

Activity-dependent Neuronal Control of Gap-junctional Communication in Astrocytes

Nathalie Rouach, Jacques Glowinski, and Christian Giaume

INSERM U114, Collège de France 11, 75231 Paris Cedex 05, France

Abstract. A typical feature of astrocytes is their high degree of intercellular communication through gap junction channels. Using different models of astrocyte cultures and astrocyte/neuron cocultures, we have demonstrated that neurons upregulate gap-junctional communication and the expression of connexin 43 (Cx43) in astrocytes. The propagation of intercellular calcium waves triggered in astrocytes by mechanical stimulation was also increased in cocultures. This facilitation depends on the age and number of neurons, indicating that the state of neuronal differentiation and neuron density constitute two crucial factors of this interaction. The effects of neurons on astrocytic communication and Cx43 expression were reversed completely after

neurotoxic treatments. Moreover, the neuronal facilitation of glial coupling was suppressed, without change in Cx43 expression, after prolonged pharmacological treatments that prevented spontaneous synaptic activity. Altogether, these results demonstrate that neurons exert multiple and differential controls on astrocytic gap-junctional communication. Since astrocytes have been shown to facilitate synaptic efficacy, our findings suggest that neuronal and astrocytic networks interact actively through mutual setting of their respective modes of communication.

Key words: neuro–glial interaction • gap junctions • glial cells • connexin 43 • synaptic activity

Introduction

Glial cells represent the largest cell population in the brain. Typically, three major groups of glial cells are distinguished: macroglial cells, which include oligodendrocytes and astrocytes; and microglial cells, which share features with immune cells. For a long time, glial cells have been thought to provide mainly a structural, trophic, and metabolic support to neurons. A typical feature of macroglial cells, in particular the astrocyte, is that they establish cell–cell communication *in vitro* and *in situ*, through intercellular channels forming specialized membrane areas defined as gap junctions (see Dermietzel and Spray, 1998). Gap junction channels span two plasma membranes and are formed by the alignment of two hemichannels, each consisting of an oligomer of structural subunit proteins, called connexins (Cxs)¹. These junctional proteins consti-

tute a multigene family whose members are distinguished according to their predicted molecular weight in kilodaltons (see Bruzzone et al., 1996). These intercellular channels are permeable to ions and small molecules with a molecular weight up to 1–1.5 kD. The pattern of Cx expression differs for the two types of macroglial cells and is developmentally and regionally regulated. Cx43 is the major Cx in astrocytes from embryonic to adult stages, although other types of Cx have been detected in culture, as well as *in situ* (see Dermietzel and Spray, 1998). Functional studies performed in cultures and brain slices have demonstrated that astrocytic gap-junctional communication (GJC) is controlled by neurotransmitters, cytokines, growth factors, and other bioactive compounds (see Giaume and McCarthy, 1996; Spray et al., 1999). Thus, GJC provides the basis for functional networks that could constitute sophisticated and plastic pathways, allowing intercellular communication between defined groups of astrocytes (Giaume and McCarthy, 1996).

A major step in understanding the active role played by astrocytes in several brain functions has been achieved by

Address correspondence to Dr. Christian Giaume, INSERM U114, Collège de France 11, Place Marcelin Berthelot, 75231 Paris Cedex 05, France. Tel.: 33 144271222. Fax: 33 144271260. E-mail: christian.giaume@college-de-france.fr

¹Abbreviations used in this paper: [Ca²⁺]_i, intracellular calcium concentration; A/N cocultures, secondary plating of astrocytes on neuron cultures; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione disodium; Cx, connexin; Cx43, connexin 43; DAPI, 4',6-diamino-2-phenylindol; GABA, γ -amino-butyric acid; GFAP, glial fibrillary acid protein; GJC, gap-junctional communication; LY, Lucifer yellow; MAP 2, microtubule-

associated protein; N/A cocultures, cocultures of neurons plated on confluent astrocytes; NMDA, N-methyl-D aspartic acid; SSIC, spontaneous synaptic inward currents; TTX, tetrodotoxin.

demonstrating that calcium signaling is an important feature in astrocyte physiology and represents a form of cell excitability (Verkhatsky et al., 1998). Indeed, these cells can sense, integrate, and respond to external stimuli released by neurons through changes in intracellular calcium concentration ($[Ca^{2+}]_i$). Since astrocytes do not generate action potentials and are devoid of synaptic contacts, the existence of an elaborate calcium signaling mechanism may allow these cells to communicate with each other. This concept is based on the pioneering observations of intercellular calcium waves in astrocytes and the concept of a glial long-range signaling system (Cornell-Bell et al., 1990). Several studies have demonstrated that gap junction channels and/or the release of an active factor in the extracellular space are involved in the propagation of astrocytic calcium waves (see Charles, 1998; Giaume and Venance, 1998). Moreover, Cx seems to play a role in the external component of calcium waves, since Cx expression regulates the release of ATP from astrocytes, a process that may be involved in the extracellular component of calcium waves (Cotrina et al., 1998a).

During the last decade, the proposal for an active role of astrocytes in brain function has gained a lot of interest, thanks to several studies that have established that glial cells may participate in processing information. There is strong evidence that astrocytes contribute to the modulation of neuronal properties and synaptic activity. Indeed, calcium increases in astrocytes induce calcium responses in neighboring neurons (Parpura et al., 1994; Bezzi et al., 1998) and astrocytes are required for oscillatory activity in cultured neurons (Verderio et al., 1999). Coculture of neurons with glial cells enhances synaptic efficiency, and inefficient synapses require glial signals to become fully functional (Pfrieger and Barres, 1997). Direct stimulation of astrocytes potentiates inhibitory transmission in hippocampal slices (Kang et al., 1998). Electrical stimulation of an astrocyte induces neuronal responses and an increase in synaptic transmission through a glutamate-dependent process (Araque et al., 1998). Finally, astrocytes regulate the appearance of postsynaptic currents by contributing to the differentiation of specific excitable membrane properties (Li et al., 1999).

The present study was undertaken to investigate whether neurons can regulate a main intercellular communication process in astrocytes reciprocally. Several different models of cocultures were used to study the neuronal influence on GJC, Cx43 expression, and calcium wave propagation in astrocytes. We report here that neurons upregulate gap junction-mediated communication, Cx43 expression, and increase the extent of propagation of calcium waves in astrocytes. This neuronal-induced facilitation can be modulated differently depending on the functional state of the neurons.

Materials and Methods

Cell Cultures

Pregnant OFA (oncins franche souche A) rats (IFFA Credo) were killed by prolonged exposure to high concentrations of carbon dioxide. Embryos were rapidly removed from the uterus and placed in PBS supplemented with glucose (33 mM). Striata were dissected from 18-d-old rat embryos and mechanically dissociated in PBS-glucose solution. Depending on the

type of experiment, cells were plated in culture dishes of various diameters. The culture medium (except for neuronal cultures) consisted of a 1:1 mixture of MEM and F-12 nutrient (GIBCO BRL) supplemented with glutamine (2 mM), $NaHCO_3$ (13 mM), Hepes (5 mM, pH 7.4), glucose (33 mM), penicillin-streptomycin (5 IU/ml and 5 mg/ml, respectively), and 10% Nu-serum (Collaborative Research). Once plated, cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2 . Three combinations of astrocyte/neuronal cocultures were made from these dissociated cells.

Spontaneous Cocultures

Spontaneous cocultures of astrocytes and neurons were obtained after seeding of 10^6 dissociated cells in poly-L-ornithine-coated (1.5 μ g/ml) 35-mm diam culture dishes (NUNC) and by changing the culture medium once a week. To provide an internal control of cultures enriched in astrocytes, the culture medium was changed daily in half the dishes and cells were repetitively rinsed with PBS-glucose solution from the second through the sixth day, a procedure that eliminates neurons.

Neurons/Astrocytes (N/A) Cocultures

N/A cocultures were obtained by adding dissociated cells to 2-wk-old primary cultures of confluent astrocytes. The internal control was provided by incubating sister dishes of confluent astrocytes originating from the same culture. Primary astrocytic cultures were prepared as previously described (Venance et al., 1997) with slight modifications. Poly-L-ornithine-coated (1.5 μ g/ml) 35-mm diam culture dishes (2.10^6 cells/dish; NUNC) were used for scrape-loading, electrophysiology, and immunoblot experiments. 12-mm coverslips (3.10^5 cells/coverslip) were employed for immunocytochemistry and electrophysiology. Glass slides (3.10^6 cells/slide; Rouvier-Gassalem), previously coated with poly-L-ornithine (15 μ g/ml) and natural mouse laminin (1 μ g/ml), were used for calcium imaging experiments. The culture medium was changed once a week, and on day 8, cytosine arabinoside (5 μ M) was added for 48 h. Under these conditions, after 21 d in culture, 95% of the cells stained positive for glial fibrillary acid protein (GFAP; Sigma-Aldrich; Table I). Unless otherwise stated, the number of dissociated cells added to the primary cultures of 2-wk-old astrocytes represented half the number of first-plated cells. This second plating resulted mainly in neurons, as the number of astrocytes was similar in astrocytes cultures and N/A cocultures (Table I). 7 d in coculture under these conditions, 62% of the cells stained positive for GFAP and 35% for microtubule-associated protein 2 (MAP 2; Table I), GFAP and MAP 2 being taken as astrocytic and neuronal markers, respectively (Matus et al., 1981; Raff, 1989).

Astrocytes/Neurons (A/N) Cocultures

A/N cocultures were obtained by replating the 19–21-d-old cultured astrocytes on 3- or 8-d-old neuronal primary cultures. Internal controls were provided by replating astrocytes originating from the same primary culture. Cell replating was achieved by washing the primary confluent astrocytes (Venance et al., 1997) in PBS and incubating in a trypsin-EDTA (0.05 and 0.02%, respectively) solution for 5 min at 37°C. Dissociated cells were harvested and trypsin was saturated with 20% FCS. After 10 min centrifugation (1,000 rpm), the pellet was resuspended in 1 ml of culture medium and the cells were replated at high density (6×10^5 cells/35-mm dish) on 3- or 8-d-old neuronal primary cultures, or into sister dishes without neurons. Cultures were stored at 37°C in a humidified atmosphere of 92% air and 8% CO_2 and were used 2 d later.

This procedure resulted in various proportions of the two cell types, as summarized in Table I. To quantify these proportions, immunocytochemistry was performed (see below). In addition, optic microscopy identification of typical morphology of astrocytes and neurons was facilitated by safranin staining. For this purpose cells were exposed to safranin (1%) during 5 min and then rinsed with water and alcohol.

Neuronal Primary Cultures

Primary neuronal cultures were prepared using the method described by El Etr et al. (1989) with slight modifications. Dissociated cells were plated on 35-mm dishes (10^6 cells; NUNC) successively coated with poly-L-ornithine (15 μ g/ml) and culture medium containing 10% FCS (Dutcher). After removing the last coating solution, cells were seeded in a serum-free medium consisting of 1:1 mixture of DME and F-12 nutrient (GIBCO BRL), supplemented with glutamine (2 mM), $NaHCO_3$ (13 mM), Hepes

Table I. Density of Astrocytes and Neurons in the Three Culture Model

	Density of cells					
	Immunocytochemistry GFAP, MAP2, and DAPI*		Safranin staining [‡]		INDO-1 fluorescence [§]	
	A	N	A	N	A	N
Spontaneous coculture Astrocytes	–	–	98 ± 6	44 ± 4	–	–
7-d coculture N/A Astrocytes	30 ± 1 28 ± 1	17 ± 2 1 ± 1	125 ± 9 121 ± 10	59 ± 3 3 ± 1	55 ± 4 61 ± 3	27 ± 3 2 ± 1
10-d coculture Astrocytes	–	–	117 ± 6 112 ± 3	52 ± 7 1 ± 1	–	–

Data are expressed as mean ± SEM and were obtained from four independent experiments. A, Astrocytes; N, neurons.

