before age 3 years 8 months, child produced proportionally more complex gesture sentences than mother ($P < 0.02$, sign test[24]). In only 6 of the 20 sessions did mothers' production of complex sentences rise above 4%, compared to 18 of 20 for the children ($z = 3.55$, $P < 0.001$). Thus, American children were unlikely to have learned complex gesture sentences from their mothers.

Overall, American mothers' gestures did not resemble their children's. Indeed, American children's gestures had more in common with Chinese children's gestures than with their own mothers'. American children thus appear to be responsible for the structural aspects of their systems. In contrast, Chinese mothers' gestures did resemble their children's, at least in part. Chinese children may therefore have learned segments of their systems from their mothers or, more likely given that Chinese and American children's gestures follow the same patterns, the mothers may have learned them from their children. If so, we ask why Chinese (but not American) mothers copy gesture patterns from their deaf children. The answer might involve cultural differences in attitudes toward children's communications, or the languages themselves (it may be easier to produce complex gesture sentences while speaking Mandarin than while speaking English). Whatever the reason, the fact remains that American children took the lead in creating their gesture systems, a lead their mothers did not follow.

Given the salient differences between Chinese and American cultures[8–12], the structural similarities in the children's gesture systems are striking. These structural properties—consistent marking of semantic elements by deletion and by ordering, and concatenation of propositions within a single sentence—are developmentally robust in humans (but not, apparently, in chimpanzees[25]). Their development is buffered against large variations in environmental conditions and in this sense can be considered 'innate'[26]. □

Methods

Children's and mothers' gestures were coded according to a system developed previously[23]. Criteria for isolating gestures grew out of a concern that the gestures meet the minimal requirements for a communicative symbol: first, the gesture must be directed to another individual; the gesturer must establish eye contact with a communication partner, or be assured of the partner's attention, before acting; and second, gesture must not itself be a direct manipulation of some relevant person or object; it must be empty-handed[17]. Using these criteria, we isolated gestures from the stream of motor behaviour. We characterized the form of the gestures following guidelines established for coding conventional sign languages, and divided gestures into sentence strings on the basis of motoric criteria. We then characterized the meaning of the gestures, deciding how many and what type of propositions were conveyed in a sentence, and identifying individual semantic elements.

Reliability was established between two trained coders who independently transcribed a portion of the videotapes. Two native Mandarin speakers (one born and raised in Taiwan) who were bilingual in English coded the Chinese videotapes, and two native English speakers coded the American videotapes. Reliability was 87% and 89% agreement between coders (for the Chinese and American samples, respectively) for describing gesture form, 100% and 93% for identifying sentence boundaries, 87% and 85% for identifying types of propositions, and 92% and 90% for identifying semantic elements.

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Correspondence and requests for materials to S.G.-M. (e-mail: sgsg@ccp.uchicago.edu).

Prostaglandins stimulate calcium-dependent glutamate release in astrocytes

Paola Bezzi*, Giorgio Carmignoto†, Lucia Pasti†, Sabino Vesce*, Daniela Rossi*, Barbara Lodi Rizzini*, Tullio Pozzan† & Andrea Volterra*

* *Institute of Pharmacological Sciences, University of Milan, Via Balzaretti 9, 20133 Milan, Italy*

† *Department of Experimental Biomedical Sciences and CNR Center for the Study of Biomembranes, University of Padova, Viale G. Colombo 3, 35121 Padova, Italy*

Astrocytes in the brain form an intimately associated network with neurons. They respond to neuronal activity and synaptically released glutamate by raising intracellular calcium concentration ($[Ca^{2+}]_i$)[1,2], which could represent the start of back-signalling to neurons[3–5]. Here we show that coactivation of the AMPA/kainate and metabotropic glutamate receptors (mGluRs) on astrocytes stimulates these cells to release glutamate through a Ca^{2+}-dependent process mediated by prostaglandins. Pharmacological inhibition of prostaglandin synthesis prevents glutamate release, whereas application of prostaglandins (in particular PGE_2) mimics and occludes the releasing action of GluR agonists. PGE_2 promotes Ca^{2+}-dependent glutamate release from cultured astrocytes and also from acute brain slices under conditions that suppress neuronal exocytotic release. When applied to the CA1 hippocampal region, PGE_2 induces increases in $[Ca^{2+}]_i$ both in astrocytes and in neurons. The $[Ca^{2+}]_i$ increase in neurons is mediated by glutamate released from astrocytes, because it is abolished by GluR antagonists. Our results reveal a new pathway of regulated transmitter release from astrocytes and outline the existence of an integrated glutamatergic cross-talk between neurons and astrocytes *in situ* that may play critical roles in synaptic plasticity and in neurotoxicity.

