Cell coupling and Cx43 expression in embryonic mouse neural progenitor cells

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Summary

Embryonic neural progenitors isolated from the mouse striatal germinal zone grow in vitro as floating cell aggregates called neurospheres, which, upon adhesion, can be induced to differentiate into the three main cell types of the central nervous system (CNS), that is, astrocytes, neurons and oligodendrocytes. To study the possible role of connexins and junctional communication during differentiation of neural progenitors, we assessed cell-to-cell communication by microinjecting Lucifer Yellow into neurospheres at various times after adhesion. Cells located in neurospheres were strongly coupled, regardless of the differentiation time. Microinjections performed on the cell layers formed by differentiated cells migrating out of the neurosphere established that only astrocytes were coupled. These observations suggest the existence of at least three distinct communication compartments: coupled proliferating cells located in the sphere, uncoupled cells undergoing neuronal or oligodendrocytic differentiation and coupled differentiating astrocytes. A blockade of junctional communication by 18-β-glycyrrhetinic acid (βGA) reduced, in a concentration-dependent manner, the viability of undifferentiated neural progenitor cells. This effect appeared to be specific, inasmuch as it was reversible and that cell survival was not affected in the presence of the inactive analog glycyrrhyzic acid. Addition of βGA to adherent neurospheres also decreased cell density and altered the morphology of differentiated cells. Cx43 was strongly expressed in either undifferentiated or differentiated neurospheres, where it was found both within the sphere and in astrocytes, the two cell populations that were dye coupled. Western blot analysis further showed that Cx43 phosphorylation was strongly increased in adherent neurospheres, suggesting a post-translational regulation during differentiation. These results point to a major role of cell-to-cell communication and Cx43 during the differentiation of neural progenitor cells in vitro.

Key words: Astrocyte, CNS, Connexin, Gap junction, Neuron, Oligodendrocyte

Introduction

Connexins are a multigenic family of proteins that can be distinguished on the basis of their molecular weight. Six oligomerized connexins form a hemichannel (or connexon) that can bind to another hemichannel present in the membrane of an adjacent cell, resulting in a complete intercellular channel. These channels, which accumulate at specific membrane areas defined as gap junctions, allow cells to share small molecules (<1 kDa) such as metabolites and ions. Such an exchange of signals (or intercellular coupling) has been implicated in various cellular mechanisms, including ionic homeostasis, electrical synchronization, proliferation and migration (Bruzzone et al., 1996; Kumar and Gilula, 1996).

Connexins are expressed in the three main cell types of the central nervous system (CNS), that is, astrocytes, oligodendrocytes and neurons. Increasing evidence suggests that connexins regulate different aspects of neuronal networks and neuron-glia interactions, in both the developing and mature brain (for a review, see Dermietzel and Spray, 1998). This would require the establishment of communication compartments that isolate groups of coupled cells engaged in a coordinated activity from other populations that participate in distinct processes. Such a dynamic regulation of cell-cell communication seems particularly well suited to respond to the instructive cues operating during CNS development (Kandler and Katz, 1995; Nadarajah et al., 1997; Naus and Bani-Yaghoub, 1998). Thus, in vivo studies have shown that, in the developing neocortex, neuroblasts and proliferating cells located in the ventricular zone are coupled, whereas differentiating or migrating neurons are not (LoTurco and Kriegstein, 1991; Bittman et al., 1997). Evidence for coupling territories has also been obtained in other areas of neurogenesis, such as the subventricular zone and rostral migratory stream (Miragall et al., 1997; Menezes et al., 2000). Furthermore, there is an inverse correlation between the expression of connexin43 (Cx43) and Cx26, and cell proliferation (Miragall et al., 1997; Bittman and LoTurco, 1999), suggesting that coupling and cell cycle of neural progenitors may be interdependent.

Studies in vitro have shown that Cx43 expression and coupling are downregulated as differentiation proceeds in either immortalized rodent neuroblasts (Rozental et al., 1998) or cell lines derived from rat peripheral neurotumoral cells (Donahue at al., 1998). In a human teratocarcinoma cell line consisting of precursors able to differentiate into post-mitotic neurons, Cx43 protein expression is decreased after neuronal

differentiation, and a pharmacological blockade of gap junctions antagonizes the acquisition of the neuronal phenotype (Bani-Yaghoub et al., 1999a; Bani-Yaghoub et al., 1999b). Taken together, these observations strongly suggest that, on the one hand, Cx43 plays a role in mediating intercellular coupling during proliferation of neural precursors and, on the other hand, a downregulation of coupling and Cx43 expression occurs with cell differentiation.

