral sclerosis (21, 22). To explore inflammatory mechanisms in spinal cord, we used rat and mouse spinal astrocytes in culture. FGF-1 induced the release of ATP and the opening of Px1 HCs via P2X7-Rs. This opening underlay an early (2 h) increase in membrane perme-

ability by autocrine/paracrine action of the released ATP. By 7 h, both Px1 HCs and Cx43 HCs contributed to the increased membrane permeability. These changes were accompanied by reduction in dye coupling via (connexin) GJs, which also depended on ATP release. Autocrine/paracrine effects of ATP are likely relevant to disease processes in the spinal cord in neurodegenerative and after trauma.

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age of ions and small molecules that coordinate numerous cell functions (2). Cx hemichannels (HCs) in nonjunctional membrane can be “functional” in that they can open connecting cell interior and extracellular milieu (3). In cultured cortical astrocytes, Cx43 HCs provide a pathway for uptake and release of small molecules, including tracers to which GJs are permeable and small organic molecules such as ATP, NAD+, glutamate, glucose, and prostaglandins (4). Opening of HCs appears involved in many physiological and pathological cell responses, including volume regulation, proliferation, calcium wave propagation by extracellular messengers, and cell death during metabolic inhibition (3).

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nexons, that open in nonjunctional surface membrane in several vertebrate cell types, including Xenopus oocytes (6), mammalian neurons (7), and HEK293 cells (8). Expression of Px1 can induce electrical coupling of Xenopus oocytes (6) and C6 glioma cells (9), presumably via GJs, although ultrastructural data are lacking.

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The authors declare no conflict of interest.

1M.V.L.B. and V.A. contributed equally to this work.

2To whom correspondence may be addressed. E-mail: michael.bennett@einstein.yu.edu or abudara@fmed.edu.uy.

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Results

FGF-1 Induces Spinal Astrocytes to Take up Etd⁺ and Lucifer Yellow⁻ (LY⁻). Two GJ Permeant Tracers. Confluent cultures of spinal astrocytes were exposed to 10 ng/mL FGF-1 and 5 IU/mL heparin for varying periods. We used a single application; activity FGF-1 with heparin lasts over 24 h, although only 30-60 min in its absence (23). Initially, we evaluated membrane permeability by dye uptake using a 5-min simultaneous application of LY⁻ and Etd⁺ (0.5 mM) and Etd⁺ (5 μM) followed by washing and fluorescence imaging. Cultures showed increased uptake of Etd⁺ and LY⁻ after 2- and 7-h FGF-1 treatments (Fig. S1A). The two dyes were partially colocalized in single cells. Uptake depended on FGF-1 concentration, and responses to 10 ng/mL were near maximal (24). Whereas control or 2-h FGF-1-treated cells were flat, most cells after 7 h of FGF-1 treatment were reactive, i.e., their cytoplasm was retracted around the nucleus leaving thin connections between cells (Fig. S1, black arrows in 7-h phase image and Fig. S7A) (see ref. 21).

FGF-1–Induced Increase in Membrane Permeability Is at First Mediated by Px HC; by 7-h Treatment, both Px and Cx HCs Contribute. In our initial studies, cultures were treated with FGF-1 for varying times and LY⁻ uptake was measured as for Fig. S1. By this method uptake was significant after 0.5–2 h of FGF-1 treatment, maximal after 4–7 h, and decreased to near control after 15 h and 40 h, the longest interval tested (Fig. S4). Because of the high fluorescence of LY⁻ in solution, we did not attempt to measure uptake in the presence of LY⁻ in the medium, an approach that is convenient for Etd⁺, which shows very little fluorescence. Uptake was measured as for Fig. S1. By this method, uptake was significantly greater than control at 7 h (Fig. 1A) and the fluorescence decay was 5.5 ± 1.5 times control at 2 h and 6.8 ± 0.8 times control at 7 h (Fig. 1B and C). After both 2-h and 7-h FGF-1 treatment, carbonoxolone (CBX) (0.2 mM), a Cx/Px HC blocker (8), reduced decay rates to the control level. Thus, at this time both Px and Cx HCs appear to contribute to dye uptake, although the degree of block with Cx HC blockers varied somewhat in the different experiments.

An inhibitor of FGFR1 tyrosine kinase, PD170374 (27), blocked both the Etd⁺ uptake after 2 h and 7 h of FGF-1 treatment (application 45 min before FGF-1, Fig. S2) and the morphological changes induced by FGF-1 (as also shown by ref. 21, using the related compound, PD166866).

siRNA Knockdown of Px1 and Genetic Ablation of Cx43 Attenuate the Permeability Increase Induced by FGF-1. Cx43 is the main connexin expressed by spinal astrocytes in culture, and Px1 is also expressed by these cells (5) (Fig. S3). To establish the contribution of P1x HC and Cx43 HC to the increase in membrane permeability, we used Cx43 KO mice (28), and a small interfering RNA against Pax1 (siRNA Pax1), which effectively blocks Pax1 expression in mouse cortical astrocytes (26). Immunofluorescence and Western blot analysis established that this siRNA Pax1 greatly reduced expression of Pax1 in...
rat spinal astrocytes (Fig. S3). A proprietary Silencer Select Negative Control oligonucleotide (Ambion) was used as a negative control.