The release of endogenous glutamate from cultured cortical astrocytes was monitored continuously by means of an enzymatic assay[6]. Coapplication of (*S*)-α-amino-3-hydroxy-5-methyl-4-

isoxazolepropionic acid (AMPA) and (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid (*t*-ACPD), agonists of AMPA/kainate and mGluRs, respectively, induced potent and rapid release of glutamate from the astrocytes (Fig. 1a) in a dose-dependent manner (Fig. 1b). *t*-ACPD alone, but not AMPA alone, also stimulated the release, although to a much lower extent. Glutamate release was mediated by AMPA receptors (AMPARs) and mGluRs linked to the inositol-1,4,5-trisphosphate ($Ins(1,4,5)P_3$)–Ca^{2+} cascade[7] because: (1) it was potentiated by cyclothiazide and reduced by GYKI 52466, two agents selective for the AMPAR subtypes; (2) stimulation with the selective group I mGluR agonist (*R*,*S*)-3,5-dihydroxyphenylglycine (DHPG) was as effective as *t*-ACPD, whereas groups II (L-CCGI) and III (L-AP4) mGluR agonists were much less effective (Fig. 1c).

Astrocytes can release glutamate through multiple mechanisms, both Ca^{2+}-independent (reversed glutamate transport[8] and swelling-evoked release[9]) and Ca^{2+}-dependent[4]. The effect of AMPA + *t*-ACPD was: (1) insensitive to the inhibitory action of dihydrokainate (2–5 mM) on astrocyte glutamate transport[10]; (2) additive to glutamate release through the transporters induced by *t*-pyrrolidine-2,4-dicarboxylic acid (PDC)[11] (+83.5 ± 8.4%; $n = 3$; Fig. 1d); and (3) not associated with astrocyte swelling (98.8 ± 0.5% of the fluorescence emitted from unstimulated cells by the dye 2′7′-bis(2-carboxyethyl)-5(6)-carboxy fluorescein/AM at its isosbestic point[12] ($n = 3$). In contrast, it was drastically reduced either by stimulating in a medium without Ca^{2+} or upon increasing the intracellular buffering capacity by cell loading with the Ca^{2+}-chelator BAPTA/AM. Substitution of external Na^+ with *N*-methyl-D-glucamine (NMDG) also decreased the release (Fig. 1c). When bradykinin was used to evoke Ca^{2+}-dependent release, coadministration of AMPA + *t*-ACPD did not produce further stimulation (+6 ± 2%, $n = 3$; Fig. 1d). The release process induced by AMPA + *t*-ACPD in analogy with that of bradykinin[4,13], was sensitive to the anion transport inhibitor furosemide (− 71 ± 3.5%; $n = 7$) and to tetanus neurotoxin (TeNT, 2 μg ml^{-1}), a blocker of neuronal exocytotic release[14] (Fig. 1c). Importantly, TeNT inhibition developed slowly: first observed after 8 h of exposure (18.5 ± 1.2%, $n = 3$), it progressively increased to 72.5 ± 5% ($n = 6$) after 20 h. In parallel, release through the glutamate transporters with PDC was unaffected (not shown). We conclude that the release mechanism activated by GluR agonists in astrocytes is totally distinct from swelling- or transporter-mediated release and it apparently shares common properties with the Ca^{2+}-dependent release process elicited by bradykinin[13,15].

In striatal neurons, associative activation of AMPARs and mGluRs stimulates phospholipase A_2 (PLA_2) and arachidonic acid release[16]. We investigated whether the same mechanism plays a role in the glutamate release process of astrocytes. Indeed: (1) AMPA + *t*-ACPD (and *t*-ACPD alone, to a lower extent) rapidly enhanced ^{3}H-arachidonic acid liberation through a Ca^{2+}-dependent