To gain insight into the role of connexins and junctional communication during the early steps of CNS differentiation, we have taken advantage of a primary culture of neural progenitor cells (Reynolds and Weiss, 1992; Reynolds et al., 1992). These cells have the ability to self-renew in the presence of epidermal growth factor (EGF) (Reynolds and Weiss, 1996) and basic fibroblast growth factor (Ciccolini and Svensden, 1998), forming spheric cell aggregates called neurospheres. Moreover, upon removal of growth factors and adhesion onto a coated support, cells migrate out of the sphere and form layers of differentiated neurons, astrocytes and oligodendrocytes. Neurospheres can be regarded, therefore, as a simplified in vitro model of CNS differentiation. Finally, neural progenitors expanded in vitro can be grafted into the brain of recipient animals and have been shown to differentiate in vivo (Svensden et al., 1996; Vescovi et al., 1999; Temple, 2001). It is not known whether multipotent neural progenitor cells need to be coupled and whether coupling territories are modulated during cell differentiation. To address these issues, we have isolated neural progenitors from the mouse embryonic striatal ventricular zone (SVZ) and found that proliferating cells located in adherent neurospheres exhibited a strong intercellular coupling. By contrast, only astrocytes were coupled among the outgrowth of differentiating and migrating cells. In addition, we found that the two cell populations that were coupled expressed Cx43, which became predominantly phosphorylated after differentiation. Finally, treatment of neurospheres with the gap junction inhibitor 18-β-glycyrrhetinic acid (βGA), strongly reduced the viability of both proliferating and differentiating neural cells. These observations demonstrate that junctional coupling is widespread among CNS progenitors and substantiate the hypothesis for an active role of Cx43 and intercellular communication during proliferation and differentiation of neural progenitors.

Materials and Methods

Reagents

The tissue culture medium consisted of DMEM-F12 (BioMedia, Boussens, France), supplemented with B27 (Gibco-BRL, Eragny, France). Human recombinant epidermal growth factor (EGF), penicillin, streptomycin, poly-D-lysine, fibronectin, 18-β-glycyrrhetinic acid (βGA), glycyrrhyzic acid (GZA), bromodeoxyuridine (BrdU), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were from Sigma (St. Louis, MO). Plasticware and tissue culture flasks were from Costar (Cambridge, MA) or Falcon (Plymouth, UK). Equipment for electrophoresis was from Bio-Rad Laboratories (Richmond, CA). Calf intestinal alkaline phosphatase was from Roche Molecular Biochemicals (Meylan, France); Trasylol® was from Bayer-France (Puteaux, France). The micro BCA protein assay and the SuperSignal West chemiluminescent substrate kits were obtained from Pierce (Rockford, IL). Mice were purchased from IFFA-CREDO (Lyon, France) and killed in accordance with the currently approved guidelines for animal handling.

Preparation, growth and differentiation of neurospheres

Pregnant C57BL/6J mice at a gestational age of 14.5 to 15 days were killed by cervical dislocation, and embryos were transferred in Petri dishes containing ice-cooled HBSS (Hanks balanced solution without Ca^{2+} and Mg^{2+}). Striata were dissected from the embryos and mechanically dissociated as previously described (Reynolds et al., 1992; Ben-Hur et al., 1998). The viability of the single cell suspension was assessed using a 0.4% Trypan blue solution (Gibco-BRL). Viable cells were seeded at a concentration ranging from 10^5 to 2.5×10^5 cells/ml in uncoated T75 tissue culture flasks. Culture medium (hereafter referred to as standard medium) consisted of DMEM-F12 supplemented with B27 (1/50 dilution) and contained 0.524 mg/ml stabilized glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin and 20 ng/ml human recombinant EGF. Cells were cultured at 37°C in a humidified atmosphere of 90%O_2/10%CO_2, and fresh EGF was added every two days for the first 8 days, during which progenitors grew into floating neurospheres. Subsequently, two thirds of the culture medium was replaced twice a week. Neurospheres were passaged by centrifugation at 200 ***g*** for 5 minutes; the pellet was resuspended in one third of the final volume, gently dissociated by pipetting and transferred into flasks containing two thirds of the volume of fresh medium. Each neurosphere preparation was kept in culture up to a maximum of one month, which corresponded to four cell passages.