*Number of cells counted per field at ×40.

[‡]Number of cells counted per field at ×10.

[§]Number of cells counted per field at ×20.

(5 mM, pH 7.4), glucose (33 mM), penicillin-streptomycin (5 IU/ml and 5 µg/ml, respectively), and a mixture of salt and hormones containing insulin (25 µg/ml), transferrin (100 µg/ml), progesterone (20 nM), putrescine (60 µM), and sodium selenite (Na₂SeO₃, 30 nM). Cells were cultured at 37°C in a humidified atmosphere of 92% air and 8% CO₂.

Morphometric Analysis

MAP 2 immunostained neurons (revealed with an HRP-conjugated goat anti-mouse IgG, diluted 1:1,000) in N/A cocultures were examined under a light microscope at ×20 and ×40. Field images were captured and analyzed on computer image analysis software (NIH Image) using a CCD camera connecting the microscope to the computer. The perimeter of the cell body, the number of primary neurites, the total length of neurites, and the number of branching points were measured. 6 neurons were analyzed per coverslip.

Electrophysiology

Currents and voltages were recorded from astrocytes or neurons using the whole-cell configuration of the patch clamp technique and an inverted microscope equipped with Hoffmann optics (Diaphot; Nikon). Recordings were obtained with an Axopatch-1D amplifier and filtered at 5 kHz. Data were digitized and analyzed using pClamp6 software (Axon Instruments). Bath solution contained NaCl (140 mM), KCl (5.5 mM), CaCl₂ (1.8 mM), MgCl₂ (1 mM), glucose (10 mM), and Hepes (10 mM), pH 7.3. Recording pipettes (3–5 MΩ) were filled with internal solution containing KCl (140 mM), EGTA_{KOH} (5 mM), MgCl₂ (1 mM), Hepes (10 mM), and ATP Mg²⁺ (3 mM) with pH fixed at 7.3. For recording neuronal Na⁺ currents, CsCl (140 mM) was substituted for KCl in the pipette solution. The identification of neurons was confirmed electrophysiologically by their ability to generate tetrodotoxin (TTX)-sensitive Na⁺-mediated action potentials and by the presence of spontaneous synaptic currents. The analysis of synaptic currents was performed offline using Origin software (Microcal) to generate amplitude histograms.

Determination of Gap-junctional Permeability

Dye was injected into individual astrocytes during recording of the cell in the whole-cell configuration for 2 min with a patch pipette filled with Lucifer yellow (LY) 0.2% (dipotassium salt; Sigma-Aldrich). The pipette was then withdrawn and the microscopic field around the recorded cell was photographed 5 min later under epifluorescence illumination using appropriate filters. Gap-junctional permeability was quantified by determining the number of adjacent neighboring fluorescent astrocytes. GJC was also studied using the scrape-loading technique, as previously described (Venance et al., 1997). For each trial, data were quantified by measuring the fluorescence areas in 5 consecutive fields from digitized negatives using image analysis software (NIH Image). Quantification of the effects induced by different agents on GJC was performed by comparing the computed fluorescence areas between test and control conditions.

Calcium Wave Imaging

Measurements of [Ca²⁺]_i in rat cultured striatal astrocytes were made as previously described (Venance et al., 1997), under dual emission microfluorimetry using a cell-permeant fluorescent calcium probe, Indo1-AM (Sigma-Aldrich). All experiments were performed at room temperature, in a standard solution containing NaCl (140 mM), KCl (5.5 mM), CaCl₂ (1.8 mM), MgCl₂ (1 mM), glucose (10 mM), and Hepes (10 mM; Calbiochem-Novabiochem), with a pH of 7.3. The calcium-free solution contained NaCl (140 mM), KCl (5.5 mM), MgCl₂ (1.5 mM), Hepes (10 mM), glucose (10 mM) and EGTA (2 mM), with a pH of 7.3. Mechanical stimulation was performed using a patch clamp pipette driven by a hydrolic micromanipulator to gently touch the top of an astrocyte while ionomycin was superfused using a multichannel perfusion apparatus.

Immunocytochemistry

Cells were fixed with 2% paraformaldehyde at 4°C for 20 min and permeabilized with Tween-20 (0.05%, pH 7.5). Immunostaining for GFAP, MAP 2, and Cx43 was performed by incubating the cultures with antibodies diluted in the Tween-20 buffer. Cultures were simultaneously incubated with primary antibodies against GFAP (monoclonal rabbit, diluted 1:200; Sigma-Aldrich) and MAP 2 (monoclonal mouse, diluted 1:500; Sigma-Aldrich), or against GFAP and Cx43 (monoclonal mouse, diluted 1:200; Chemicon). Secondary antibodies were applied in different combinations and included rhodamine-conjugated goat anti-mouse IgG1 (diluted 1:250; Southern Biotechnology Associates, Inc.) and fluorescein-conjugated goat anti-rabbit IgG (diluted 1:200; Southern Biotechnology Associates, Inc.). Rinsing was performed between every incubation step. 4',6-diamino-2-phenylindol (DAPI; 0.1 mg/ml in water; Sigma-Aldrich) was used in the immunocytochemical experiments described above to stain cell nuclei in the cultures. After staining, coverslips were mounted on glass slides in moviol and examined with an upright microscope (Optiphot, Nikon) equipped with epifluorescence and appropriate filters.

Immunoblotting

Cells grown in 35-mm culture dishes were washed with Hepes buffer and lysed with 200 µl of boiled 2% SDS solution to prevent protein dephosphorylation by phosphatases. Alternatively, a cocktail of phosphatase inhibitors, orthovanadate (1 mM) and β-glycerophosphate (10 mM), was also used in complementary experiments and led to similar observations as those reported in Results. Protein concentration was determined with the bicinchoninic acid method, using BSA as the standard. Samples were separated on 10% polyacrylamide gels and transferred to nitrocellulose (Hybond-ECL; Amersham Pharmacia Biotech). Blots were tested with a monoclonal mouse Cx43 antibody diluted at 1:1,000 (Chemicon), followed by HRP-conjugated sheep anti-mouse secondary antibody diluted at 1:1,000 (Amersham Pharmacia Biotech) and developed using the chemiluminescence detection kit (Renaissance kit; NEN Life Science Products).

Semiquantitative densitometry analysis of the Western blots was performed using a CCD camera connected to a computer and image analysis software (NIH Image). Due to the addition of neurons, the total amount of proteins per 35-mm dish was higher in N/A cocultures than in astrocyte cultures. Indeed, the number of astrocytes was similar in the two cultures (Table I). To compare the same amount of astrocytic proteins collected from both culture models, samples used for immunoblotting did not contain the same protein amount, but represented the same percentage of total protein per 35-mm dishes. For astrocyte cultures, samples always contained 20 μ g protein. Similar observations were made in control experiments in which the same protein amount was analyzed from the two culture models.

Solutions and Chemicals

Except for Endothelin 1 (Neosystem), 6-cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX), and (RS)-baclofen (Tocris), all drugs were purchased from Sigma-Aldrich. All experiments were performed at room temperature (20–22°C).

Statistical Analysis

Data were expressed as mean \pm SEM. *n* refers to the number of independent experiments. All statistical analysis was performed on raw data and statistical significance was established at $P < 0.05$ and $P < 0.01$ as indicated.

Results

Neurons Increase Gap-junctional Permeability and Cx43 Expression in Astrocytes

The influence of neurons on dye coupling between conflu-

ent astrocytes was investigated using three different culture models that resulted in similar proportions of identified cell types. Indeed, cultures of astrocytes were pure at 95% and cocultures contained \sim 35% neurons and 65% astrocytes, identified with either MAP 2 and GFAP antibodies, respectively, or by their morphological features under light and fluorescence microscopy (Table I). Special care was taken to provide each model with an internal control for GJC in pure cultures of astrocytes.

GJC was first investigated in single astrocytes recorded with a patch clamp pipette loaded with LY. In cultures of astrocytes devoid of neurons (85 recorded cells in ten independent experiments), the average incidence of dye coupling per experiment was 25% for 1–10 coupled cells and 31% for >10 coupled cells, whereas in 44% of the cells, no dye coupling was observed (Fig. 1, A, B, and bottom). In spontaneous cocultures (99 recorded astrocytes among ten independent experiments), a marked increase (71%) in the incidence of dye coupling was essentially observed for astrocytes coupled to >10 cells (Fig. 1, bottom). In cocultures, the average incidence of dye coupling was 26% for 1–10 coupled cells, 53% for >10 coupled cells, and 21% for cells devoid of dye coupling (Fig. 1, C, D, and bottom).

The observations made above were supported by testing dye coupling using scrape-loading in N/A cocultures. In these conditions, junctional permeability in astrocytes was enhanced only when neurons were kept for one week or

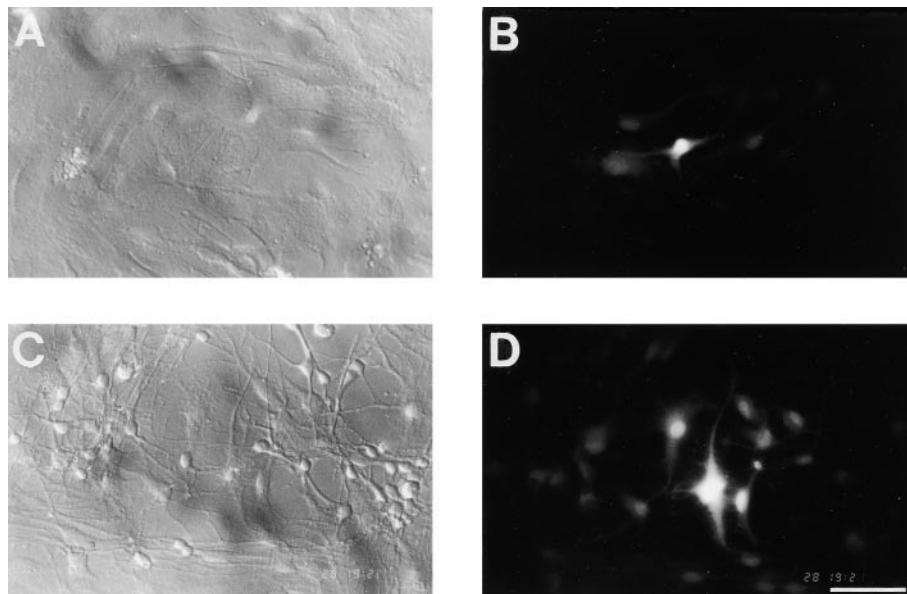


Figure 1. Dye coupling between astrocytes is upregulated by neurons. Dye coupling was determined by patch clamp recordings of astrocytes performed with a pipette solution containing 0.2% LY. A–D, Light micrographs taken with Hoffman optics of (A) astrocyte culture and (C) spontaneous coculture and fluorescence micrographs of the same fields (B and D, respectively) taken 5 min after withdrawal of the recording pipettes. The number of dye-coupled cells is increased in the presence of neurons, indicating an increase of the permeability of gap junctions. Bar, 100 μ m. Bottom, Summary diagram of dye coupling experiments obtained from 184 astrocytes filled with LY and classified in three categories, noncoupled (0), weakly coupled (1–10; stained cells), and highly coupled (>10 stained cells). The threeway χ^2 comparison test for the three categories reveals a significant difference in the distribution of astrocytic coupling measured in the absence and presence of neurons ($P < 0.01$).