Twenty-four hours after transfection with siRNA Px1, basal Etd⁺ uptake was reduced to (0.6 ± 0.2 times uptake by the control, n = 4, P < 0.05; untreated and negative-control treated wells did not significantly different, Fig. 2A). The basal uptake was not reduced significantly by applying octanol or CBX, suggesting that it was not mediated by HCs; possibly other mechanisms independent of Px HC opening but coupled to Px1 expression were involved (see ref. 8). After a 2-h FGF-1 treatment, Etd⁺ uptake by siRNA Px1 transfected cells (normalized to that by control cells transfected with siRNA and without FGF-1 treatment) was less than uptake after a 2-h FGF-1 treatment by cells transfected with negative control (normalized to that by control cells transfected with negative control without FGF-1 treatment; ratio of siRNA to negative control: 0.54 ± 0.10, n = 4, P < 0.05) and somewhat greater than the uptake by the negative control with no FGF-1 treatment (Fig. 2A; 1.25 ± 0.05 times negative control, n = 4, P < 0.05). The small residual excess uptake is ascribable to incomplete block of Px1 expression (Fig. S3). After 7 h of FGF-1 treatment, uptake by siRNA Px1 transfected cells (normalized to that by control cells transfected with siRNA and without FGF-1 treatment) was less than uptake after a 7-h FGF-1 treatment by cells transfected with negative control (normalized to that by control cells transfected with negative control without FGF-1 treatment; ratio of siRNA to negative control: 0.69 ± 0.3, n = 4, P < 0.05) but greater than uptake by siRNA Px1 transfected cells after 2 h of FGF-1 treatment (2.33 ± 0.18, n = 4, P < 0.05); this increased uptake is ascribable to Cx43 HCs.

In astrocytes from Cx43 KO mice, basal uptake was comparable to that in cells from WT (1.07 ± 0.07 times WT, n = 4, P > 0.05, Fig. 2B). Uptake after 2 h of FGF-1 treatment was only slightly less in KO cells than in WT cells (1.61 ± 0.05 in KO and 1.85 ± 0.02 in WT times control, n = 4, P < 0.05 for both), in agreement with the inference of little contribution to uptake by Cx43 HCs at this time; the small difference observed is ascribable to altered expression of other genes in the Cx43 KO and reduction in intracellular ATP leading to reduced ATP release (29). After 7 h of FGF-1 treatment, uptake in KO cells (1.4 ± 0.15 times KO control, P < 0.05) was much less than that in WT (2.8 ± 0.7 times control, P < 0.05 WT vs. KO) and about the same as that after 2 h of FGF-1 treatment, consistent with lack of Cx43 HCs and continued Px1 mediation. These molecular data confirm that the membrane permeability depends primarily on Px1 HCs after 2 h of FGF-1 treatment and on both Px1 and Cx43 HCs after 7 h of FGF-1 treatment.

FGF-1 Activation of Px1 HCs Depends on Secreted ATP. We tested whether FGF-1–induced Etd⁺ uptake by astrocytes was blocked by apyrase (APY), a soluble ATPase that would prevent autocrine and paracrine action of secreted ATP or by oxidized ATP (oATP), a general blocker of P2XRs. APY (2 μM) or oATP (0.2 mM) applied for the last 15 min of a 2-h FGF-1 treatment and during uptake measurement completely prevented the increase in Etd⁺ uptake (Fig. 3A). (The block by apyrase, which hydrolyzes ATP to ADP and then AMP, indicates that these hydrolysis products are not the active moiety.) Thus, the action of FGF-1 in opening Px HCs is ATP- and P2X-dependent and reversible during 15 min of apyrase or oATP application. Application of APY or oATP for the last 15 min of a 7-h FGF-1 treatment and during Etd⁺ uptake only partially reversed the increase in uptake (Fig. 3B). Thus, the Px HC component of uptake is reversible in ~15 min or less, but the Cx HC component of uptake, which may involve insertion of HCs into the cell surface, is not re-
versible in 15 min or, as suggested below, is not entirely dependent on ATP and P2XR for its activation.

To characterize further this residual Etd⁺ uptake after 7 h of FGF-1 with late block of ATP action, we applied 1 mM octanol after measurement of uptake for 10–20 min (Fig. 3 C and D). The component resistant to APY or brilliant blue G (BBG) was reduced to near control by 1 mM octanol. Similarly, uptake by astrocytes treated with siRNA Pxl1 and then 7 h of FGF-1 treatment was reduced to near control by octanol (Fig. 3E, arrow).

In support of FGF-1–induced ATP secretion, ATP levels in the culture medium were increased compared with control (Fig. S4A). This accumulation was due at least in part to ATP release through HCs, because APY and P2X-R and HC blockers reduced the effects of FGF-1.

To investigate the initial release of ATP in response to FGF-1, we applied botulinum neurotoxin A (BoNT A), which cleaves SNAP-25, a SNARE protein involved in vesicular release at many sites (30). BoNT A (200 ng/mL) applied 30 min before 7-h FGF-1 treatment largely prevented Etd⁺ uptake. BoNT A significantly reduced uptake when applied at the start of FGF-1 treatment, but had little effect when applied at 1 h or 6 h during the 7-h FGF-1 treatment, although P2X blockers had marked effects when applied 15 min before the end of the 7-h treatment (Fig. 4). These data suggest that FGF-1 induces vesicular release of ATP and that this ATP triggers further and regenerative ATP release by inducing the opening of Px HCs; after Px HCs are open, vesicular release is not necessary to maintain ATP concentration. The effect of BoNT A was not uniform across cells. In 27 of 40 cells (four experiments) treated with BoNT A 0.5 h before the start of a 7-h FGF-1 treatment, the uptake was similar to that in controls, i.e., the effect of FGF-1 was completely blocked. In 13 of 40 cells, the uptake was comparable (approximately two times the control) to the component insensitive to apyrase after a 7-h FGF-1 treatment (Fig. 3B and below). This result is consistent with an FGF-1–induced increase in open Cx43 HCs independent of ATP in a fraction of the cells. Variability among cells was also indicated in the experiments on efflux of LY from dye injected cells (Fig. 1 B and C). Although the astrocytes have properties in common, molecular heterogeneity in culture may arise through stochastic differences in gene expression and signaling pathways (31, 32).