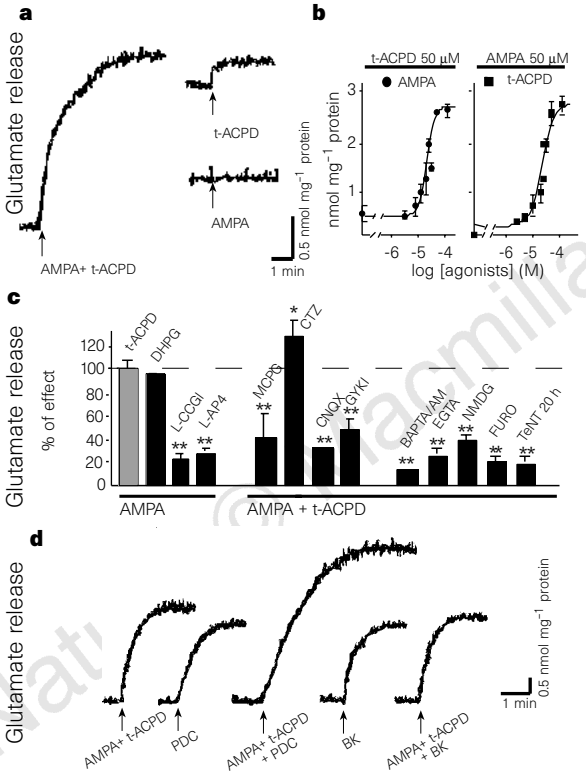

Figure 1 Ca^{2+}-dependent glutamate release from astrocytes in response to joint stimulation of AMPARs and mGluRs. **a**, Fluorescence traces representing glutamate release elicited by AMPA and *t*-ACPD together (each at 50 μM) or individually (at 100 μM). **b**, Dose–response curves of AMPA + *t*-ACPD-evoked release: each agonist curve is in the presence of 50μM of the co-agonist. Points represent mean ±s.e.m. ($n \geq 4$); **c**, release induced by 50 μM AMPA + 50 μM *t*-ACPD (=100%, striped bar) and effect of different pharmacological manipulations. Left, Substitution of *t*-ACPD with subtype-selective mGluR agonists: (*R*,*S*)-3,5-dihydroxyphenylglycine (DHPG, 100 μM); (2*S*,1′*S*,2′*S*)-2-(carboxycyclopropyl)-glycine (L-CCGI, 1 μM); *L*(+)-2-amino-4-phosphonobutyric acid (L-AP4, 150 μM). Middle, Addition of cyclothiazide (CTZ, 100 μM) or GluR antagonists: (*R*,*S*)-α-methyl-4-carboxyphenylglycine (MCPG, 500 μM) for mGluRs; 6-cyano-7-nitroquinoxaline-2,3,-dione (CNQX, 10 μM) and GYKI 52466 (GYKI, 50 μM) for AMPA/kainate receptors. Right, Pretreatment of cultures with 1,2-bis(2-aminophenoxy)-ethane-*N*,*N*,*N*′*N*′-tetraacetic acid tetrakis(acetoxymethyl)ester (BAPTA/AM, 50 μM), furosemide (FURO, 5 mM) or tetanus neurotoxin for 20 h (TeNT 20 h); stimulation in a medium free of Ca^{2+} (EGTA) or Na^+ (NMDG). $^*P < 0.05$; $^{**}P < 0.01$, one-way ANOVA and Scheffée method for multiple comparisons ($n = 3$–8). **d**, Additivity of the glutamate-releasing action of AMPA + *t*-ACPD (each at 100 μM) to that of PDC (2 mM) but not to that of bradykinin (BK) (100 nM).

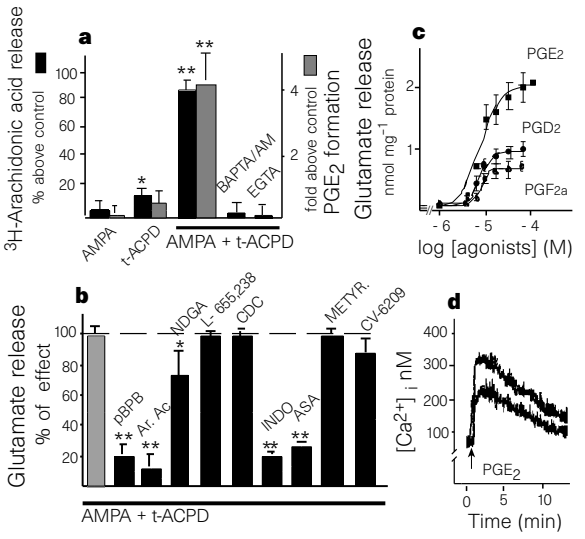

Figure 2 Activation of the arachidonate cascade and involvement of prostaglandins in the Ca^{2+}-dependent release of glutamate from astrocytes. **a**, Liberation of ^{3}H-arachidonic acid-derived material (left scale, solid bars) and formation of PGE_2 (right scale, striped bars) in response to AMPA, *t*-ACPD or AMPA + *t*-ACPD (all at 50 μM); preloading of the cells with BAPTA/AM (50 μM) or stimulation in Ca^{2+}-free medium (EGTA) prevents the response: $n = 5$–9: $^*P < 0.05$; $^{**}P < 0.01$, one-way ANOVA and Scheffée method for multiple comparisons. **b**, Glutamate release evoked by AMPA + *t*-ACPD in the absence (striped bar = 100%) and presence of arachidonic acid cascade inhibitors (solid bars; $n = 3$–10; statistics as in **a**). **c**, Dose–response curves of glutamate release stimulated by PGE_2, D_2 and $F_{2\alpha}$. **d**, $[Ca^{2+}]_i$ elevations induced by PGE_2 (20 or 50 μM) in populations of astrocytes[19]. At 20 μM, PGE_2 increased $[Ca^{2+}]_i$ (92 ± 4 nM, $n = 82$) by 145 ± 15 nM ($n = 6$); at 50 μM, by 251 ± 31 nM ($n = 7$); its effects in **c** and **d** were resistant to 1 μM indomethacin (not shown).