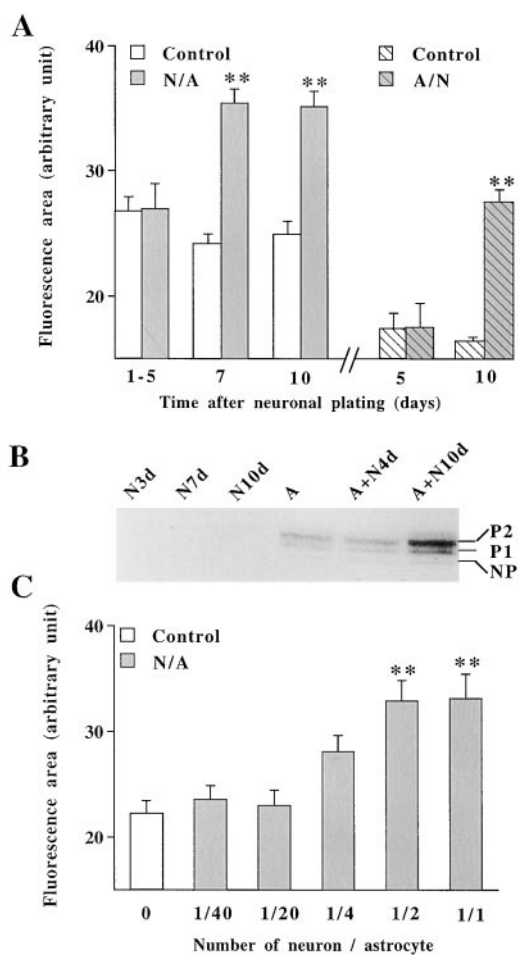


Figure 2. The increasing effect of neurons on astrocytic GJC is age- and density-dependent. **A**, The level of GJC (astrocytic coupling) was evaluated in N/A and A/N cocultures using the scrape-loading dye transfer technique, and was expressed as arbitrary units referring to the fluorescence area. A significant increase in astrocytic GJC was first detected 7 d after neuronal plating. The ratios between the fluorescence areas of tests (N/A cocultures) and internal controls (astrocyte cultures) are: 1.01, 1.47, and 1.41 for 1–5, 7, and 10 d, respectively. Similar observations were performed using A/N cocultures. The ratios between the fluorescence areas of tests and internal controls are: 1.01 and 1.68 for 5 and 10 d, respectively. The number of independent experiments shown varied from 4 to 18. Statistical analysis was carried out by *t* test. **B**, Western blot analysis of the Cx43 carried out on cellular extracts prepared, at the indicated ages, from neuronal cultures, astrocyte cultures, and cocultures (N, A, and A+N, respectively). The increasing effect of neurons on Cx43 expression was observed 1 wk after neuronal plating. Note that of the three isoforms (NP, P1, and P2) of Cx43, P2 predominated, and that the proportion was not modified by neurons. Data are representative of four to ten independent experiments. **C**, Effect of the proportion of neurons versus astrocytes on astrocytic GJC evaluated in N/A cocultures using the scrape-loading dye transfer technique. Several proportions were tested and corresponded to seeding of 50, 100, 500, 1,000, and 2,000 $\times 10^3$ neurons per 35-mm diam culture dish in which confluent astrocytes were cultured for 2 wk. The ratios between the fluorescence areas of tests and internal controls are: 1.06, 1.04, 1.27, 1.48, and 1.49 for the above indicated seeding densities, respectively. Four independent experiments were performed and statistical significance was established by one-way ANOVA, followed by post hoc Dunnett's multiple comparison.

more in cocultures (Fig. 2 A). This age dependence of neurons' influence on astrocytic GJC was confirmed using the A/N model at two critical ages, five and ten days after neuronal plating (Fig. 2 A). LY diffusion in N/A cocultures was markedly reduced by two potent inhibitors of GJC in astrocytes, 18 α -glycyrrhetic acid (10 μ M, 10 min) and endothelin-1 (0.1 μ M, 10 min). This reduction in dye diffusion reached 77 ($n = 4$) and 72% ($n = 4$), respectively, as compared with controls. This indicated that the neuron-induced increase in dye coupling is mediated by gap-junctional channels.

Since the upregulation of astrocytic GJC is related to neuron age, characterization of the maturation state of neurons in N/A cocultures was achieved by whole-cell recordings performed at two selected times after neuronal replating (Fig. 3; also see Table IV). After 4–5 days in cocultures, neurons exhibited small Na^+ inward currents in response to depolarization and infrequent, low amplitude spontaneous synaptic inward currents (SSIC; Fig. 3, A₁ and A₂). Five times larger inward Na^+ currents, and large and numerous SSIC were observed when recordings were performed one week after coculture (Fig. 3, B₁ and B₂; also see Table IV). Consistent with the well-known predominance of γ -amino-butyric acid (GABA)-ergic neurons in striatal cultures (Fraser et al., 1993), the majority of SSIC were identified as GABAergic, based on their reversal potentials at 0 mV, their kinetic properties, and their sensitivity to 10 μ M bicuculline. These data indicate that the first week of coculture may represent a turning point in the acquisition of these two electrophysiological properties that characterize their functional maturation.

To define the molecular mechanisms involved in the neuron-induced upregulation of gap junction permeability in astrocytes, Cx43 expression was investigated under various experimental conditions using the immunoblotting method. As shown by Western blot analysis performed with Cx43 antibodies, Cx43 expression could not be detected in pure neuronal cultures taken at either three, seven, or ten days after plating (Fig. 2 B). In contrast, in three-week-old astrocytic cultures, three distinct bands were detected at 42, 44, and 46 kD corresponding, respectively, to the nonphosphorylated (NP) and phosphorylated (P1 and P2) isoforms previously described for Cx43 (Musil et al., 1990). The P2 isoform was the most abundant, suggesting that the functional form of Cx43 predominates ($n = 10$). When N/A cocultures were used, no change in Cx43 expression was detected in astrocytes cocultured four days with neurons ($n = 4$), but an increase in immunoreactivity was observed ten days after neuronal plating ($n = 10$). In addition, no change in the proportion of the three Cx43 bands was detected, indicating that the protein state of phosphorylation was not modified in the presence of neurons.

Finally, several plating densities were tested in the N/A coculture model to determine whether the increase in astrocytic GJC depends on the cell proportions. As illustrated in Fig. 2 C, no increase in dye spread was observed when 7–10-d-old neurons were plated at low density, but the effect became significant at high density. This increase in astrocytic GJC was not attributed to the replating procedure since a secondary replating of astrocytes on primary confluent astrocytic cultures did not affect

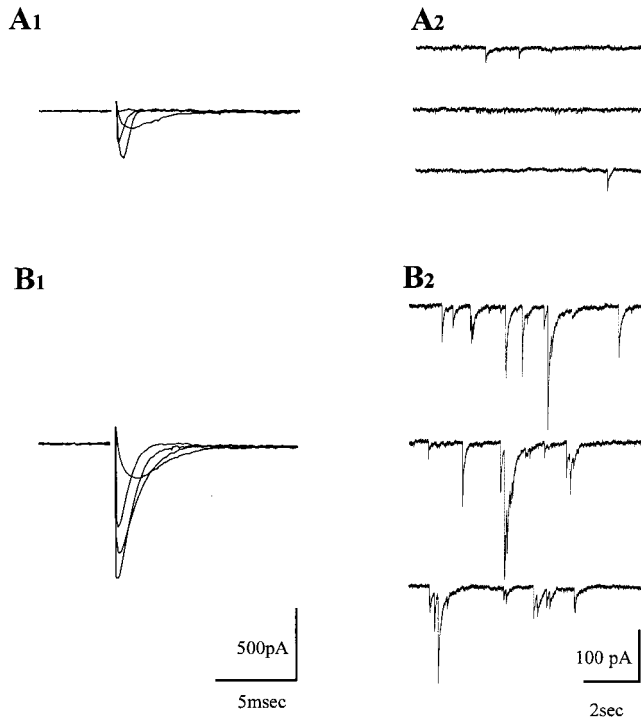


Figure 3. Patch clamp recordings of Na⁺ inward currents recorded from neurons in N/A cocultures 4 d (A₁ and A₂) and 10 d (B₁ and B₂) after neuronal plating. A₁ and B₁, Superimposed traces of currents in response to depolarizing pulses of +70, +80, +90, and +100 mV stepped from a holding potential of -100 mV. A₂-B₂, Occurrence of spontaneous synaptic inward currents illustrated by three consecutive current traces recorded at a holding potential of -60 mV. Current traces shown in A₁ and A₂ and B₁ and B₂ were from the same cells and were obtained with pipettes filled with a CsCl solution.

LY diffusion. In addition, the upregulation of astrocytic GJC induced by neurons seems specific. Indeed, replating of microglial cells on astrocytes at a density similar to that of neurons resulted in a decrease in both gap-

junctional permeability and Cx43 expression (data not shown).

Neurons Increase the Extent of Intercellular Calcium Waves in Astrocytes

Intercellular calcium waves were induced by mechanical stimulation of single astrocytes visually identified within monolayers of confluent astrocytes cultured in either the absence (Fig. 4 A) or presence (Fig. 4 B) of neurons. As indicated by changes in the ratio of Indo1 emissions in the two types of cultures, the mechanical stimulation of an astrocyte induced a large increase in [Ca²⁺]_i in the stimulated cell, which was followed by a calcium response in the neighboring cells. The proportion of cells responding to mechanical stimulation was ~65% in the investigated microscopic fields (*n* = 47) of astrocytic cultures (Table II). This percentage was not significantly modified in N/A cocultures three to four days after neuronal plating (57%, *n* = 12), but increased to 80 (*n* = 18) and 82% (*n* = 22) when experiments were performed six to seven days, and ten days, respectively, after neuronal plating (Fig. 4 C). These later increases resulted from neither a difference in the amplitude of the [Ca²⁺]_i rise generated in the stimulated cell, nor from a change in basal [Ca²⁺]_i measured in astrocytes (Table II).

Calcium release from internal stores represents an important and critical step in the cascade of events involved in the propagation of intercellular calcium waves (see Charles, 1998; Giaume and Venance, 1998). To determine whether the presence of neurons affected the quantity of mobilizable Ca²⁺ from internal Ca²⁺ pools, astrocytes were exposed to ionomycin (20 μM) in the absence of external Ca²⁺. Under these conditions, calcium responses were similar in astrocytic cultures (0.25 ± 0.02; *n* = 260) and in N/A cocultures older than six days (0.23 ± 0.01; *n* = 164). Moreover, the speed of propagation of calcium waves in astrocytes was not significantly different (Mann-Whitney test, *P* > 0.05) in either the absence (22 ± 4 μm/s, *n* = 5) or presence (28 ± 3 μm/s, *n* = 7) of neurons.