**Exogenous ATP Increases Etd⁺ Uptake.** ATP, like FGF-1, increased Etd⁺ uptake; 0.5 mM applied at 0, 2, and 4 h caused an increase in Etd⁺ uptake evaluated 2 or 7 h after the first ATP application (Fig. S4 C and D). After initial release of ATP from vesicles in response to FGF-1, ATP-activated interaction of Px1 HCs by P2X-Rs and then opening of Cx43 HCs would tend to maintain or increase ATP concentration. After a 2-h or 7-h application of 0.5 mM ATP (multiple applications as above) or 10 μM benzoyl ATP (BzATP, single application), a selective P2X agonist that is not hydrolyzed by ATPases, Etd⁺ uptake (Fig. S4 C–F) was comparable to that by astrocytes treated with FGF-1 for the same time period (Fig. 1D–G). Pharmacological evidence with the blockers used above indicated that the uptake was mediated by Pxl1 HCs at 2 h and by both Pxl1 and Cx43 HCs at 7 h. The Etd⁺ uptake induced by ATP after 2 or 7 h was not affected by reactive blue 2 (RB2), a nonselective P2Y antagonist applied 15 min before uptake measurement, suggesting that P2Y receptors do not induce uptake (Fig. S4 C and D). (RB2 may also block P2X₁, P2X₃, P2X₇, but not P2X₂ receptors; refs. 33, 34).

The ATP-induced increase in uptake was completely inhibited in astrocytes treated with ATP + oATP for 7 h (following a 5-min pretreatment with oATP; data not shown), indicating that activation of P2X receptors is required for ATP-induced permeabilization. However, FGF-1 for 7 h applied with apyrase or oATP (preceded by a 5-min pretreatment with the same agent, Fig. S4B) did not completely block the increase in Etd⁺ uptake. We presume the residual increase was Cx HC mediated and octanol sensitive, and, in agreement, the uptake after 7 h of FGF-1 treatment in siRNA Pxl1-treated cells was blocked by octanol (Fig. 3E). These data suggest that FGF-1 can activate some Cx HCs independently of ATP release and P2X-R activation.

The actions of P2X2 or Pxl1 HC blockers and Cx HC blockers after 7 h of FGF-1 treatment demonstrated occlusion of the blocking actions in that antagonists of either type of HC could each block more than half the increase in permeability. In Fig. 1B, block by either BBG or Oct each reduced the LY²⁻ decay rate by more than half. In Fig. S4 D and E, block of Etd⁺ uptake by either BBG or Oct was greater than half the block by CBX, which blocks both Px and Cx HCs. Rather than lack of specificity for the parallel uptake pathways, block of ATP release through Cx43 HCs may have reduced ATP activation of Pxl1 HCs. These results further support the inference that after 7 h of FGF-1 treatment, both Cx and Px HCs contributed to the increase in permeability.

**FGF-1 Treatment Reduces Intercellular Coupling with Little Effect on Surface Cx43.** FGF-1 modulation of coupling of spinal astrocytes was tested by iontophoretic injection of Etd⁺ and by scrape loading with LY²⁻. Astrocytes were microinjected with EtdBr (1 mM in 150 mM KCl) (Fig. S5). Control cells showed an incidence of dye coupling (percentage of cells coupled to one or more cells) of 72 ± 8.7% under control conditions (n = 4 experiments, 40 injections total). After 2- and 7-h FGF-1 treatments, the incidence of dye coupling between spinal astrocytes decreased to 38.0 ± 9.0% and 17.5 ± 9.5%, respectively (P < 0.05 vs. control for each duration, 2 h vs. 7 h, not significant).

In scrape loading, LY²⁻ spread from the edge of a cut was decreased to 27 ± 7% of control after a 7-h FGF-1 treatment, a decrease comparable to the effect of CBX (0.1 mM applied 15 min before loading) (to 22 ± 2% of control, n = 4, P < 0.001 vs. control for both FGF-1 and CBX, Fig. S6). Apyrase applied before and during a 7-h FGF-1 treatment (FGF-1 + APY) increased the extent of LY²⁻ spread to 70 ± 7% of control (n = 4, P < 0.05 vs. control, P < 0.01 vs. FGF-1 alone).

Astrocytes under control conditions or after a 7-h FGF-1 treatment displayed diffuse cytoplasmic Cx43 immunoreactivity and punctate labeling at cell interfaces (Fig. S7A). Labeling was not obviously affected by FGF-1, but some concentration around the nucleus may have been associated with activation and repositioning of cytoplasm.

Western blot analysis of biotinylated surface and total proteins showed that FGF-1 treatment (7 h) reduced the level of total Cx43 to 60 ± 10% of control (Fig. S7B, P < 0.05, n = 4) and reduced the level of surface Cx43 (biotinylation, Biot) to 80 ± 8% of control (Fig. S7B, not significant, P = 0.06, n = 4). FGF-2 has similar effects on striatal and cortical astrocytes (35).
Discussion

Here, we demonstrate that spinal astrocytes in culture become permeable to LY2+ and Etd+ when treated with FGF-1. We attribute this permeabilization primarily to: (i) activation of FGFRs, (ii) which causes vesicular release of ATP; (iii) ATP-mediated activation of P2X7Rs, which leads to (iv) opening of Pxl HCs and further release of ATP; and (v) by ~7 h, but not at 2 h, opening of Cx43 HCs as well, which also release ATP. Moreover, (vi) FGF-1 treatment causes a marked reduction in intercellular communication mediated by connexin GJs (Fig. 5). Our results may be specific to spinal astrocytes. Although FGF-2 decreases coupling in cortical as well as striatal astrocytes (35), FGF-1 did not permeabilize cortical astrocytes to Etd+, although it caused the morphological changes associated with activation (Figs. S8 and S9).