Differentiation of neural progenitors was induced by transferring floating neurospheres to an adhesive support and withdrawing EGF from standard medium. Briefly, glass coverslips, plastic flasks and Petri dishes were coated successively with poly-D-lysine (10 μg/ml in water) and fibronectin (10 μg/ml in PBS). An average of 10 floating neurospheres kept for a minimum of 10 days (one passage) and up to 3 weeks (three passages) in culture were transferred in 20 μl of standard culture medium onto freshly coated coverslips. For the large scale preparation of adherent neurospheres needed for biochemical analysis, floating neurospheres were collected by centrifugation (5 minutes at 200 ***g***), resuspended in 2 ml culture medium and transferred onto coated plasticware. Neurospheres were incubated for 2 hours to initiate cell adhesion, and culture medium without EGF was gently added. Unless otherwise mentioned, adherent neurospheres were cultured for 8 days, during which medium was changed (half the volume) twice.

Dye coupling experiments

The incidence of cell-cell communication was assessed by microinjecting the membrane-impermeant tracer Lucifer Yellow (LY; M_r 443) into single cells located in different areas of neurospheres that had been adherent for the specified times. Just before injections, neurospheres that were adherent to coated glass coverslips were transferred onto the heated (37°C) stage of an inverted microscope (Zeiss IM35), and 10 mM Hepes was added to the culture medium. Electrodes were pulled to a resistance of 50-60 MΩ and filled with a 4% solution of LY in 1 M LiCl. Following the impalement of individual cells, the tracer was injected by iontophoresis, applying square pulses of 0.1 nA amplitude, 900 msecond duration and 0.5 Hz frequency for 5 minutes. Each injection site was photographed with Ektachrome 400 daylight film (Kodak, Rochester, NY) under phase contrast and epifluorescence illumination, using appropriate filters, before and/or after pulling out the electrode. Coupling was defined as LY diffusion from the injected cell to at least one adjacent cell. Microinjected neurospheres were fixed in 4% paraformaldehyde for 30 minutes at room temperature, rinsed three times in phosphate-buffered saline (PBS) and stored at 4°C for subsequent immunocytochemical analysis (see below).

Uncoupling experiments

Single cell suspensions (3×10^5 cells/ml) of striatal progenitors in standard culture medium were aliquotted in 1.5 ml tubes containing

either the gap junction inhibitor βGA (Davidson et al., 1986) or its inactive analog GZA at the specified final concentrations. Because of their lipophilic nature, both drugs were dissolved in a mixture of dimethyl sulfoxide (DMSO) and ethanol (3:2, vol:vol), pre-warmed at 37°C and mixed by vortexing before addition to the medium (final concentrations of DMSO and ethanol did not exceed 0.1%). Solvent alone was added to control cultures. 100 μl of cell suspension per well was distributed in 96-well plates (eight wells/condition), and cell viability was measured three days later, in the absence of any medium change. Reversibility was assessed on floating neurospheres at the end of the 3 day incubation by adding two volumes of fresh standard culture medium. Four days after dilution of the drugs, neurospheres were examined with an inverted microscope and photographs were taken for each condition. To assess cell viability on adherent cells, floating neurospheres expanded in culture for 10 days were spun at 200 ***g*** for 5 minutes, dissociated as a single cell suspension and eventually aliquoted (1×10^5 cells/ml) into 96-well plates that had been previously coated with poly-D-lysine and fibronectin. For long-term treatment of adherent neurospheres, 0.25% serum albumin (BSA) was added to the culture medium to avoid the toxic effects of DMSO. Adherent neurospheres were cultured for eight days, during which βGA (50 μM) and DMSO were added every two days, and half the medium was replaced with fresh medium every three days. To test whether the uncoupling agent had an effect on differentiated cells, neurospheres were first cultured for 3 days under control conditions, to allow differentiation of neural progenitor cells, before adding βGA (50 μM) for the next 5 days, as described above.

Cell proliferation and viability assays

Adherent neurospheres were incubated for 24 hours in the presence of 10 μM BrdU, fixed for 30 minutes at room temperature in ethanol/acetic acid (95%/5%, vol/vol) and then processed for immunostaining with a mouse IgG1 antibody (1/50; Becton Dickinson, San Jose, CA), as described elsewhere (Ben-Hur et al., 1998). Viable cells in either floating or adherent neurospheres were quantified colorimetrically by the MTT assay, in which the tetrazolium salt is cleaved in the mitochondria of metabolically active cells to form a precipitable formazan dye (Mosmann, 1983). MTT was added at a final concentration of 1 μg/ml to each well for 4 hours at 37°C, and precipitates were solubilized in MTT dissolving buffer (100 μl/well). Plates were first incubated 5 minutes at 37°C, followed by a 10 minute period at room temperature on a shaking stage to allow color development. Differences in optical densities between 570 nm and 630 nm were measured spectrophotometrically.