Table II. Effects of Neurons on the Propagation of Astrocytic Calcium Waves

Experimental conditions	Astrocytes	Responding astrocytes	Neurons	Responding neurons	Total astrocytes responding before (B), during (D), or after (A) neurons*			Ratio (F ₄₀₅ /F ₄₈₀)		
					B	D	A	Before stimulation	Stimulated	2 min after stimulation
					<i>n</i>	<i>n</i>	<i>n</i>			
Astrocytes	59 ± 3 (14)	42 ± 4 (14)	—	—	—	—	—	0.10 ± 0.01 (9)	0.71 ± 0.02 (14)	0.15 ± 0.02 (9)
Coculture N(3-4j)/A	58 ± 3 (12)	33 ± 4 (12)	22 ± 3 (9)	2 ± 1 (9)	18	19	0 (9)	0.13 ± 0.01 (9)	0.68 ± 0.03 (12)	0.18 ± 0.02 (9)
Astrocytes	61 ± 4 (18)	40 ± 3 (18)	—	—	—	—	—	0.10 ± 0.01 (12)	0.77 ± 0.05 (18)	0.15 ± 0.02 (12)
Coculture N(6-7j)/A	67 ± 3 (18)	57 ± 3 (18)	27 ± 3 (11)	9 ± 2 (11)	30	48	6 (11)	0.10 ± 0.01 (12)	0.79 ± 0.04 (18)	0.17 ± 0.01 (12)
Astrocytes	61 ± 4 (15)	37 ± 4 (15)	—	—	—	—	—	0.10 ± 0.01 (12)	0.81 ± 0.08 (15)	0.14 ± 0.02 (12)
Coculture N(10j)/A	58 ± 3 (22)	47 ± 3 (22)	18 ± 2 (12)	7 ± 1 (12)	38	42	13 (12)	0.10 ± 0.01 (12)	0.80 ± 0.06 (22)	0.13 ± 0.01 (12)

Data are expressed as mean ± SEM and were obtained from four independent experiments. Statistical significance was established using the Mann-Whitney statistical test by comparing each condition to its internal control represented by astrocyte cultures. Numbers in parentheses are the number of fields.

*Data are expressed as total number of astrocytes analyzed in the corresponding number of fields. For each responding neuron, the timing of the calcium response was analyzed in three surrounding astrocytes.

[†]The same fields were analyzed to provide the average total number of astrocytes per field and the corresponding astrocytes responding to the mechanical stimulation of a single astrocyte.

[‡]The same fields were analyzed to provide the averaged total number of neurons per field and the corresponding neurons responding to the mechanical stimulation of a single astrocyte.

^{||}*P* < 0.01.

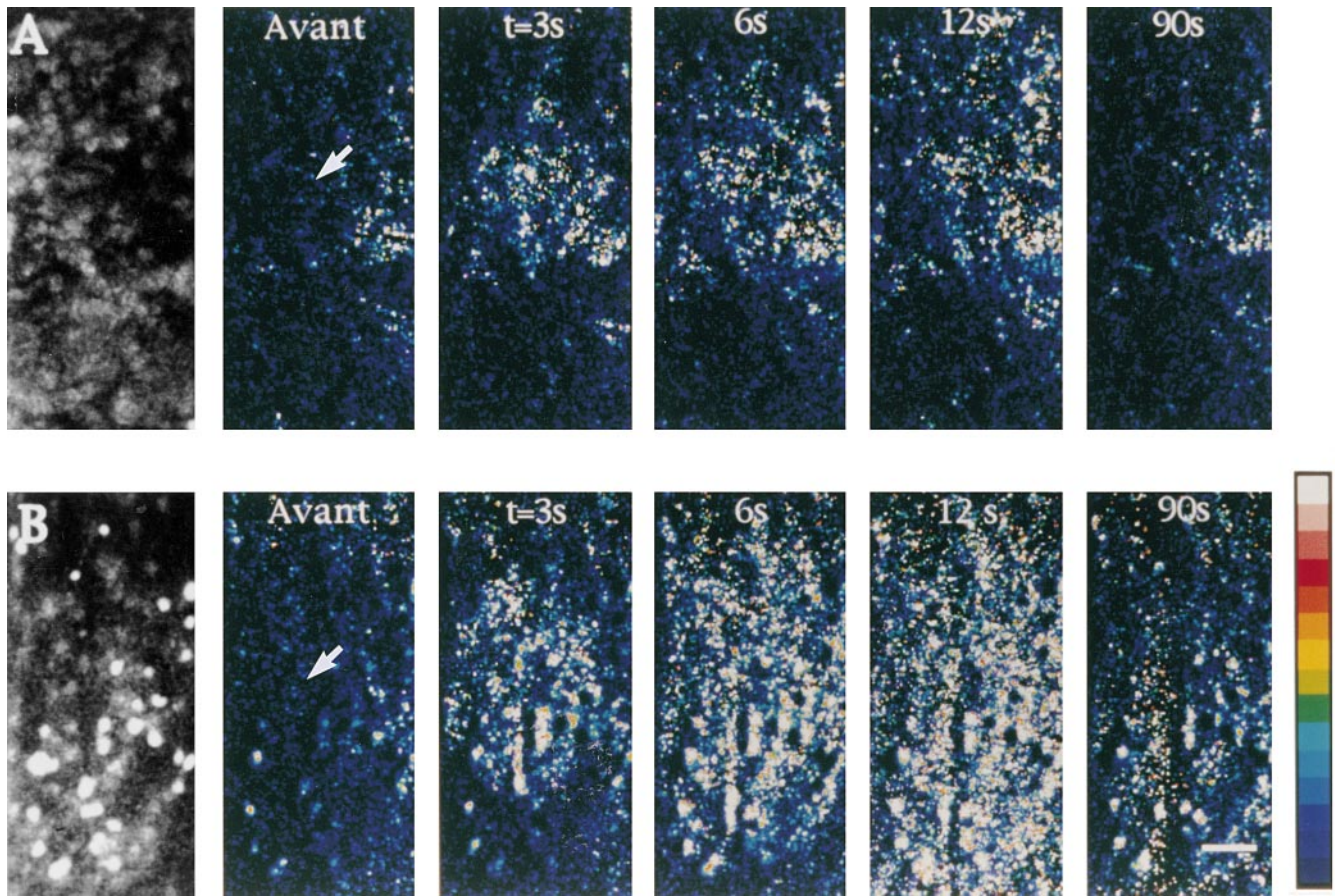
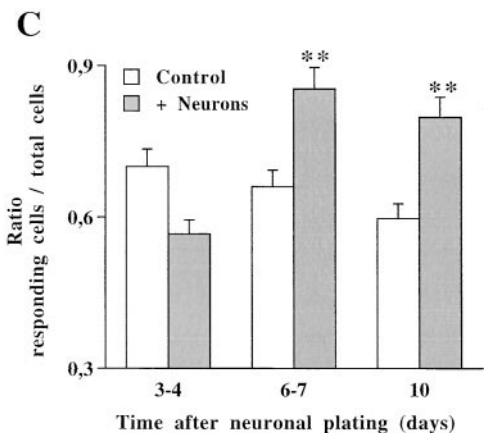


Figure 4. Effect of neurons on the extent of calcium wave propagation in astrocytes. Changes in $[Ca^{2+}]_i$ were monitored in astrocyte cultures (A) and N/A cocultures (B) by calcium imaging in cells loaded with Indo1-AM. Unitary cell stimulation (white arrows) was achieved by mechanical stimulation of a single astrocyte. The left-hand black and white images correspond to the fluorescence images taken at 480 nm. The propagation of a calcium wave is illustrated by sequential images taken at the indicated times and by pseudocolor representations of $[Ca^{2+}]_i$ expressed as the ratio of Indo1-AM emissions (F_{450}/F_{480}) caused by excitation at 355 nm. Note that in addition to the increase in astrocytic $[Ca^{2+}]_i$, a subpopulation of neurons identified as white spots in the 480-nm image also responded to the mechanical stimulation by a rise in $[Ca^{2+}]_i$. The scale refers to ratios from 0.01–1.00 that, in astrocytes, corresponded to estimated $[Ca^{2+}]_i$ values of 10–1,200 nM, respectively. Bar, 25 μ m. C, Summary diagram representing the ratio between responding astrocytes and the total number of astrocytes from microscopic fields in astrocyte cultures (Control) and in N/A cocultures (+ Neurons). Statistical significance of the effect of neurons was established for each time after neuronal plating by Student's unpaired *t* test. The ratios between tests (N/A cocultures) and internal controls (astrocyte cultures) are: 0.81, 1.29, and 1.34 for 3–4, 6–7, and 10 d, respectively. These analyses were performed in 99 fields obtained from four independent experiments detailed in Table II.



In agreement with previous observations (Nedergaard, 1994; Parpura et al., 1994; Charles, 1994; Bezzi et al., 1998), the propagation of calcium waves in astrocytes was associated with an increase of $[Ca^{2+}]_i$ in neurons (Fig. 4 B). In the N/A coculture model, the percentage of neurons responding to the mechanical stimulation of an astrocyte was found to increase after one week of coculture (Table II). As revealed by a temporal analysis, the $[Ca^{2+}]_i$ increase in astrocytes occurred mostly before or during the neuronal response (Table II). This indicated that the neu-

ron-induced increase in the propagation of calcium waves in astrocytes did not result from a secondary activation of a subpopulation of astrocytes.

N-methyl-D Aspartic Acid (NMDA)-induced Neurotoxicity Suppresses the Effect of Neurons on Astrocytic Coupling

Since the presence of mature neurons leads to an enhancement of astrocytic GJC, attempts were made to determine

whether neurodegeneration could induce a modification of GJC in astrocytes. Sustained stimulation of glutamatergic NMDA receptors in cultured striatal neurons results in dramatic neuronal death (Marin et al., 1994). Such a stimulation is not expected to exert a primary effect on astrocyte survival since glial cells, at least in culture, do not respond to NMDA (von Blanckenfeld et al., 1995).

When N/A cocultures were exposed for 60 min to a high concentration of NMDA (300 μ M) in the absence of Mg^{2+} , a significant neuronal death was observed 24 h later. This was demonstrated by the loss of neuritic processes and by cell body aggregation and shrinkage (Fig. 5, A and B). As determined by the extent of dye spread, when compared with untreated N/A cocultures, the astrocytic GJC in cocultures previously exposed to NMDA was reduced 24 h after NMDA application and was found to be close to basal levels measured in astrocytic cultures. In contrast, LY diffusion in pure culture of astrocytes was not modified by this NMDA treatment (Fig. 5 C).

Western blot analysis indicated that Cx43 expression was similar in astrocyte cultures and in cocultures examined 24 h after NMDA treatment, while it was higher in untreated cocultures ($n = 4$) (Fig. 5 D). In addition, no

change was observed in the proportion of the three isoforms (NP, P1, and P2) detected with the Cx43 antibody, suggesting that the phosphorylation state of Cx43 was not modified by the NMDA treatment.

Change in the Resting Membrane Potential of Astrocytes Is Not Involved in the Neuron-induced Upregulation of Astrocytic Coupling

It was recently reported that astrocytes exhibit a wide range of resting membrane potentials and are dynamically coupled by gap junction channels (McKhann et al., 1997). In addition, depolarization of astrocytes by high extracellular potassium enhances GJC in astrocytes (Enkvist and McCarthy, 1994), whereas other treatments that result in the opening of potassium channels are followed by a reduction in GJC (Granda et al., 1998). Accordingly, to determine whether the upregulation of astrocytic GJC was related to a change in the resting membrane potential in astrocytes, the membrane potential was monitored under current clamp conditions during the first minute after rupture of the patch membrane in both spontaneous cocultures and control culture of astrocytes.