action (peak at 1 min; Fig. 2a and legend for details); and (2) inhibitors of the eicosanoid-forming enzymes[17] affected AMPA + *t*-ACPD-evoked glutamate release (Fig. 2b). In particular, release was drastically reduced by blocking either PLA_2 (with *p*-bromophenacylbromide (pBPB, 10 μM) or aristolochic acid (Ar.Ac., 50 μM)) or cyclooxygenase (with indomethacin (INDO, 1 μM) or aspirin (ASA, 10 μM)). In contrast, inhibitors of lipoxygenases (nordihydroguaiaretic acid (NDGA, 10 μM), L-655,238 (1 μM) or cinnamyl-3,4-dihydroxy-α-cyanocinnamate (CDC, 0.3 μM)), of epoxygenase (metyrapone, 100 μM) and of platelet aggregating factor (PAF) receptors (CV-6209, 1 μM) were practically ineffective. Prostaglandins D_2, E_2 and $F_{2\alpha}$, the major cyclooxygenase products of astrocytes[18], all rapidly stimulated glutamate release (Fig. 2c). PGE_2, which is formed in response to AMPA + *t*-ACPD (Fig. 2a), was the most potent, with a plateau effect approaching that of the GluR agonists. Moreover, when coapplied, the effect of the two stimuli was not additive (+8 ± 2% of the effect of PGE_2 alone; $n = 3$), suggesting that they activate a common pathway. Interestingly, the releasing action of PGE_2, like that of AMPA + *t*-ACPD, was prevented by cell loading with BAPTA/AM (− 80 ± 9%; $n = 6$). In addition, PGE_2 (1–50 μM) produced marked elevation of $[Ca^{2+}]_i$ in astrocytes loaded with the dye Fura-2/AM[19] (Fig. 2d). Most of this response (82.6 ± 5.3%; $n = 7$) was insensitive to the blocking of GluRs by a cocktail of mGluR, AMPAR and NMDAR antagonists (1 mM (*R*,*S*)-α-methyl-4-carboxyphenylglycine (MCPG), 50 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 50 μM 2-amino-5-phosphonopentanoic acid (D-AP5)). Therefore, PGE_2 mediates glutamate release from astrocytes in response to GluR stimulation by triggering Ca^{2+}-dependent events that are essential for release.

We next investigated whether the same phenomenon is expressed by astrocytes from acute cortical or hippocampal slices preincubated for 40 min in TeNT[20] (100 μg ml^{-1}). This treatment abolished neuronal exocytosis but, at least in culture, left Ca^{2+}-dependent release from astrocytes nearly intact (− 9.2 ± 2.7; $n = 4$). The suppression of synaptic glutamate release by TeNT was confirmed by: (1) inhibition of excitatory post-synaptic currents from CA1 neurons evoked by Schaffer collateral stimulation (not shown); (2) abolishment of the massive glutamate release upon high K^+ stimulation otherwise observed in control slices (− 98 ± 2%, $n = 7$; Fig. 3a, see also b and c). Both AMPA + *t*-ACPD and PGE_2 stimulated glutamate release from TeNT-treated slices (Fig. 3b and c, upper panels) with comparable efficacy in the cortex and hippocampus (not shown). As in cultured astrocytes, indomethacin inhibited the effect of AMPA + *t*-ACPD (− 68.8 ± 8%, $n = 4$; Fig. 3b) and BAPTA that of PGE_2 (− 61.2 ± 6%, $n = 4$; Fig. 3c).