To summarize the evidence, uptake by Pxl HCs at 2 h and both Pxl and Cx43 HCs at 7 h is indicated by the actions of HC blockers (Fig. 1); CBX blocked both classes of HC, whereas octanol and La3+ blocked only Cx HCs. The inferences were confirmed by the effects of siRNA Pxl and use of cells from Cx43 KO mice (Fig. 2). Mediation by ATP acting on P2XR was indicated by block by apyrase and P2XR antagonists. Also, ATP or BzATP alone produced similar changes in permeability with comparable pharmacology (Fig. S4). FGF-1 initiated the permeability changes, and the permeability changes were prevented by PD170374, an FGR-1 blocker (Fig. S2). FGF-1 caused vesicular release of ATP, indicated by block of permeabilization by BoNT A applied before FGF-1. However, BoNT A applied 1 h after FGF-1 had much less effect (Fig. 4), and evidently, ATP release became self-maintaining once Pxl HCs had opened; ATP released from Pxl HCs caused activation of P2X7 receptors, opening of Pxl HCs, and further release of ATP that further activated P2X7 receptors. Rise in Ca2+ caused by FGF-1 (36) or influx through P2XR may have contributed to Pxl HC opening (11). Also, there may have been some opening of CxHCs by FGF-1 in a parallel, ATP-independent pathway (Fig. S4). FGF-1 caused a smaller reduction in dye coupling in the presence of apyrase, indicating a contribution by extracellular ATP (Fig. S6).

The action of ATP to open Pxl HCs is rapid in onset and rapidly reversible (<1 min) as indicated by electrophysiological measurement (8). In our experiments, apyrase or P2X-R blockers applied 15 min before uptake abolished Pxl HC permeability, also indicating reversal of Pxl HC opening but at a poorer time resolution. However, the component blocked by Cx HC blockers was not greatly changed by this pretreatment. Thus, Cx HC opening is not as rapidly reversible. What mechanisms might explain the late involvement of Cx HCs in FGF-1 induced permeabilization? The response of P2X7Rs to ATP desensitizes (37). However, the transient increase in [Ca2+]i, due to influx through P2X7 receptors or release from intracellular stores activated via FGF-1 receptors can activate intracellular signaling proteins, such as kinases (36). In HeLa Cx43 transfectants, this early [Ca2+]i signaling appears to be essential for the increase in membrane permeability and surface expression of Cx43 HCs observed after 7 h of FGF-1 treatment, because increase in both is abolished by intracellular Ca2+ chelation with BAPTA (38). However, in spinal astrocytes, increased permeability was accompanied by a minor reduction in surface Cx43 (Fig. S7), suggesting increase in Cx43 HC open probability. In cortical astrocytes, cytokines such as TNF-α and IL-1β induce cellular permeabilization but decrease the amount of surface Cx43 HCs; thus, the HC open probability must be increased; this process is mediated through a p38 MAPK-dependent mechanism (16). Activation of P2X or P2Y receptors can increase [Ca2+]i, leading to activation of p38 MAPK-dependent pathways (39, 40). In spinal astrocytes, activation of blockers of P2YRs (i.e., RB2) and P2XRs (e.g., BBG) indicate that P2X7Rs are involved in permeabilization (Fig. 3 and Fig. S4). FGF-1 transiently reduced dye coupling in spinal astrocytes.

Cytochrome and growth factors decrease connexin-mediated intercellular communication in astrocytes from some but not all brain areas (35). FGF-1 had much less effect on dye coupling in the presence of apyrase, implicating ATP as a major autocrine/paracrine mediator of the effect. In HeLa cells, decrease in coupling occurs without significant change in total Cx43 levels (38), and changes in phosphorylation state of Cx43 could decrease open probability of GJ channels as previously demonstrated for other growth factors (41). Decrease in the amount of Cx43 in GJs would not increase availability of Cx43 HCs, because junctional degradation involves proteolysis of entire cell–cell channels and not separation of HCs making them available for reuse (42).

GJs between astrocytes allow cooperation between the cells in maintaining the composition of the extracellular milieu of active neurons (43). Moreover, Pxl and Cx HCs play a number of important roles under physiological conditions, although opening of Cx HCs may accelerate cell death following insults such as metabolic inhibition (3), and Pxl HCs have been implicated in cell damage as well (7). We saw no reduction in astrocyte viability resulting from activation of P2X7Rs and opening of Pxl and Cx43 HCs by FGF-1 or ATP; LY2+ uptake returned to near control levels after 15–40 h of FGF-1 treatment (Fig. L4). However, in vivo extracellular ATP released from astrocytes could induce release of more ATP from microglia, at least in part through HCs (4), and enhance the inflammatory response acting as a paracrine signal to a different type of cell. Activated microglia also release other proinflammatory molecules including TNF-α and IL-1β that could act on astrocytes in concert with FGF-1 (which may be released by dying neurons) (21). Astrocyte Pxl and Cx HCs could release bioactive molecules and reduce net uptake of extracellular K+ and glutamate, leading to excitotoxicity (44); also, decreased coupling would hinder spatial buffering by astrocytes. Together, these changes could increase vulnerability of neighboring neurons, even those not directly affected by the initial insult (45). Thus, inhibition of P2X7-Rs and Pxl and Cx HCs may be therapeutic in spinal cord inflammation, and, recently, the P2X7-R antagonist, brilliant blue G, was shown to be neuroprotective in a rodent model of traumatic spinal cord injury (46).