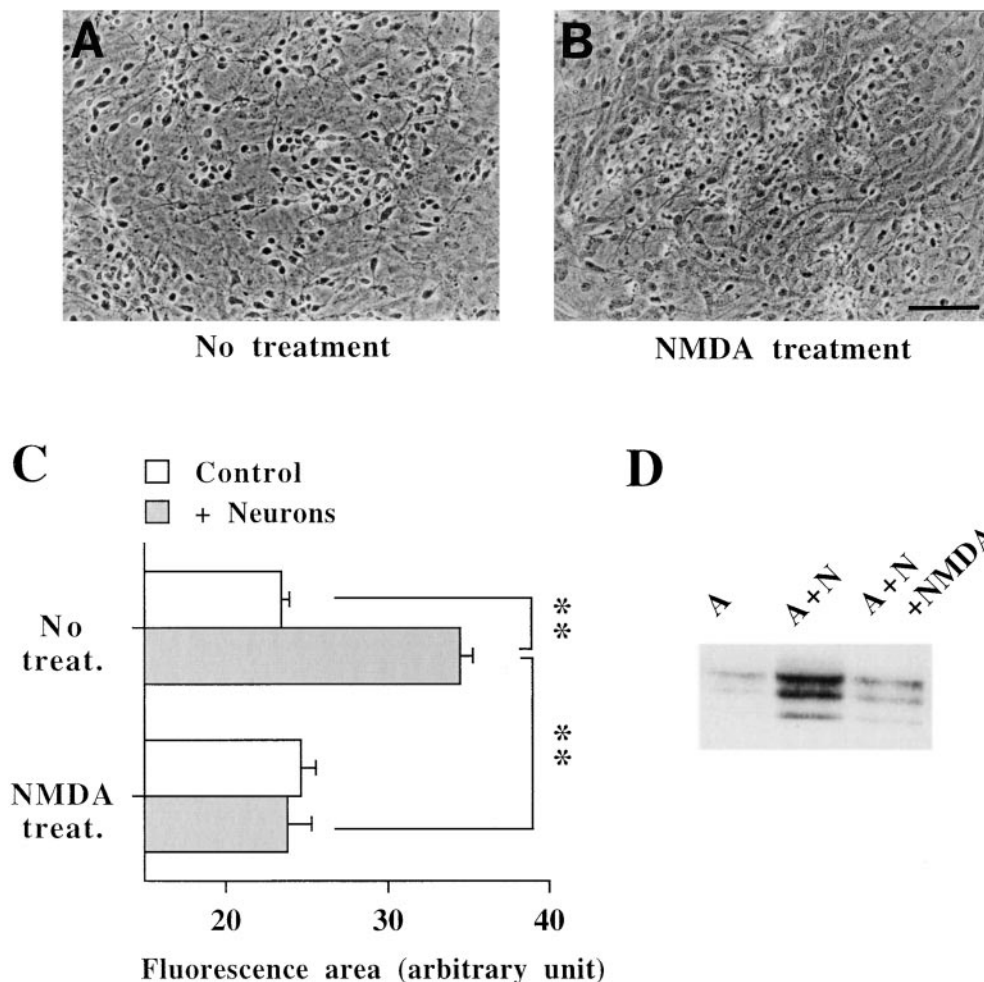


Figure 5. Reversal of the neuronal upregulation of astrocytic GJC by NMDA neurotoxic treatment. Phase-contrast micrographs of control N/A cocultures 11 d after neuronal plating without (A) and with (B) exposure to 300 μ M NMDA in the absence of external Mg^{2+} . Cells were incubated for 1 h and then fixed 24 h later. Bar, 100 μ m. C, Summary diagram of the effect of NMDA treatment on astrocytic GJC measured using the scrape-loading technique in astrocyte cultures (Control) and in N/A cocultures (+ Neurons). Statistical analysis was carried out by one-way ANOVA, followed by post hoc Bonferroni's multiple comparison. The ratios between the fluorescence areas of tests (N/A cocultures) and internal controls (astrocyte cultures) are: 1.47 in nontreated cells and 0.97 in NMDA-treated cells. These analysis were carried out from four to six independent experiments. D, Western blot analysis of Cx43 expression in astrocyte cultures (A), untreated N/A cocultures 11 d after neuronal plating (A+N), and in NMDA-treated cocultures (A+N+NMDA).

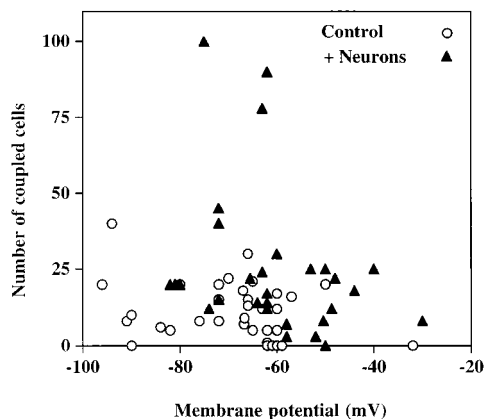


Figure 6. Lack of correlation between dye coupling and resting membrane potential in astrocytes. Whole cell recordings were performed with a pipette filled with LY 0.2% in cultures of astrocytes (Control) and in spontaneous cocultures (+ Neurons). Resting membrane potential was measured under current clamp conditions 1 min after rupture of the membrane, whereas the number of coupled cells was counted after 6 min. The plotting of these two parameters indicated that there is no relationship between the membrane potential of the recorded astrocytes and the number of coupled cells.

In these experiments, the number of coupled cells was increased from 11 ± 2 ($n = 36$) in astrocytes cultures to 26 ± 4 ($n = 30$) in N/A cocultures. In agreement with previous observations (McKhann et al., 1997), a great heterogeneity was found in the resting membrane potentials of astrocytes in both astrocytic cultures (from -32 to -96 mV) and N/A cocultures (from -30 to -82 mV). Moreover, the average resting membrane potential in astrocytes from astrocytic cultures (-69 ± 2 mV; $n = 36$) was higher than that observed in 10–14-d-old spontaneous cocultures (-61 ± 2 mV; $n = 30$; Mann-Whitney test, $P > 0.05$). This could suggest that the increase in astrocytic GJC in spontaneous cocultures is due to cell depolarization. However, no correlation was found between the membrane potential of the recorded cell and its level of dye coupling (Fig. 6). In fact, astrocytes with a low membrane potential (-30 to -50 mV) appeared less coupled (14 ± 3 coupled cells, $n = 9$) than those exhibiting a high membrane potential (-70 to -90 mV, 22 ± 5 coupled cells, $n = 22$).

Long-term Blockade of Synaptic Activity Prevents the Stimulatory Effect of Neurons on Astrocytic Coupling, but Not on Cx43 Expression

Since the neuron-induced upregulation of astrocytic GJC occurs when neurons exhibit large sodium inward currents and frequent spontaneous synaptic activity (Fig. 3), attempts were made to determine whether the pharmacological blockade of neuronal excitability prevents the neuronal-induced response. Inhibition of Na^+ inward currents and GABAergic SSIC was achieved by exposing N/A cocultures to $1 \mu\text{M}$ TTX and $10 \mu\text{M}$ bicuculline or $100 \mu\text{M}$ picrotoxin, respectively. Finally, although glutamatergic synaptic currents were not observed in our cocultures, the possible involvement of glutamate was also investigated. The hypothesis of an accumulation of extracellular glu-

tamate and subsequent stimulation of astrocytic glutamate receptors was tested since several processes could result in an accumulation of glutamate in the extracellular space of striatal cocultures (see Mailly et al., 1999). Indeed, glutamate can be released from neuronal axons (see Steinhäuser and Gallo, 1996) or from astrocytes (Parpura et al., 1994; Bezzi et al., 1998) and thus contributes to the external concentration of this excitatory amino acid. Accordingly, N/A cocultures were treated with CNQX, an antagonist of the AMPA/kainate subtype of glutamate receptors that was previously reported to prevent glutamate effect on GJC in astrocytes (Enkvist and McCarthy, 1994).

It is of particular interest that these different pharmacological treatments (TTX, bicuculline, and CNQX) significantly reduced GJC when they lasted for 24 h. These effects were still observed after 72 h (Fig. 7). However, no modification of GJC was observed when N/A cocultures were exposed for either 10 min or 6 h to TTX, bicuculline, picrotoxin, and CNQX ($10 \mu\text{M}$). Finally, controls were carried out to demonstrate that all of these long-term treatments (>24 h) had no effect on astrocytic GJC in astrocyte cultures devoid of neurons ($n = 4$).

No change in neuronal density was observed in all of these long-term pharmacological conditions, indicating that the decrease in astrocytic GJC observed in treated N/A cocultures did not result from a loss of neurons. In addition, several morphological criteria used as an index of neuronal differentiation were found to be constant before and after long-term treatments, suggesting that the decrease of astrocytic GJC was not due to a loss of neuronal maturation (Table III).

Surprisingly, the reducing effects of prolonged treatments with TTX, bicuculline, picrotoxin, or CNQX on GJC were not correlated with a change in Cx43 expression (Fig. 7, insert). Indeed, according to Western blot analysis performed 24–72 h after the beginning of these treatments, Cx43 expression in the treated N/A cocultures was similar to that found in untreated cells and no change in the proportion of the three Cx43 isoforms could be observed ($n = 4$). Attempts were also made to determine whether the subcellular localization of the Cx43 immunoreactivity was modified in treated cocultures. The abundant and heterogeneous distribution of this protein within the cytoplasm and at the plasma membrane in control and treated cocultures did not allow the detection of a clear difference in the pattern of immunofluorescence between these cocultures (data not shown).

A Direct Effect on Astrocytes of Released GABA, Glutamate, or a Soluble Factor Is Not Involved in the Neuron-induced Upregulation of Astrocyte Coupling

To determine whether the bicuculline- and picrotoxin-sensitive neuron-induced upregulation of GJC in astrocytes was linked directly to the effect of GABA released from striatal neurons on astrocytes, astrocyte cultures were exposed for either a brief (10 min) or a long (72 h) period to various concentrations of GABA (0.01 – 1 mM) in the presence of nipecotic acid ($10 \mu\text{M}$), an inhibitor of GABA uptake. The agonists of GABA_A and GABA_B receptors, muscimol (30 – $500 \mu\text{M}$, 20 min to 24 h) and baclofen (100 – $400 \mu\text{M}$, 20 min to 24 h), respectively, were also used in