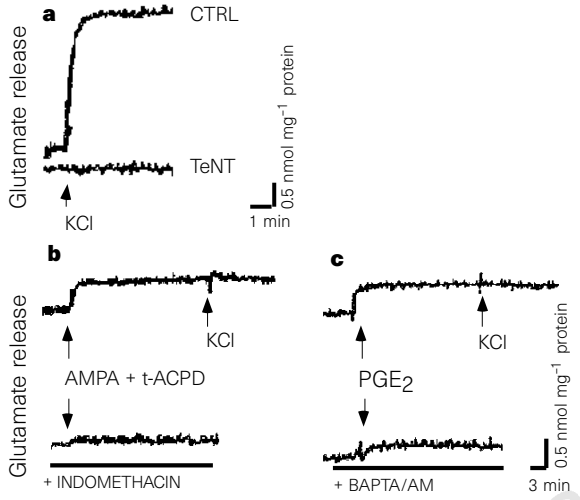

Figure 3 AMPA + *t*-ACPD and PGE_2 stimulate glutamate release from hippocampal slices with blocked neuronal exocytosis. **a**, Glutamate release evoked by high KCl (20–40 mM) in naive slices (CTRL; 2.2 ± 0.2 nmol mg^{-1} protein, $n = 4$) and in TeNT-treated slices (100 μg ml^{-1}, 40 min, $n = 7$). **b** and **c**, all slices pre-incubated in TeNT: lack of response to KCl confirms effective blockade of synaptic glutamate release; upper and lower traces, each representative of 4–7 determinations, are from separate slices. **b**, Release evoked by AMPA + *t*-ACPD (each at 50 μM, upper trace) and inhibition by indomethacin (3 μM, lower trace). **c**, Response to PGE_2 (20μM, 0.48 ± 0.1 nmol mg^{-1} protein, $n = 6$, upper trace), attenuated in slices loaded with BAPTA/AM (50 μM, lower trace).

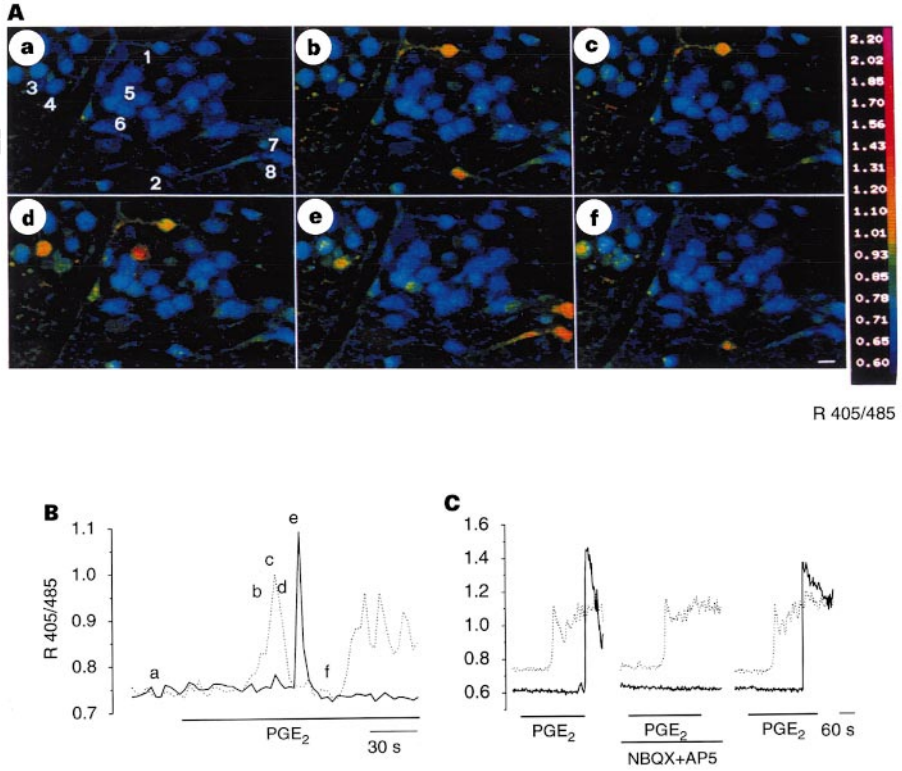

Figure 4 Effects of PGE_2 on the $[Ca^{2+}]_i$ of Indo-1-loaded hippocampal cells. **A**, Pseudocolour images of the $[Ca^{2+}]_i$ changes in the hippocampal CA1 region of a 7-day-old rat. The sequence shows $[Ca^{2+}]_i$ transients in astrocytes 1 and 2 and pyramidal neurons 3–8 upon slice perfusion with 10 μM PGE_2. Four additional neurons in the field showed delayed $[Ca^{2+}]_i$ elevations (not shown) in correspondence to the second $[Ca^{2+}]_i$ peak in the astrocytes (see trace in **B**). Acquisition rate, 2 s. Scale bar, 10 μm. **B**, Kinetics of PGE_2-induced $[Ca^{2+}]_i$ changes in the astrocyte 1 (dotted line) and in the neuron 8 (continuous line). Letters a–f correspond to the value of R405/485 as measured from cells in the images a–f in **A**. **C** $[Ca^{2+}]_i$ increases in one pyramidal neuron (continuous line) and one adjacent astrocyte (dotted line) following three consecutive PGE_2 (10 μM) applications. In the presence of NBQX and D-AP5 (both at 50 μM) the PGE_2-induced $[Ca^{2+}]_i$ increase in the neuron was abolished, whereas that of the astrocyte was unchanged. Acquisition rate: 2 s.