Antibodies

Differentiating oligodendrocytes were detected with the O4 mouse monoclonal antibody (IgM 1:20; a kind gift from Susan Barnett, Glasgow, Scotland). Neurons were identified with TujI, a mouse IgG2a antibody raised against the neuron specific β3-tubulin (1:500; Babco, Richmond, CA). Astrocytes were labeled with either a rabbit polyclonal (1/200; DAKO, Glostrup, Denmark) or a mouse monoclonal IgG1 (1:100; Chemicon, Temecula, CA) antibody raised against glial fibrillary acidic protein (GFAP). A mouse monoclonal IgG1 and a rabbit polyclonal IgG against Cx43 (both from Zymed, San Francisco, CA) were employed for immunocytochemistry (at a dilution of 1:250 and 1:500, respectively) and western blotting (at a dilution of 1:3,000). Fluorescein-conjugated goat anti-mouse IgG and anti-rabbit IgG, rhodamine-conjugated goat anti-mouse IgG, biotin-conjugated anti-rabbit IgG and streptavidin-7-amino-4-methylcoumarin-3-acetic acid (AMCA)-conjugated donkey anti-rabbit IgG were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

Immunocytochemistry

Floating neurospheres were fixed 4 hours at 4°C in 2% paraformaldehyde in PBS, washed in PBS, incubated overnight at 4°C in a 15% sucrose solution and eventually embedded in a drop of Tissue-Tek compound (Miles Scientific, Naperville, IL). Specimens were immediately frozen on dry ice and stored at –20°C until frozen sections, cut at the 10 μm setting of a CM-3000 cryostat (Leica, Nussloch, Germany), were collected on coated slides and processed for immunocytochemistry. Adherent cells were fixed in 4% paraformaldehyde, rinsed repeatedly in PBS and incubated with primary and secondary antibodies as indicated below. For Cx43 labeling, cells were fixed in 2% paraformaldehyde, then washed and permeabilized with 0.1% Triton X-100 in blocking solution for 30 minutes.

All samples were incubated overnight at 4°C in a humidified chamber with primary antibodies diluted in blocking solution (PBS supplemented with 5% normal goat serum and 5% normal donkey serum), rinsed repeatedly in PBS and then stained for 30 minutes at room temperature with the cognate secondary antibodies diluted in blocking solution. Triple immunolabeling was carried out using primary mouse antibodies of the IgM and IgG classes and a rabbit antibody, as previously described (McKinnon et al., 1990; Ben-Hur et al., 1998). After several washes in PBS, immunostained cells were preserved in Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA). The specificity of the immunofluorescent staining was assessed for each experimental condition by performing the first incubation in the absence of primary antibodies. No staining was observed under such conditions.

Photographs were taken with a Leica microcope (DMRB model) equipped with a Coolsnap camera system (Princeton Instruments, Evry, France), stored on a PC and processed with Photoshop 5.5 software (Adobe Systems, San Jose, CA). Confocal microscopy was performed using an Axiovert LSM 510 microscope (Zeiss, Jena, Germany) equipped with 63× 1.4 NA and 100× 1.4 NA objective lenses and three separate laser beams. Samples were labeled with fluorescein (FITC)- and/or rhodamine (TRITC)-conjugated secondary antibodies. Double-labeled samples were analyzed either simultaneously or sequentially. In either case, FITC was excited by the blue beam and detected through an interferential narrow band filter (BP 505-550 nm), whereas TRITC was excited by the red beam and detected through a long pass filter (LP 650 nm).

Western blotting and dephosphorylation assay

Protein extracts were prepared in a lysis buffer containing 50 mM NaCl, 50 mM Tris-HCl (pH 8), 0.1% SDS, 1% NP-40, 0.5% deoxycholate, supplemented with 10 μg each chymostatin, leupeptin and pepstatin and 10 KU/ml Trasylol. Neurospheres were solubilized in 2 ml lysis buffer (for either a pellet of floating neurospheres or neurospheres adherent to a T75 flask) by repeated aspiration (20 times) through a 23-gauge needle. Crude extracts were centrifuged at 4°C for 30 minutes at 10,000 ***g***, and supernatants were then submitted to protein quantification using the Micro BCA protein assay kit, according to the manufacturer's recommendations. Equal amounts of total protein were incubated overnight at 37°C in the presence of 100 units of calf intestinal alkaline phosphatase. Control reactions were carried out with phosphatase buffer only or in the presence of phosphatase inhibitors (2 mg/ml Na-orthovanadate, 10 mM EDTA and 10 mM PO_4), as previously described (Musil et al., 1990a). Samples were quenched by addition of gel loading buffer (0.025 M Tris pH 6.8, 0.5% SDS, 1% β-mercaptoethanol, 0.025% bromophenol blue, 17.5% glycerol) and aliquots were loaded on a 12.5% polyacrylamide SDS-gel. After separation, proteins were transferred to nitrocellulose membranes (Protran; Schleicher & Schuell, Keene, NH). Membranes were saturated for 30 minutes in PBS containing 5% non-fat dried milk and 0.1% Tween and subsequently incubated overnight with either the monoclonal or polyclonal anti-Cx43