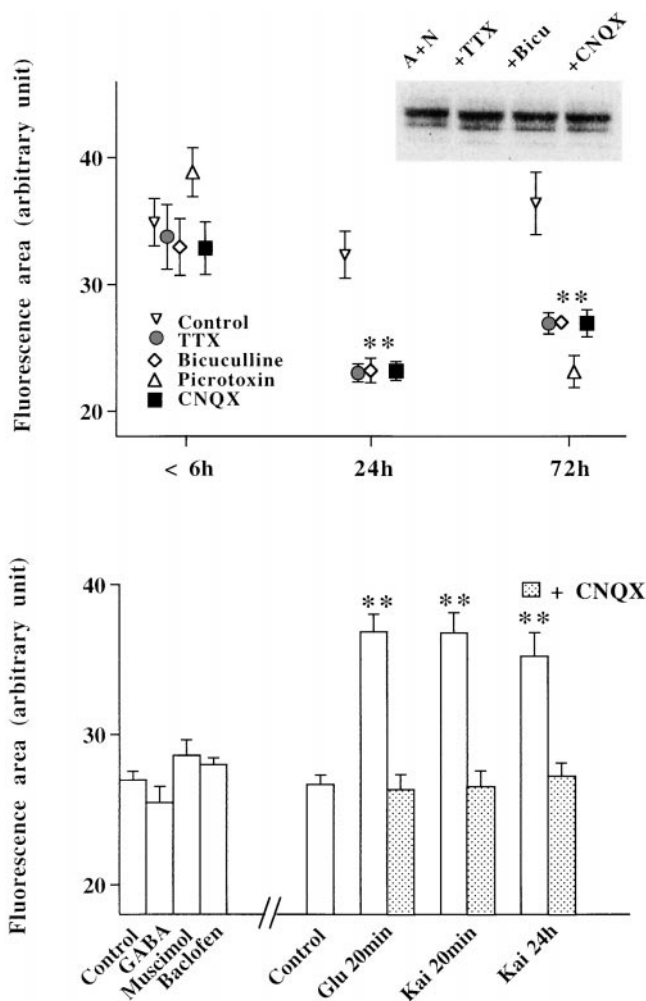


Figure 7. Effect of Na⁺ channel blocker, receptor antagonists, and neurotransmitters on the astrocytic GJC measured using the scrape-loading technique. Top, Treatments of N/A cocultures performed during the indicated times in the presence of either 1 μ M TTX, 10 μ M bicuculline (Bicu), 100 μ M picrotoxin, or 10 μ M CNQX; controls refer to nontreated N/A cocultures. For each indicated treatment duration, statistical significance of the effect of treatments was established by one-way ANOVA, followed by post hoc Dunnett's multiple comparison. The ratios between the fluorescence areas of internal controls (nontreated N/A cocultures) and tests (TTX, bicuculline, picrotoxin, and CNQX) are: 0.97, 0.94, 0.94, and 1.11 for <6 h; 0.71, 0.72, and 0.72 (picrotoxin nontested) for 24h; 0.74, 0.74, 0.74, and 0.64 for 72 h. These analyses were carried out from four to ten independent experiments. Insert, Western blot analysis of Cx43 expression in untreated N/A cocultures (A+N) after 24–72 h exposure to the indicated compounds. Note that there was no change in the level and proportion of the three Cx43 isoforms. Data are representative of four to eight independent experiments. Bottom, Effect of agonists and antagonists of the GABA_A, GABA_B, and AMPA/kainate receptors on astrocytic GJC studied in astrocyte cultures. Unless indicated, the compounds were used at the following concentration and incubation duration: 0.01–1 mM GABA (10 min to 72 h) with 10 μ M nipecotoc acid; 30–500 μ M muscimol (20 min to 24 h); 100–400 μ M baclofen (20 min to 24 h); 400 μ M glutamate; 400 μ M kainate; 10 μ M CNQX. Statistical significance of the effect of treatments was established by one-way ANOVA, followed by post hoc Dunnett's multiple comparison. The ratios between the fluorescence areas of tests and internal controls are: 0.94 for GABA, 1.06 for muscimol, 1.04 for baclofen, 1.38 for glutamate,

these experiments. None of these treatments resulted in a change in GJC.

To test whether the reducing effect of CNQX on GJC resulted from a direct effect of glutamate on astrocytes, cultures of astrocytes were exposed to glutamate or kainate. In agreement with previous reports (Enkvist and McCarthy, 1994), the incubation of astrocytic cultures with glutamate (400 μ M, 20 min) was followed by a large increase in the diffusion of LY (Fig. 7). Long-term application of glutamate was not performed since this excitatory amino acid is taken up in astrocytes, and the inhibitor of glutamate uptake, trans-PDC (1 mM), had a toxic effect when applied for 24 h (data not shown). However, the increasing effect of glutamate on GJC was mimicked by kainate (400 μ M) when this agonist of the AMPA/kainate receptor subtype was applied for either 20 min or 24 h (Fig. 7). Interestingly, in both cases, the effect of kainate could be prevented by a 20-min exposure of CNQX (10 μ M). This indicates that the increase of astrocytic GJC induced by the stimulation of glutamate receptors corresponds to a tonic and rapidly reversible response. Thus, this process seems to be different from that involved in the CNQX effect on cocultures since, in this case, prolonged (at least 24 h) exposure of the cells to the antagonist was required to reduce GJC.

Finally, no significant change in LY diffusion was observed when conditioned media from neuronal cultures ($n = 9$) or N/A cocultures older than seven days ($n = 9$) were added for 3, 24, or 48 h on either three-week-old astrocyte cultures or six-day-old N/A cocultures. This strongly suggests that a soluble factor (distinct from GABA) released from mature striatal neurons in the external medium, through a TTX-sensitive process, is not responsible for the neuron-induced upregulation of GJC in astrocytes.

Effect of TTX and Antagonists of GABA_A and AMPA Receptors on Spontaneous Neuronal Firing and Synaptic Activity

To study in detail the reducing effects of TTX and receptor antagonist long-term treatments on astrocytic GJC, spontaneous synaptic and spiking activities in neurons were recorded in N/A cocultures treated for 24 to 72 h with either TTX (1 μ M), bicuculline (10 μ M), or CNQX (10 μ M; Fig. 7). Spontaneous firing of neurons was studied under current clamp conditions for two- to five-minute periods. As summarized in Table IV, in control and bicuculline treatments, recorded cells exhibited a sustained firing of action potentials. In contrast, neurons exposed to either TTX or CNQX were characterized by the lack of spontaneous action potentials. Whereas TTX treatment did not affect the membrane potential, frequent depolarizations (22 ± 3 mV, 100 events recorded from five neurons) without firing of action potentials were monitored in the presence of CNQX. However, resting membrane potentials in neurons were similar in control and CNQX-

0.99 for glutamate+CNQX, 1.38 for kainate (20 min), 0.99 for kainate (20 min)+CNQX, 1.32 for kainate (24h), and 1.02 for kainate (24h)+CNQX. The number of independent experiments shown varied from four to 14.

Table III. Effects of Drug Treatments on Neuronal Density and Morphology

Drug	Density*	Cell body perimeter	Primary neurites	Branch points	Total neurite length
		μm			μm
Control	16 ± 1.1	31.7 ± 0.9	2.4 ± 0.1	1.1 ± 0.2	221.4 ± 17.9
TTX	16.8 ± 1.3	32.2 ± 0.8	2.5 ± 0.2	1.3 ± 0.3	233.8 ± 20
Bicuculline	14.2 ± 1.3	33.6 ± 0.6	2.7 ± 0.1	1.2 ± 0.2	240.2 ± 23.6
CNQX	15.9 ± 1.4	34.5 ± 0.8	2.6 ± 0.2	2.1 ± 0.4	255.3 ± 15.8

Data are expressed as mean ± SEM and were obtained from four independent experiments in which neurons were cocultured for 8–10 d with astrocytes (N/A cocultures). Treatments with TTX (1 μM), bicuculline (10 μM), and CNQX (10 μM) were performed for 24–72 h.

*Number of cells per field counted at $\times 40$.

treated cocultures (-55 ± 3 mV, $n = 8$ and -52 ± 2 mV, $n = 5$, respectively).

Continuous recordings of 10–12-min duration obtained in voltage clamp conditions were also performed to determine the frequency of SSIC in control and treated neurons. Amplitude histograms were obtained by analyzing 300 events in each situation (Fig. 8, A, B, and D), except for bicuculline (Fig. 8 C) since very few events were detected with this antagonist ($n = 47$, for an overall recording duration of 34 min). In TTX, bicuculline, and CNQX experiments, the SSIC frequency was diminished by 63, 97, and 43%, respectively, when compared with controls (Table IV). When changes in SSIC amplitude were compared, a drastic reduction was observed after bicuculline, TTX, and CNQX treatments (83, 86, and 65%, respectively; Table IV).

Altogether, these observations indicate that TTX and the receptor antagonists tested drastically reduced the spontaneous synaptic activity, whereas these compounds have a differential effect on the firing activity of striatal neurons. This suggests that the neuron-induced upregulation of astrocytic GJC is not directly related to neuronal firing, but rather is correlated with their synaptic activity.

Discussion

This study provides several lines of evidence indicating that neurons control the level of intercellular communication between astrocytes through gap junction channels. Indeed, both the function and expression of the main astrocytic Cx (Cx43) were found to be increased in the presence of neurons. These results confirm and extend previous observations made on rat cerebellar cocultures (Fisher and

Kettenmann, 1985) and on the intact optic nerve from the frog (Marrero and Orkand, 1996) suggesting that neurons facilitate glial communication. Using several models of cultures from the rat striatum, we were able to show that the neuronal density and neuron differentiation are two important factors in this neuroglial interaction. The stimulatory effect of neurons on astrocytic GJC was prevented in several conditions in which functional neuronal properties were altered. Altogether, these observations indicate that Cxs in astrocytes represent an important target in the neuroglial partnership and lead to the concept that, thanks to tight interactions, these two cell types set their level of communication reciprocally.

Regulations of Connexin 43 Expression and GJC in Astrocytes

Our results indicate that the neuronal-induced upregulation of Cx43 and associated GJC in astrocytes is dependent on the density and the age of the neurons since neuronal maturation is required for this process to occur. This effect of neurons was reversible since the NMDA-induced neurotoxicity resulted in a reduction of Cx43 immunodetection, without modification in the proportion of the three Cx43 isoforms, as well as in a decrease of GJC. This reversibility was complete after neuronal death since levels of Cx43 expression and gap junction permeability returned to those of control astrocytes. It is noteworthy that the neurotoxic NMDA treatment decreased, but did not abolish, the astrocytic GJC, extending a previous observation indicating that gap junction channels remain open during ischemic conditions (Cotrina et al., 1998b). Therefore, due to the persistence of gap junction channel perme-

Table IV. Electrophysiological Properties of Neurons

	INa amplitude	Synaptic activity			
		Frequency	Amplitude	Range	Spiking activity
			pA	pA	
$n\text{A}$	Hz				
4–5 d neuron	0.20 ± 0.08* (7)	0.03 ± 0.01* (7)	-27.7 ± 5.4* (7)	-6 to -52 (7)	-
7–10 d neuron	1.04 ± 0.21 (8)	0.82 ± 0.13 (10)	-91.8 ± 4.8 (10)	-5.6 to -456.8 (10)	Yes (6)
+TTX	0 (7)	0.30 ± 0.1* (7)	-12.6 ± 0.5* (7)	-5.3 to -95.2 (7)	No (7)
+Bicuculline	-	0.03 ± 0.01* (9)	-15 ± 1.7* (9)	-6 to -57.6 (9)	Yes (5)
+CNQX	-	0.47 ± 0.17‡ (11)	-32.1 ± 2.5* (11)	-5 to -329.8 (11)	No (5)

Data are expressed as mean ± SEM. Statistical significance was established by comparing all the conditions tested to their internal control, represented by 7–10 d neuron and using the Mann-Whitney statistical test. Treatments with TTX (1 μM), bicuculline (10 μM), and CNQX (10 μM) were performed for 24–72 h. Number in parentheses equals the number of independent experiments.

* $P < 0.01$

‡ $P < 0.05$.