To address the possible physiological significance of glutamate release by astrocytes, we then studied the effects of PGE_2 on the $[Ca^{2+}]_i$ of astrocytes and neurons from the CA1 hippocampal region of acute brain slices after incubation with the fluorescent Ca^{2+} indicator Indo-1/AM and TeNT. In the confocal images, CA1 neurons loaded with Indo-1 could be clearly distinguished by their typical pyramidal shape and large soma size, whereas astrocytes were distinguished by their small size, stellate shape and, occasionally, end feet processes that typically contact blood vessels (Fig. 4A, see cell labelled 1). In patch-clamp experiments, more than 90% of these cells revealed features consistent with those of astrocytes, that is, absence of action potential discharges in response to depolarizing current pulses, highly negative resting potentials and dye coupling upon Indo-1 filling through the patch pipette.

PGE_2 induced significant $[Ca^{2+}]_i$ increases in astrocytes, such as in cells labelled 1 and 2 in Fig. 4A, and in a number of neurons, such as cells labelled 3–8 (Fig. 4B). In a series of similar experiments, 67% of the pyramidal neurons and 76% of the astrocytes loaded with Indo-1 responded to PGE_2 (0.5–50 μM). To investigate whether the $[Ca^{2+}]_i$ increases in neurons and astrocytes were due to glutamate release and activation of GluRs, PGE_2 was applied in the presence of 6-nitro-7-sulphamoyl benzo[*f*]quinoxaline-2,3-dione (NBQX) and D-AP5, antagonists of ionotropic GluRs. Although in most astrocytes (27/33) the response to PGE_2 was unchanged, in a large number of neurons (31 out of 58, $n = 8$) it was abolished by NBQX and D-AP5, but recovered after washout (Fig. 4C). These data demonstrate that the $[Ca^{2+}]_i$ increase induced by PGE_2 in neurons is mediated by glutamate, most probably released from astrocytes. This conclusion is strengthened by the observation that, in the presence of either MCPG (1 mM), NBQX and D-AP5 or MCPG alone, the response to PGE_2 was blocked in all the responsive neurons ($n = 22$ and 14, respectively). In the presence of NBQX, D-AP5 and MCPG, the astrocyte response was also affected: 7 out of the 19 PGE_2-responding cells failed to respond, whereas in the remaining astrocytes PGE_2 still largely increased $[Ca^{2+}]_i$ ($86.3 \pm 9.1\%$ of the response amplitude without antagonists; $n = 12$). Likewise, with MCPG alone, 6 out of 12 astrocytes failed to respond to PGE_2 whereas in the remaining astrocytes the amplitude of the $[Ca^{2+}]_i$ increase was only slightly reduced ($86.8 \pm 4.5\%$ of the response without MCPG; $n = 6$). These results suggest that the PGE_2-induced $[Ca^{2+}]_i$ elevation in astrocytes depends on two sequential processes: the first one is directly activated by PGE_2, and the second one follows the release of glutamate and the stimulation of mGluRs on the astrocytes themselves. Apparently, this positive feedback mechanism, while present also in culture, is of major relevance *in situ*.

Our results show that astrocytes respond to joint stimulation of the AMPARs and mGluRs by releasing glutamate through a new Ca^{2+}-dependent process mediated by prostaglandins. Indeed, the potent and direct elevation of astrocyte $[Ca^{2+}]_i$ induced by PGE_2 is apparently essential for activation of release. The mechanism by which PGE_2 increases $[Ca^{2+}]_i$ and the steps that specifically link $[Ca^{2+}]_i$ rise to transmitter release await further elucidation. We also demonstrate that glutamate released from astrocytes upon PGE_2 stimulation triggers $[Ca^{2+}]_i$ elevations in a large number of neurons from the CA1 hippocampal region. As $[Ca^{2+}]_i$ rises are crucial in a variety of neuronal functions, including phenomena such as long-term potentiation or depression, the astrocyte modulation of the $[Ca^{2+}]_i$ in neurons might disclose an unexpected participation of glial cells in the plasticity of synaptic transmission. Moreover, because GluRs on astrocytes are activated by synaptically released glutamate[1,2,21,22], the present data outline the existence of continuous bidirectional communication between neurons and astrocytes, based on a reciprocal glutamatergic signalling[22]. This communication may integrate with classic synaptic transmission in the processing of information in the brain. Finally, as prostaglandins can be products of altered central nervous system functions[23–25] and are formed by glia in response to pro-inflammatory agents[26], the prostaglandin-mediated glutamate release from astrocytes may also play a pathophysiological role in a number of brain diseases or injuries. □

Methods

Tissue preparations. Confluent monolayers of cultured astrocytes from the cerebral cortex of newborn rats and transverse thin slices (150–250 μm) either from the visual cortex or the hippocampus of 7–12-day-old rats were prepared as described[11,27]. At least 97% of the cultured cells were positive to an antibody against the astrocyte-specific glial fibrillary acidic protein (GFAP). Cultures depleted of microglia retained responses to AMPA + *t*-ACPD and PGE_2.