Materials and Methods

Details are provided in SI Materials and Methods.

Materials. Culture media and serum were from Gibco BRL. FGF-1 (Sigma-Aldrich) was prepared as a 10 μg/mL stock solution in 5,000 UI/mL heparin.

Cell Cultures. Astrocytes from spinal cords of 1-d-old rat pups were prepared and cultured as described with minor modifications (21). Cultures were used after 15–20 d.

Fig. 5. Proposed reactions initiated by FGF-1. (i) FGF-1 binds to its (dimeric) receptor, likely causing a rise in cytoplasmic Ca2+, (ii) ATP is released from vesicles, an action blocked by BoNT A. (iii) The ATP released acts on P2X7-Rs, which allows Ca2+ to enter. (iv) Activation of P2X7-Rs leads to opening of Pxl HCs (green arrow), allowing ATP release and Ca2+ entry. (v) Cx43 HCs are opened hours later, either through an action of FGF-1 or of P2X7-Rs and Pxl HCs. (vi) Gap junctional communication is reduced, an effect largely prevented by apyrase.
Lucifer Yellow and Ethidium Permeability Assay. Dye uptake was measured under control conditions and after treatment with FGF-1 and other reagents for various times. Uptake of Etd- and LY2- was measured by brief application after treatment, or Etd+ uptake was measured in time lapse in the presence of Etd-. In other experiments, cells not in contact with other cells were coated with LY2- through a pipette, and decay of fluorescence was monitored.

ATP Measurement. ATP in the culture medium was determined using a luciferin-luciferase kit ( Molecular Probes) and lumimenter (LUMistar; BMG Labtech).

Dye Coupling. Coupling between confluent astrocytes in culture was evaluated in two ways, by isotonicphoretic injection of EtdBr into single cells and by scrape loading of lucifer yellow (LY2-), ion WM 457.2) as described (47).

Immunofluorescence. Primary antibodies, a rabbit polyclonal anti-Cx43 antibody (48) and a chicken anti-Px1 C-terminal antibody (Millipore) were applied to ethanol-fixed astrocytes.

Surface Biotinylation and Western Blotting. Surface proteins were characterized by biotinylation, pull down with NeutrAvidin beads, and Western blotting as described (49).

siRNA Pxl and Cx43 KO. Cells were treated with siRNA targeting mouse pannexin 1 mRNA (sequence no. 3 in ref. 26). Cx43 KO mice (28) were obtained from D. C. Spray and E. Scemes (Albert Einstein College of Medicine, Bronx, NY).

Statistics. Data are presented as means ± SEM with the usual statistical tests and criterion P < 0.05 for significance. N was taken as the number of cells evaluated rather than the number of cultures, because the separate experiments gave the same results.

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Supporting Information

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SI Materials and Methods

Materials. Culture medium and serum were from Gibco BRL. FGF-1 and other reagents were from SIGMA unless otherwise specified.

Cell Cultures. Astrocytes from spinal cords of 1-d-old rat pups were prepared and cultured as described with minor modifications (1). Cells were plated (at 2.0 × 10^4 cells/cm²) on 12-mm glass coverslips or on 60-mm plastic culture dishes and kept in a maintenance medium composed of DMEM supplemented with 10% FBS (1.2 g/L), NaHCO₃ (1.2 g/L), penicillin (100 U/mL), and streptomycin (100 μg/mL). Cultures were grown at 37 °C in a humidified atmosphere of 5% CO₂ and 95% O₂. Astrocytes in confluent monolayers after 15–20 d in culture were >98% pure as determined by GFAP immunoreactivity, and there were no cells positive for OX42, a microglial marker.

Drugs and Solutions. Solution composition: Tyrode’s: NaCl (140 mM), KCl (4 mM), CaCl₂ (1.8 mM), MgCl₂ (1 mM), Heps (5 mM), glucose (5 mM), pyruvate (2 mM), (pH = 7.4); Hank’s: NaCl (137 mM); KCl (5 mM); CaCl₂ (1.8 mM); MgCl₂ (0.95 mM); KH₂PO₄ (0.4 mM); MgSO₄ (0.4 mM); NaHCO₃ (4 mM); NaH₂PO₄ (0.3 mM); glucose (5 mM), pH = 7.40. Lucifer yellow used for dye uptake experiments was prepared from a 5% stock solution in Tyrode’s solution, 5 μM EtdBr was added as indicated, and the cation, 314) were applied at room temperature to cultures for 5 min at 500 μM and 5 μM final concentrations, respectively. Astrocytes were rinsed three times at the end of the dye exposure to stop uptake and reduce background labeling and held in Tyrode’s solution for the brief period before photomicrographs were taken. Coverslips with labeled cells were mounted in a perfusion chamber and visualized in a Nikon microscope (Optiphot) equipped with epifluorescence illumination and appropriate filters for LY₂⁺ (excitation wavelength peak, 426 nm; emission wavelength peak, 528 nm); Etd (excitation wavelength peak, 528 nm; emission wavelength peak, 598 nm). Fluorescence and phase-contrast images were captured with a DC290 Zoom Kodak Digital Camera (exposure time = 0.7 s). Fluorescence was digitized in arbitrary units (AU) with image processing software (Adobe Photoshop 7.0). Regions of interest including single cells were evaluated for overall fluorescence and background subtracted using areas where the cultures were thin or where there were no cells. Dye uptake was expressed as the difference Fᵢ – F₀, where Fᵢ represents the average fluorescence from 10–15 astrocytes and F₀ corresponds to the background fluorescence evaluated in the same field in regions where the cultures were very thin or nonconfluent. At least three fields were selected at random near the center of each coverslip, and every cell in each field was measured.