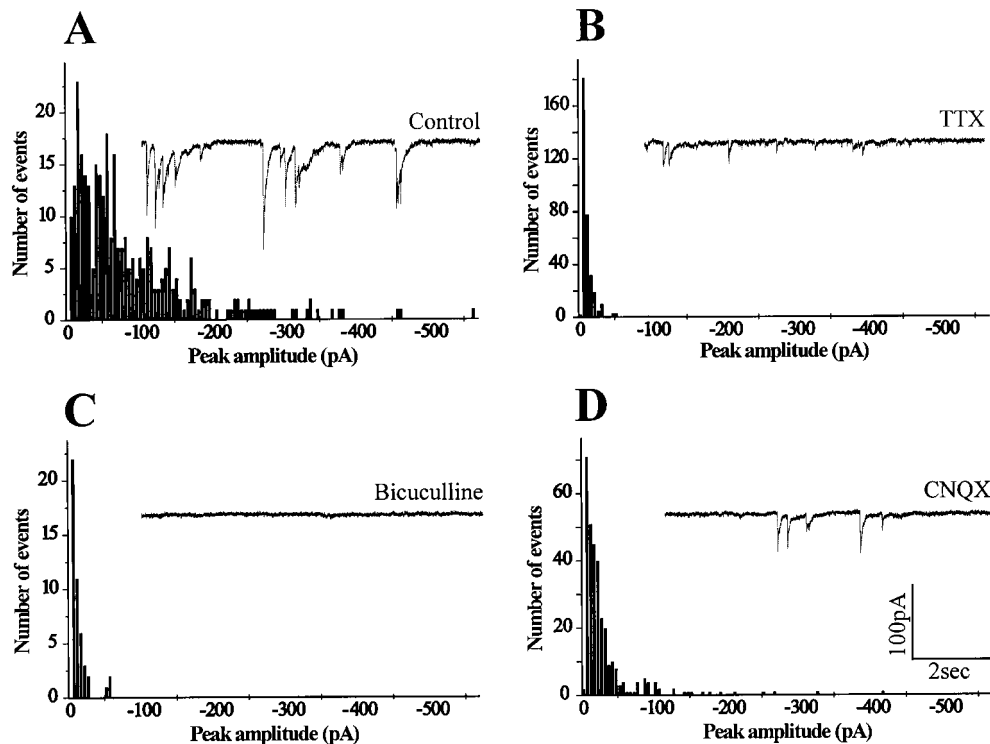


Figure 8. Effect of Na⁺ channel blocker and receptor antagonists on spontaneous synaptic activity recorded from neurons in N/A cocultures. A–D, Neurons were clamped at –60 mV and studied 24–72 h after treatments with either 1 μM TTX, 10 μM bicuculline, or 10 μM CNQX. Amplitude histograms were obtained by analyzing 300 events in each situation (A, B, and D), except in C since very few events were detected in the presence of bicuculline ($n = 47$, for an overall recording duration of 34 min). Only transient with uninterrupted rising phases were included to eliminate any signals involving multiple discharges.

ability in astrocytes, direct intercellular exchanges of death signals could occur and thus participate in the extension of neuronal damages (Lin et al., 1998).

The good correlation of the level of Cx43 expression with the extent of dye coupling and calcium waves in astrocytes strongly suggest that the neuron-induced facilitation of GJC likely resulted from an enhanced number of functional channels in astrocytes. However, different neuronal signals seem to contribute to the control of Cx43 expression and GJC. Indeed, several pharmacological treatments, including prolonged exposure of the N/A cocultures to a blocker of Na⁺ channels, or to antagonists of GABA_A and AMPA/kainate receptors, abolished the neuronal effect on astrocytic GJC, but did not affect the neuronal-induced expression of Cx43. In addition, the phosphorylation state of Cx43 does not seem to be implicated in this GJC regulation since these long-term pharmacological treatments did not modify the proportion of the three Cx43 isoforms. This later observation confirms that besides the well-documented regulation of Cx43 channels by phosphorylation processes (Saez et al., 1993; Bruzzone et al., 1996), this protein can be subjected to other posttranslational regulations.

Characteristics of the Upregulation of Astrocytic Coupling by Neurons

Several mechanisms known to regulate the permeability of gap junction channels in astrocytes have been considered to elucidate the process involved in the neuron-induced facilitation of GJC. The lack of correlation between the resting membrane potential of astrocytes and the number of dye-coupled astrocytes indicates that there is no direct relationship between these two parameters. A change in cal-

cium homeostasis does not seem to be involved in the neuron-induced increase in astrocytic GJC since the resting [Ca²⁺]_i and the filling of internal Ca²⁺ pools in astrocytes were found to be similar in astrocytic cultures and in N/A cocultures. GJC in astrocytes can be regulated by modifications in the astrocytic volume induced by hypoosmotic solutions (see Spray et al., 1999), and morphological changes have been observed in a subpopulation of astrocytes from the olfactory bulb cocultured with neurons (Matsutani and Yamamoto, 1997). However, based on the examination of LY injections or GFAP immunofluorescence stainings we failed to detect a neuronal influence on the shape or volume of the astrocytes. An eventual direct implication of GABA released from striatal neurons in the upregulation of astrocytic GJC in N/A cocultures is excluded since the applications on pure astrocytic cultures for short or long periods of either GABA or muscimol and baclofen, the agonists of GABA_A and GABA_B receptors, respectively, had no incidence on the level of intercellular communication. According to Enkvist and McCarthy (1994), the external application of glutamate increases dye coupling in cultured astrocytes from the rat cerebral cortex. A similar CNQX-sensitive glutamate-induced facilitation of GJC was observed in cultured striatal astrocytes. However, excluding a direct consequence of a tonic action of external glutamate on astrocytes, short applications of this AMPA/kainate receptor antagonist, which were efficient in blocking the stimulatory effects of glutamate or kainate on GJC in pure astrocytic cultures, did not suppress the neuronal-induced upregulation of GJC in astrocytes. Prolonged (>24 h) incubations with CNQX were indeed required to observe such a suppression. ATP has been reported to help astrocytic propagation of calcium waves (Cotrina et al., 1998a) and can be released by neu-

rons. However, such a process is unlikely to be involved since ATP has been shown to inhibit GJC in astrocytes (Enkvist and McCarthy, 1992). Finally, as observed for the induction of the glutamate transporter L-glutamate transporter type 1 (GLT1) in astrocyto-neuronal cultures, changes in the astrocytic phenotype can be mediated by a soluble factor released from neurons (Gegelashvili et al., 1997). However, conditioned media from either pure cultures of striatal neurons or N/A cocultures taken several times after neuronal plating had no effect on GJC in astrocyte cultures. Altogether, these observations suggest that the neuronal-induced increases in Cx43 expression and GJC rather could be due to membrane-associated factors. Such an implication of membrane-associated factors has already been reported for the inhibition of proliferation (Nakatsuji and Miller, 1998) and morphological changes (Matsutani and Yamamoto, 1997) of astrocytes, two processes that require direct contact with neurons.

The Facilitation of Astrocytic Communication Depends on Synaptic Activity

Whereas short-term applications of the sodium channel blocker, TTX, or the GABA_A and AMPA/kainate receptor antagonists, bicuculline and CNQX, respectively, were ineffective, the long-term applications (>24 h) of these pharmacological treatments all suppressed the neuronal stimulatory effect on astrocytic GJC. This suggested that either nerve firing or synaptic activity is required in the neuron-induced facilitation of GJC. Patch clamp recordings of striatal neurons cocultured with astrocytes revealed that the spontaneous firing of action potentials was suppressed by TTX and CNQX, but not by bicuculline long-term treatments. This is in agreement with other studies indicating that TTX prevents neuronal firing, CNQX blocks oscillating spiking activity in hippocampal neurons (Bacci et al., 1999), whereas bicuculline does not affect the firing properties of differentiated PC19 neurons (Lin et al., 1996). In contrast, these three pharmacological treatments led to a drastic reduction of synaptic activity. Indeed, as expected, only miniature postsynaptic events due to the spontaneous release at synaptic terminals were recorded under TTX treatment. Moreover, in agreement with the GABAergic nature of the synaptic activity in cultured striatal neurons (Fraser et al., 1993), spontaneous postsynaptic currents were abolished by bicuculline. Finally, while glutamatergic postsynaptic responses were absent in striatal cocultures, the blocking effect of CNQX on spontaneous GABAergic synaptic activity could indirectly result from a reduced amplitude of action potential due to a decrease in Na⁺ currents, as previously suggested (see O'Brien et al., 1998). Alternatively, a reduction of synaptic GABA release due to the inhibition of the AMPA/kainate receptor mediated rises in neuronal [Ca²⁺]_i induced by accumulated extracellular glutamate (Araque et al., 1998) could account for the blocking effect of CNQX on SSIC. Altogether, our observations suggest that neurons may control GJC in astrocytes through an activity-dependent mechanism more likely related to synaptic function than to spiking properties. They also reveal that the blockade of neuronal spontaneous synaptic activity reduces the functional properties of the Cx43 channels

without modification of their expression and phosphorylation states.

Besides the well-documented action of several regulatory factors on transcription, translation, gating, and degradation processes of Cx, the formation and function of these proteins also depend on extrinsic molecules that are not part of the channel itself. For instance, specific cell-adhesion molecules are required since liver cell adhesion molecule (LCAM) expression has been shown to be crucial for the establishment of functional gap junction channels (Musil et al., 1990) and the presence of Cx alone is not sufficient to induce communication (Mège et al., 1988). A correlation has also been observed between the lack of neuronal cell adhesion molecule (NCAM) and the deficiency of communication in either cell lines (Musil et al., 1990) or glial cells (Hofer et al., 1996). Accordingly, the synaptic activity-dependent regulation of astrocytic GJC described in our study could be related to the expression of proteins or to the assembly of membrane proteins modulating neuroglial interactions. Interestingly, carbohydrate polysialic acid and heparan sulfate proteoglycan have been reported to act as important activity-dependent regulators of neuronal and glial remodeling (Lauri et al., 1999; Theodosis et al., 1999). These two molecules are thought to act by regulating the assembly of active signaling complexes at the cell surface by interacting with several molecules, including CAMs that are expressed in neurons and astrocytes. Consequently, the expression of cell-cell association molecules dependent on neuronal activity could be involved in the reducing effect of channel blocker and receptor antagonists on astrocytic GJC.

Gap-junctional Communication in Astrocytes: A Target for Neuroglial Interaction

An important consequence of the upregulation of astrocytic GJC by neurons is the increase in the propagation of intercellular calcium waves. This finding demonstrates that neuronal activity controls a major modality of communication between nonexcitable glial cells. As recently reported, astrocytes enhance synaptic efficacy in collicular neurons (Pfrieger and Barres, 1997) and facilitate the appearance of specific excitable properties in spinal neurons (Li et al., 1999). Accordingly, neurons and astrocytes tightly and actively interact through a reciprocal setting of their specific mode of communication: synaptic transmission for neurons and calcium waves for astrocytes. Such a mutual control reinforces the concept of a collaboration between neuronal and astrocytic networks in information processing (Smith, 1992). Indeed, astrocytes respond to neuronal activity by an elevation in [Ca²⁺]_i, which triggers the release of gliotransmitters and in turn contributes to the regulation of synaptic activity (see Araque et al., 1999). Consequently, the magnitude of these neuroglial interactions likely depends upon the functional status of the astrocytic networks and particularly of their state of GJC, which is submitted to multiple neuronal regulations. In fact, in addition to synaptic activity, these later regulations may affect other astrocytic functions as well since GJC contributes to several of their properties. This includes for example, extracellular and intracellular homeostasis, trafficking and supply of energy metabolites, protection of

neurons against oxidative stress, and propagation of death signals (see Bruzzone and Giaume, 1999). Most of these astrocytic properties are involved directly in neuroglial interactions. Thus, neuronal damage or dysfunction could provide and/or amplify their effect by affecting the level of astrocytic GJC, which in turn may alter astrocytic properties. In agreement with this statement, changes of Cx43 topographical distribution in astrocytes have been reported to occur in vivo in response to traumatic or pathological situations (see Giaume and McCarthy, 1996).