Enzymatic assay of endogenous glutamate release. Efflux of endogenous glutamate was monitored in continuous culture using an enzymatic assay[6] with modifications. Cultured astrocytes plated on glass coverslips or acute slices gently fixed to a mesh holder were lodged in a 1×1 cm cuvette (2 ml volume) inside a Perkin-Elmer LS50B computerized spectrofluorometer at 37 °C under stirring in (in mM): NaCl 120, KCl 3.1, NaH_2PO_4 1.25, HEPES–Na 25, glucose 4, $MgCl_2$ 1, $CaCl_2$ 2 at pH 7.4, added with glutamate dehydrogenase (GDH, 40 U ml^{-1}; Sigma G2626, batch 64H7130) and 1 mM $NADP^+$. Glutamate released from the preparations was immediately oxidized by GDH to α-ketoglutarate (thereby preventing the re-uptake process[10]) with formation of NADPH and fluorescence emission at 430 nm (delay <1 s; excitation light 335 nm). Release was quantified referring to standard curves constructed with exogenous glutamate and by normalizing for the protein content of each sample. Agents were added directly in the cuvette through a microsyringe, except those acting intracellularly, that required pre-incubation (30 min, unless otherwise specified).

Monitoring of ^{3}H-arachidonic acid release and PGE_2 formation. Release of ^{3}H-arachidonic acid-derived material from cultures pre-incubated overnight with ^{3}H-arachidonic acid (1 μCi ml^{-1}; 209 Ci $mmol^{-1}$; Amersham) and exposed to stimulants for different times (often 1 min) was monitored according to ref. 28. Data were expressed as percentage increase above basal release (in 1 min = $0.85 \pm 0.18\%$ of the incorporated radioactivity; $n = 7$). PGE_2 formation was detected using a highly sensitive and specific ^{125}I-radioimmunoassay[26] (Amersham kit RPA 530). Cell supernatants were collected after 3-min incubations with stimulants and the data expressed as fold increase above basal level ($= 4.5 \pm 0.6$ pg mg^{-1} protein, $n = 5$).

Confocal fluorescence microscopy. Slices were incubated in the acetoxymethyl derivative of Indo-1 (Indo-1/AM, 20 μM; Molecular Probes, Eugene, OR, USA), 0.02% pluronic acid and purified TeNT (100 μg ml^{-1}) added to the physiological saline (in mM): NaCl 120, KCl 3.1, NaH_2PO_4 1.25, $NaHCO_3$ 25, dextrose 4, $MgCl_2$ 2, $CaCl_2$ 1, Na-pyruvate 2, Myo-inositol 0.75, ascorbic acid 0.1, at pH 7.4 with 5% CO_2, 95% O_2. Incubation was performed at 37 °C for 40 min under continuous mild stirring. Slices were then mounted in a chamber and placed on the stage of a Nikon inverted microscope (Diaphot 300), equipped with a ×40 immersion objective (NA = 1.1) connected with a real-time confocal system (RCM8000). The ratio of the intensity of the light emitted at the two wavelengths (405/485) was displayed as a pseudocolour scale. During recordings at room temperature, slices were superfused (3 ml min^{-1}) with (in mM): NaCl 120, KCl 3.1, NaH_2PO_4 1.25, $NaHCO_3$ 25, dextrose 5, $MgCl_2$ 1, $CaCl_2$ 2, at pH 7.4 with 5% CO_2, 95% O_2.

Patch-clamp recordings. Standard procedures for pipette preparation and whole-cell patch-clamp recordings in slices were used[27,29]. Indo-1 was included in the patch pipette at 500 μM. Schaffer collaterals were stimulated with 50 μs pulses (50 to 200 μA at 0.2 Hz) delivered by a bipolar tungsten electrode (5 μm tip, Roboz, Maryland).

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Correspondence and requests for materials should be addressed to A.V. (e-mail: Andrea.Volterra@unimi.it).