For time-lapse measurements fluorescence signals were acquired using UltraVIEW software for image acquisition and analysis (Perkin-Elmer Life Sciences). To measure permeability, single astrocytes that were not in contact with other cells were injected from a patch pipette in whole cell recording mode containing ATP (oATP) were dissolved in recording solution at a final concentration of 0.2 mM. Brilliant blue G (BBG, 5 μM) and reactive blue (RB2, 50 μM) were dissolved in stock solutions of 5 mM and 30 mM, respectively, and the final concentration was applied to the cells in recording solution, or a small volume was added to the culture dish to reach the desired final concentration. Apyrase was dissolved in PBS at 2 U/mL and diluted to the final concentration of 2 μU/mL. ATP was prepared as a 0.5-mM solution in recording medium, and the pH was adjusted to 7.4 before recording. Benzoyl-ATP (Bz-ATP) was prepared in a 10-nM stock solution and applied at the final concentration of 10 μM. The bath volume was 2 mL.

FGF-1 was prepared as a 10 μg/mL stock solution in 5,000 U/mL heparin at least 1 h before each experiment. Final concentrations were 10 ng/mL for FGF-1 and 5 U/mL for heparin. Confluent astrocyte cultures were washed three times in Tyrode’s solution. Then, the maintenance medium (see Cell Cultures) was replaced by DMEM with 2% horse serum. Under these conditions, cells were incubated with FGF-1 for different time periods at 37 °C in a humidified atmosphere of 5% CO₂ and 95% O₂. Dye uptake (evaluated as fluorescence intensity in arbitrary units, AU) of astrocytes treated with heparin alone for all exposure times used did not differ from that by cells maintained in basal conditions, i.e., incubated in DMEM with 2% horse serum (P > 0.05).

Lucifer Yellow and Ethidium Permeability Assays. Dye uptake was measured in cultures under control conditions and after treatment with FGF-1 for various times. FGF-1 solution was washed out before recording. Then, the gap junction (GJ) permeable fluorescent tracers, lucifer yellow (LY₂⁺, molecular weight of the fluorescent ion, 457) or ethidium as the bromide (Etdb⁺, MW of 314) were applied at room temperature to cultures for 5 min at 500 μM and 5 μM final concentrations, respectively. Astrocytes were rinsed three times at the end of the dye exposure to stop uptake and reduce background labeling and held in Tyrode’s solution for the brief period before photomicrographs were taken. Coverslips with labeled cells were mounted in a perfusion chamber and visualized in a Nikon microscope (Optiphot) equipped with epifluorescence illumination and appropriate filters for LY₂⁺ (excitation wavelength peak, 426 nm; emission wavelength peak, 528 nm); Etd (excitation wavelength peak, 528 nm; emission wavelength peak, 598 nm). Fluorescence and phase-contrast images were captured with a DC290 Zoom Kodak Digital Camera (exposure time = 0.7 s). Fluorescence was digitized in arbitrary units (AU) with image processing software (Adobe Photoshop 7.0). Regions of interest including single cells were evaluated for overall fluorescence and background subtracted using areas where the cultures were thin or where there were no cells. Dye uptake was expressed as the difference Fᵢ – F₀, where Fᵢ represents the average fluorescence from 10–15 astrocytes and F₀ corresponds to the background fluorescence evaluated in the same field in regions where the cultures were very thin or nonconfluent. At least three fields were selected at random near the center of each coverslip, and every cell in each field was measured.

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ATP Measurement. Levels of ATP were measured using a luciferin-luciferase kit (Molecular Probes). Cultured astrocytes (cell density, 25,000 cells/cm²) were washed three times in Tyrode’s solution and then maintained in 500 μL of Tyrode’s solution for 30 min to allow for accumulation of ATP in the extracellular medium. A sample of 250 μL of the medium was collected and centrifuged at 1,000 rpm before recording. A total of 300 μL of a reaction mixture containing a final concentration of 0.1 μg/mL of luciferase and 150 μg/mL of luciferin and the culture medium was added to the plate reader. Baseline [blank] measurements were carried out on Tyrode’s solution according to the same procedure. The photon flux was counted during 15 min using a luminometer (LUMItstar; BGMB Labtech). The reader was calibrated between 0.01–10 μM from a standard solution of ATP (Molecular Probes); the relation between luminescence and ATP concentration was linear in this range.

Dye Transfer. Functional coupling between connuent astrocytes in culture was evaluated in two ways, by iontophoretic injection of EtdBr into single cells and by scrape loading. During measurements following dye injection, cells were kept in Hanks’ solution in the absence or presence of La³⁺ (0.2 mM), which blocks Ca²⁺/H⁺ transporters to reduce possible dye leakage through the nonjunctional cell membrane; no difference was noted due to presence of La³⁺ (2). Coverslips to which the cells adhered were placed in a perfusion chamber that was mounted on the stage of an inverted microscope (TE 200; Nikon). Glass micropipettes (6030, 30–70 MΩ; A-M Systems) filled with EtdBr (1 mM in 150 mM of KCl) were used to microinject cells using depolarizing current pulses (10 nA intensity, 200 ms duration). The incidence of dye coupling was calculated as the percentage of injected cells from which the dye transferred to at least one adjacent cell within 1 min.