The authors wish to thank Drs. F. Blomstrand and L. Venance, and Prof. K.D. Peusner for their helpful comments on the manuscript. We also wish to thank Drs. C.F. Calvo, J.A. Girault, and R. Bychkov, and J. Cordier and M. Alirezai for discussion and technical help during this work.

Submitted: 13 January 2000

Revised: 28 April 2000

Accepted: 18 May 2000

References

- Araque, A., V. Parpura, R.P. Sanzgiri, and P.G. Haydon. 1998. Glutamate-dependent astrocyte modulation of synaptic transmission between cultured hippocampal neurons. *Eur. J. Neurosci.* 10:2129-2142.
- Araque, A., V. Parpura, R.P. Sanzgiri, and P.G. Haydon. 1999. Tripartite synapses: glia, the unacknowledged partner. *TINS.* 22:208-215.
- Bacci, A., C. Verderio, E. Pravettoni, and M. Matteoli. 1999. Synaptic and intrinsic mechanisms shape synchronous oscillations in hippocampal neurons in culture. *Eur. J. Neurosci.* 11:389-397.
- Bezzi, P., G. Carmignoto, L. Pasti, S. Vesce, D. Rossi, B. Lodi Rizzini, T. Pozzan, and A. Volterra. 1998. Prostaglandins stimulate calcium-dependent glutamate release in astrocytes. *Nature.* 391:281-285.
- Bruzzone, R., and C. Giaume. 1999. Connexins and information transfer through glia. In *The Functional Roles of Glial Cells in Health and Disease*. R. Matsas and M. Tsacopoulos, editors. Kluwer Academic/Plenum Publishers, New York. 321-337.
- Bruzzone, R., T.W. White, and D.L. Paul. 1996. Connections with connexins: the molecular basis of direct intercellular signaling. *Eur. J. Biochem.* 238:1-27.
- Charles, A. 1994. Glia-neuron intercellular calcium signaling. *Dev. Neurosci.* 16:196-206.
- Charles, A. 1998. Intercellular calcium waves in glia. *Glia.* 24:39-49.
- Cornell-Bell, A.H., S.M. Finkbeiner, M.S. Cooper, and S.J. Smith. 1990. Glutamate induces calcium waves in cultured astrocytes: long-range glial signaling. *Science.* 247:470-473.
- Cotrina, M.L., J.H.C. Lin, A. Alves-Rodrigues, S. Liu, J. Li, H. Azmi-Ghadimi, J. Kang, C.C.G. Naus, and M. Nedergaard. 1998a. Connexins regulate calcium signaling by controlling ATP release. *Proc. Natl. Acad. Sci. USA.* 95:15735-15740.
- Cotrina, M.L., J. Kang, J.H.C. Lin, E. Bueno, T.W. Hansen, L. He, Y. Liu, and M. Nedergaard. 1998b. Astrocytic gap junctions remain open during ischemic conditions. *J. Neurosci.* 18:2520-2537.
- Dermietzel, R., and D.C. Spray. 1998. From neuro-glue (Nervenkitt) to glia: a prologue. *Glia.* 24:1-7.
- El Etr, M., J. Cordier, J. Glowinski, and J. Prémont. 1989. A neuro-glial cooperativity is required for the potentiation by 2-chloroadenosine of the muscarinic sensitive phospholipase C. *J. Neurosci.* 9:1473-1480.
- Enkvist, M.O.K., and K.D. McCarthy. 1992. Activation of protein kinase C blocks astroglial gap junctions and inhibits the spread of calcium waves. *J. Neurochem.* 59:519-526.
- Enkvist, M.O.K., and K.D. McCarthy. 1994. Astroglial gap junction communication is increased by treatment with either glutamate or high K⁺ concentration. *J. Neurochem.* 62:489-495.
- Fischer, G., and H. Kettenmann. 1985. Cultured astrocytes form syncytium after maturation. *Exp. Cell Res.* 159:273-279.
- Fraser, D.D., K. Hoehn, S. Weiss, and B.A. MacVicar. 1993. Arachidonic acid inhibits sodium currents and synaptic transmission in cultured striatal neurons. *Neuron.* 11:633-644.
- Gegelashvili, G., N.C. Danbolt, and A. Schousboe. 1997. Neuronal soluble factors differentially regulate the expression of the GLT1 and GLAST glutamate transporters in cultured astroglia. *J. Neurochem.* 69:2612-2615.
- Giaume, C., and K.D. McCarthy. 1996. Control of junctional communication in astrocytic networks. *TINS.* 19:319-325.
- Giaume, C., and L. Venance. 1998. Intercellular calcium signaling and gap junctional communication in astrocytes. *Glia.* 24:50-64.
- Granda, B., A. Taberner, L.I. Sanchez-Abarca, and J.M. Medina. 1998. The K-ATP channel regulates the effect of Ca²⁺ on gap junction permeability in cultured astrocytes. *FEBS Lett.* 427:41-45.
- Hofer, A., J.C. Saez, C.C. Chang, J.E. Trosko, D.C. Spray, and R. Dermietzel. 1996. C-erbB2/neu transfection induces gap junctional communication in competence in glial cells. *J. Neurosci.* 16:4311-4321.
- Kang, J., J. Li, S.A. Goldman, and M. Nedergaard. 1998. Astrocyte-mediated potentiation of inhibitory synaptic transmission. *Nat. Neurosci.* 1:683-692.
- Lauri, S.E., S. Kaukinen, T. Kinnunen, A. Ylinen, S. Imai, K. Kaila, T. Taira, and H. Rauvala. 1999. Regulatory role and molecular interactions of a cell-surface heparan sulfate proteoglycan (N-syndecan) in hippocampal long-term potentiation. *J. Neurosci.* 19:1226-1235.
- Li, Y.X., A.E. Schaffner, and J.L. Barker. 1999. Astrocytes regulate the developmental appearance of GABAergic and glutamatergic postsynaptic currents in cultured embryonic rat spinal neurons. *Eur. J. Neurosci.* 11:2537-2551.
- Lin, J.H.-C., H. Weigel, M.L. Cotrina, S. Liu, E. Bueno, A.J. Hansen, T.W. Hansen, S. Goldman, and M. Nedergaard. 1998. Gap-junction-mediated propagation and amplification of cell injury. *Nat. Neurosci.* 1:494-501.
- Lin, P., K. Kusano, Q. Zhang, C.C. Felder, P.M. Geiger, and L.C. Mahan. 1996. GABA_A receptors modulate early spontaneous excitatory activity in differentiating P19 neurons. *J. Neurochem.* 66:233-242.
- Maily, F., P. Marin, M. Israel, J. Glowinski, and J. Prémont. 1999. Increase in external glutamate and NMDA receptor activation contribute to H₂O₂-induced neuronal apoptosis. *J. Neurochem.* 73:1181-1188.
- Marin, P., M. Maus, S. Desagher, J. Glowinski, and J. Prémont. 1994. Nicotine protects cultured striatal neurons against N-methyl-D-aspartate receptor-mediated neurotoxicity. *Neuroreport.* 5:1977-1980.
- Marrero, H., and R.K. Orkand. 1996. Nerve impulses increase glial intercellular permeability. *Glia.* 16:285-289.
- Matsutani, S., and N. Yamamoto. 1997. Neuronal regulation of astrocyte morphology in vitro is mediated by GABAergic signaling. *Glia.* 20:1-9.
- Matus, A., R. Bernhardt, and T. Hugh-Jones. 1981. High molecular weight microtubule-associated proteins are preferentially associated with dendritic microtubules in brain. *Proc. Natl. Acad. Sci. USA.* 78:3010-3014.
- McKham II, G.M., R. D'Ambrosio, and D. Janigro. 1997. Heterogeneity of astrocyte resting membrane potentials and intercellular coupling revealed by whole-cell and gramicidin-perforated patch recordings from cultured neocortical and hippocampal slice astrocytes. *J. Neurosci.* 17:6850-6863.
- Mège, R.-M., F. Matsuzaki, W.J. Gallin, J.I. Goldberg, B.A. Cunningham, and G.M. Edelman. 1988. Construction of epithelioid sheets by transfection of mouse sarcoma cells with cDNAs for chicken cell adhesion molecules. *Proc. Natl. Acad. Sci. USA.* 85:7274-7278.
- Musil, L.S., B.A. Cunningham, G.M. Edelman, and D.A. Goodenough. 1990. Differential phosphorylation of the gap junction protein connexin 43 in junctional communication-competent and -deficient cell lines. *J. Cell Biol.* 111:2077-2088.
- Nakatsuji, Y., and R.H. Miller. 1998. Homotypic cell contact-dependent inhibition of astrocyte proliferation. *Glia.* 22:379-389.
- Nedergaard, M. 1994. Direct signaling from astrocytes to neurons in cultures of mammalian brain cells. *Science.* 263:1768-1771.
- O'Brien, R.J., S. Kamboj, M.D. Ehlers, K.R. Rosen, G.D. Fischbach, and R.L. Huganir. 1998. Activity-dependent modulation of synaptic AMPA receptor accumulation. *Neuron.* 21:1067-1078.
- Parpura, V., T.A. Basarsky, F. Liu, K. Jęftinija, S. Jęftinija, and P.G. Haydon. 1994. Glutamate-mediated astrocyte-neuron signalling. *Nature.* 369:744-747.
- Pfrieger, F.W., and B.A. Barres. 1997. Synaptic efficacy enhanced by glial cells in vitro. *Science.* 277:1684-1687.
- Raff, M.C. 1989. Glial cell differentiation in the rat optic nerve. *Science.* 243:1450-1455.
- Saez, J.C., V.M. Berthoud, A.P. Moreno, and D.C. Spray. 1993. Gap junctions: multiplicity of controls in differentiated and undifferentiated cells and possible functional implications. In *Advances in Second Messenger and Phosphoprotein Research*. S. Shenolikar and A.C. Nairn, editors. Raven Press, New York. 163-198.
- Smith, S.J. 1992. Neuromodulatory astrocytes. *Curr. Biol.* 4:807-810.
- Spray, D.C., H.S. Duffy, and E. Scemes. 1999. Gap junctions in glia. In *The Functional Roles of Glial Cells in Health and Disease*. R. Matsas and M. Tsacopoulos, editors. Kluwer Academic/Plenum Publishers, New York. 339-359.
- Steinhäuser, C., and V. Gallo. 1996. News on glutamate receptors in glial cells. *TINS.* 19:339-345.
- Theodosis, D.T., R. Bonhomme, S. Vitiello, G. Rougon, and D.A. Poulain. 1999. Cell surface expression of polysialic acid on NCAM is a prerequisite for activity-dependent morphological neuronal and glial plasticity. *J. Neurosci.* 19:10228-10236.
- Venance, L., N. Stella, J. Glowinski, and C. Giaume. 1997. Mechanism involved in initiation and propagation of receptor-induced intercellular calcium signaling in cultured rat astrocytes. *J. Neurosci.* 17:1981-1992.
- Verderio, C., A. Bacci, S. Coco, E. Pravettoni, G. Fumagalli, and M. Matteoli. 1999. Astrocytes are required for the oscillatory activity in cultured hippocampal neurons. *Eur. J. Neurosci.* 11:2793-2800.
- Verkhatsky, A., R.K. Orkand, and H. Kettenmann. 1998. Glial calcium: homeostasis and signaling function. *Physiol. Rev.* 78:99-141.
- von Blanckenfeld, G., K. Enkvist, and H. Kettenmann. 1995. Gamma-aminobutyric acid and glutamate receptors. In *Neuroglia*. H. Kettenmann and B.R. Ransom, editors. Oxford University Press, Oxford, UK. 335-345.