Induction of epithelial tubules by growth factor HGF depends on the STAT pathway

Carla Boccaccio, Margherita Andò, Luca Tamagnone, Alberto Bardelli, Paolo Michieli, Carlo Battistini* & Paolo M. Comoglio

Institute for Cancer Research, University of Torino Medical School, Strada Provinciale 142, 10060 Candiolo, Italy
** Pharmacia & Upjohn, Preclinical Research, 20014 Nerviano, Italy*

Hepatocyte growth factor (HGF) induces a three-phase response leading to the formation of branched tubular structures in epithelial cells[1,2]. The HGF receptor tyrosine kinase works through a Src homology (SH2) docking site that can activate several signalling pathways[3]. The first phase of the response (scattering), which results from cytoskeletal reorganization, loss of intercellular junctions and cell migration[4], is dependent on phosphatidylinositol-3-OH kinase and Rac activation[5,6]. The second phase (growth) requires stimulation of the Ras–MAP kinase cascade[7]. Here we show that the third phase (tubulogenesis) is dependent on the STAT pathway. HGF stimulates recruitment of Stat-3 to the receptor, tyrosine phosphorylation, nuclear translocation and binding to the specific promoter element SIE. Electroporation of a tyrosine-phosphorylated peptide, which interferes with both the association of STAT to the receptor and STAT dimerization, inhibits tubule formation *in vitro* without affecting either HGF-induced 'scattering' or growth. The same result is obtained using a specific 'decoy' oligonucleotide that prevents STAT from binding to DNA and affecting the expression of genes involved in cell-cycle regulation (*c-fos* and *waf-1*). Activation of signal transducers that directly control transcription is therefore required for morphogenesis.

Various intracellular signalling pathways have been shown to be activated by receptor tyrosine kinases[8]. The precise role of each of these pathways must be resolved to understand the molecular basis of cell signalling specificity. The three biological responses mediated by the HGF receptor (scatter, growth and branching morphogenesis) are triggered by the tyrosine phosphorylation of a single multifunctional docking site located in the receptor's carboxy-terminal tail[3]. This sequence, containing two phosphotyrosines (Y^{1349}VHVNATY^{1356}VNV), interacts with several cytoplasmic signal transducers either directly or indirectly through molecular adaptors such as Grb2 (ref. 7), Shc (ref. 9) and Gab1 (ref. 10). We have previously shown that, after HGF stimulation, the receptor binds and activates phosphatidylinositol-3-OH kinase (PI(3)K)[11,12] and recruits the Grb2–SoS complex, stimulating Ras[13]. The PI(3)K pathway is responsible for 'scattering' through the activation of Rac, and this in turn stimulates actin reorganization and other cytoskeletal changes that are required for cell motility[5,6]. Mutagenesis of Asn 1358 in the docking site (necessary for recruiting the Grb2–SoS complex) generates an HGF receptor that can activate PI(3)K but not Ras. This mutant elicits cell scattering but not proliferation[7]. The multifunctional docking site is also directly responsible for branching morphogenesis. Insertion of the Y^{1349}VHVNATY^{1356}VNV sequence into the nerve growth factor (NGF) receptor, which otherwise lacks this biological response, makes it competent for induction of epithelial tubules[2]. However, the signalling pathway(s) critical for tubulogenesis are not yet known. Here we show that the HGF receptor binds and phosphorylates Stat-3, and that the ensuing nuclear signalling is required to trigger differentiation for branching morphogenesis.

Association of STAT proteins with non-kinase receptors, such as those for interferons and cytokines, results in their phosphorylation on a conserved tyrosine residue by the cytoplasmic Jak kinases. This phosphorylation promotes the dimerization of STAT proteins through intermolecular interactions between phosphotyrosines and SH2 domains. STAT dimers translocate into the nucleus, where they act as transcriptional factors binding specific promoter sequences[14,15]. We found that after HGF stimulation Stat-3 was transiently phosphorylated on tyrosine, with a peak between 15 and 30 min. Phosphorylated STAT was found predominantly in the nuclear fraction (Fig. 1 a–c), and its relocalization following HGF treatment was visualized by immunofluorescence (Fig. 1 d, e). STAT phosphorylation is likely to be mediated by the transient association of Stat-3 (through its SH2 domain) with the tyrosine-phosphorylated HGF receptor tail. This hypothesis is supported by three observations: first, co-precipitation of Stat-3 and the tyrosine-phosphorylated HGF receptor from intact cells (Fig. 2a); second, *in vitro* binding of endogenous Stat-3 with the purified recombinant cytoplasmic domain of the receptor (Fig. 2b); and third, binding of Stat-3 to the phosphorylated synthetic peptide Y^{P}VNV, corresponding to the sequence following Y^{1356} in the HGF-receptor docking site (Fig. 2c). The interaction between Stat-3 and the receptor is both direct and indirect, through the adaptor Gab1, as shown by co-precipitation of this molecule with both Stat-3 (Fig. 2a) and the