Scrape loading was used to introduce the GJ permeant fluorescent dye, Lucifer yellow (LY), into cells. Lucifer yellow (LY) solution according to the method described above. Protein concentration was measured by Bradford assay. Biotinylated proteins were precipitated with avidin (immunopure immobilized avidin cross-linked to agarose, NeutraVaiden; Pierce) for 1 h at 4 °C (3 μg of biotinylated protein per microliter of avidin as recommended by Pierce). Precipitated proteins were washed three times at 4 °C in Hanks’ solution, SDS 0.1%, Nonidet P-40 1%, pH 7.2. Protein samples were eluted in 0.1 M glycine-HCl at pH 2.8; 10 μL of Tris pH 7.4 was added to each sample (70 μL total volume) to neutralize the pH. The tubes containing a sample were held in boiling water for 3 min in 1 M of Tris buffer + 5% β-mercaptoethanol to cleave the biotin moiety from the labeled protein and then analyzed by immunoblotting with primary anti-Cx43.

Small Interfering RNA Against Px1 (siRNA Px1) and Cx43 Knockout. Spinal astrocytes were treated with 1 μM of siRNA Px1 targeting mouse pannexin 1 mRNA (sequence no. 3 in ref. 4; GGUGCUUGAGAACKAUAAAtt). The effectiveness of this siRNA was demonstrated by others (4) and is confirmed in Fig. S3. Confluent cultures (90%) were transfected overnight with 10 μL/mL lipofectamine reagent (Invitrogen), transfection reagents were removed, and cells incubated for 4–6 h in DMEM, 2% FBS medium before treatment with GNG-1, ATP, or other agents. A silencer negative sequence from Ambion was used as a control (4). Connexin 43 knockout (KO) mice (originally reported in ref. 5) were obtained from Drs. D. C. Spray and E. Scemes (Dominick P. Purpura Department of Neuroscience, Albert Einstein College of Medicine, Bronx, NY) and cultured astrocytes were prepared and cultured as described (1, 6).

Statistics. Data are presented as mean ± SEM; N expresses the number of independent experiments in each of which multiple cells were measured. Means for each group of cells treated in the same way were compared using a nonparametric test for continuous and unpaired variables, the Mann-Whitney U test. Differences were considered significant at P < 0.05. Statistics were performed using Graph Pad Prism 4 (2003) software. Graphics were prepared using Sigma Plot (2000).


Fig. S1. FGF-1 increases Ly²⁻ and Etd⁺ uptake by spinal astrocytes in culture. Control astrocytes (Top) were relatively impermeable. A total of 500 μM of Ly²⁻ and 5 μM of Etd⁺ were applied for 5 min to cultures in control conditions or after 2- or 7-h FGF-1 treatment (Middle and Bottom), and the cells were rapidly washed and photographed. Ly²⁻ and Etd⁺ were taken up by cells treated for 2 h or 7 h with FGF-1 (10 ng/mL). The labeling was heterogeneous and colocalized to a moderate degree. After a 7-h treatment, cytoplasm was more retracted to a perinuclear position leaving processes between cells (black arrows in the phase image). Ly²⁻ and Etd⁺ puncta may be cellular domains with higher density of dye binding sites. Some puncta do not label with both Ly²⁻ and Etd⁺; see white arrows.

Fig. S2. The FGF-1–induced Etd⁺ uptake in spinal astrocytes requires kinase activity of the FGF-1 receptor. (A and B) Uptake was measured in spinal astrocytes under control conditions (open circles) or after 2-h (A) or 7-h (B) application of FGF-1 (filled circles). Pretreatment with PD170374 (1 μM, PD), a blocker of the kinase activity of activated FGF-1 receptor, applied 45 min before the start of and during FGF-1 treatment completely blocked Etd⁺ uptake in both A and B (stars). PD alone did not alter control uptake (A, red plus signs). (C) Quantitation. *P < 0.05, ***P < 0.005 FGF-1 vs. control (n = 3 experiments).
Control and FGF-1–treated spinal astrocytes express Px1. (A) Px1 detected with an anti–C-terminal antibody in rat spinal astrocytes under control conditions was largely in the perinuclear cytoplasm or Golgi. Nuclei were labeled with Hoechst 33258 stain. (B) siRNA against Px1 virtually abolished immunolabeling. (C and D) After 2- or 7-h treatment with 10 ng/mL FGF-1 (typical of n = 4) Px1 immunoreactivity was prominent at the cell periphery. (E) Western blot detection of Px1 in astrocyte homogenates from cultures under control conditions and 24 h after transfection with siRNA Px1. A single band with a mobility of ~50 kDa was prominent in control astrocytes and greatly reduced in astrocytes 24 h after transfection with siRNA (to 20% of control, n = 3, P < 0.01).
FGF-1–treated spinal astrocytes release ATP, and exogenous ATP mimics the FGF-1–induced astrocyte permeabilization. (A) Determined by a luciferin-luciferase assay, levels of ATP in the medium of spinal astrocyte cultures were increased after treatment with FGF-1 for 2 h or 7 h (**P < 0.01 vs. control, 2 h vs. 7 h, P > 0.05, n = 6). (B) Normalized rate of Etd⁺ uptake under control conditions, after a 7-h FGF-1 treatment and after a 7-h FGF-1 treatment with APY (2 mU/mL), BBG (5 μM), oATP (200 μM), or reactive blue 2, a P2Y blocker, (RB2, 50 μM). Blockers were applied 15 min before the onset and during the FGF-1 treatment and during uptake measurement. APY, BBG, and oATP partially blocked uptake. RB2 did not have a significant effect. Thus, FGF-1 may have some effect independent of ATP release. (C and D) Etd⁺ uptake was evaluated in cells treated for 2 h (C) or 7 h (D) with 500 μM of ATP. To compensate for ATP hydrolysis by ecto-ATPases, ATP was applied at 0, 2, and 4 h during a 7-h treatment. Effects on uptake by CBX (200 μM), BBG (5 μM), RB2 (50 μM), or octanol (Oct, 1 mM) applied 15 min before and during uptake measurement were determined. (C) After 2 h of ATP, uptake was markedly reduced by CBX and BBG but not by RB2 or Oct, consistent with mediation by P2X7Rs and Px1 HCs. (D) After 7 h of ATP, CBX block was the greatest although incomplete; BBG and Oct blocked somewhat less (but not significantly so) and RB2 did not have a significant effect. (E and F) Effect of a single application of 10 μM BzATP (an activator of P2X7Rs and blocker of P2YRs) on Etd⁺ uptake 2 h (E) and 7 h (F) later with blocker treatment as for ATP. (E) After 2 h of BzATP, CBX and BBG markedly reduced uptake; Oct had no effect, consistent with Px1 mediation. (F) After 7 h of BzATP, CBX produced nearly complete block; BBG and Oct each produced partial block, consistent with uptake through both Px1 and Cx HCs (four experiments, 7–15 cells in each experiment). *P < 0.05, **P < 0.01 vs. control; *P < 0.05, **P < 0.01 vs. FGF-1, ATP, or BzATP alone.
**Fig. S5.** Intercellular transfer of Etd* injected into spinal astrocytes under control conditions and after FGF-1 treatment; transfer is less after FGF-1 treatment. (A) The dye spread to as many as three cells in the control and possibly one after a 7-h FGF-1 treatment (which activated the astrocytes). Phase contrast images are shown above and the corresponding fluorescence images below. (B) The incidence of dye coupling (defined as the percentage of injected cells from which dye spread to at least one neighboring cell) was decreased at 2-h and 7-h FGF-1 treatment (four experiments for each duration; in each experiment 10–15 cells microinjected within 15 min of the end of treatment). *P < 0.05 vs. control. Coupling after 2 h and 7 h of FGF-1 treatment did not differ significantly.

**Fig. S6.** FGF-1 reduces intercellular dye transfer via gap junctions in spinal astrocytes, an action reduced by APY. Transfer measured by scrape loading. (A) Loading and intercellular transfer of LY2− in control medium, in control medium after application of 0.1 mM CBX 15 min before loading, after 7-h treatment with FGF-1 (7 h FGF-1), and after 7 h of FGF-1 treatment with apyrase (APY) for 15 min before and during FGF-1 treatment. (B) Percentage of cells containing dye relative to control in experiments as shown in A. *P < 0.05 vs. control, ***P < 0.005 vs. control, **P < 0.01 vs. FGF-1 alone, n = 4. Both CBX and FGF-1 reduced dye transfer. APY partially protected dye transfer during FGF-1 treatment.
White arrows: Cx43 at cell-cell contacts. GJs?

Green arrows: intracellular Cx43

Red arrows: clear signs of activation

**Fig. S7.** Cx43 distribution determined by indirect immunofluorescence and by Western analysis of total and biotinylated cell surface proteins. (A) Cx43 distribution in spinal astrocytes under control conditions (Left) and after 7 h of FGF-1 (Right). Both punctate and diffuse staining appeared somewhat reduced by FGF-1. White arrows: probable gap junctions. Green arrows: probable internalized junctions. Red arrows: processes indicating activation of astrocytes. (B) Western blot of Cx43 on the cell surface isolated by biotinylation (Biot) and in total cell homogenates (total) under control conditions and after a 7-h FGF-1 treatment (Cx43, n = 4). Both surface and total Cx43 were reduced by FGF-1 with no marked change in phosphorylation as indicated by mobility of the several bands. Heart homogenate (Left) was used as a standard for phosphorylation. P2 and P3 denote phosphorylated forms and NP, the unphosphorylated form.
Fig. 58. FGF-1 does not permeabilize cortical astrocytes, unlike its effect on spinal astrocytes. (A and B) Etd uptake by cortical astrocytes (Cort) is not affected by FGF-1 at 2 or 7 h of treatment. Faster basal uptake may be due to greater surface-to-volume ratio relative to the larger spinal astrocytes. (C and D) In contrast, uptake by spinal astrocytes (Sp) is increased by 2-h and 7-h FGF-1 treatment. (E and F) Summary data for cortical and spinal astrocytes from three experiments with comparable cultures of cortical and spinal astrocytes (∼20 DIV). Cultures of cortical and spinal cells were prepared from P1 pups from the same mothers and examined at the same age. Uptake rate after FGF-1 is normalized to that by the respective untreated cortical or spinal cord astrocytes. *P < 0.05, ***P < 0.001.
Fig. S9. FGF-1 causes greater permeabilization of spinal than cortical astrocytes. Uptake of 5 μM Etd+ in response to 10 ng/ml FGF-1. First through third columns: Control, 2-h FGF-1 treatment, and 7-h FGF-1 treatment. (A) Cortical astrocytes and (B) spinal astrocytes in phase and fluorescence micrographs (Upper and Lower). Cortical astrocytes are smaller than spinal astrocytes and show greater basal uptake of Etd+ with little effect of FGF-1. Labeling was heterogeneous. After 7 h of FGF-1, the phase images of both cortical and spinal astrocytes show retraction of cytoplasm around the cell bodies, leaving processes between cells a sign of activation.