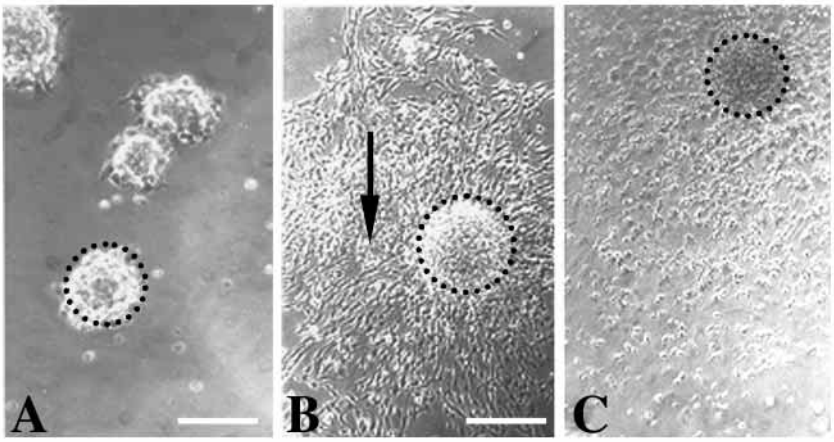

Fig. 1. Adherent neuropsheres form layers of migrating cells. Phase-contrast micrographs show that, 30 minutes after adhesion of neurospheres on coated coverslips (A), few cells have started to migrate out of the sphere. 24 hours after adhesion (B), outgrowing cells formed layers (arrow) surrounding the sphere (dotted circle). After 7 days (C), the number of cells decreased in the sphere (dotted circle), which was flattened, and increased in the outgrowth. Bar, 150 μm (A); 300 μm (B,C).

antibody (Zymed) diluted 1:3,000 in saturation buffer. After extensive washes in PBS containing 0.1% Tween, membranes were probed for 1 hour with the corresponding horseradish-peroxidase-conjugated secondary antibodies (Chemicon) diluted in saturation buffer (anti-rabbit: 1:10,000; anti-mouse: 1:5,000), rinsed again in PBS-Tween and revealed with the SuperSignal West chemiluminescent substrate kit, according to the manufacturer's instructions. The time of incubation in ECL detection reagents and exposure (typically 1 minute) to Hyperfilm (Amersham-Pharmacia, Buckinghamshire, UK) were identical for all experimental conditions.

Results

Dye coupling in neurospheres

LY was microinjected either into the center of the neurosphere or into the layers composed of migrating cells (Fig. 1), using neurospheres that had been adhering for times ranging between 45 minutes and 23 days (Table 1). At each adhesion time, strong coupling was detected between the cells located in the sphere (Table 1), so that microinjection of LY resulted in the rapid transfer of the tracer to at least 20 neighboring cells (Fig. 2A). By contrast, only 36 out of the 68 cells that were microinjected in the sphere outgrowth were coupled. These migrating cells were identified according to their morphology and/or their immunocytochemical labeling (Figs 2 and 3). On the basis of their diamond-shaped cell body (Fig. 2B) and expression of the cytoskeletal protein GFAP (Fig. 3), we established that all the communicating cells of the outgrowth were astrocytes. Thus, in differentiating neurospheres, astrocytes form coupled clusters of migrating and differentiating cells, which communicate exclusively among themselves. Differentiating oligodendrocytes, identified as

Table 1. Cell coupling in neurospheres

Injection site		Adhesion time: 45 min to 3 days	4-8 days	16-23 days
Neurosphere		+ (25)	+ (3)	+ (4)
Outgrowth of the neurosphere	Astrocyte	+ (8)	+ (14)	+ (14)
	Neuron	– (2)	– (9)	– (6)
	Oligodendrocyte	– (2)	– (4)	– (9)

Floating neuropsheres were transferred to coated coverslips and, after the indicated adhesion times, individual cells were microinjected with Lucifer Yellow (LY) in two distinct sites: the neurosphere, containing round progenitor and precursor cells, and the layers composed of the outgrowth of differentiated cells, which were identified according to morphology and/or immunocytochemistry. The plus sign denotes the presence of cell coupling (i.e., LY diffusion from the injected cell to at least an adjacent one); the minus sign indicates the absence of LY diffusion. The number of microinjections is indicated in parentheses.

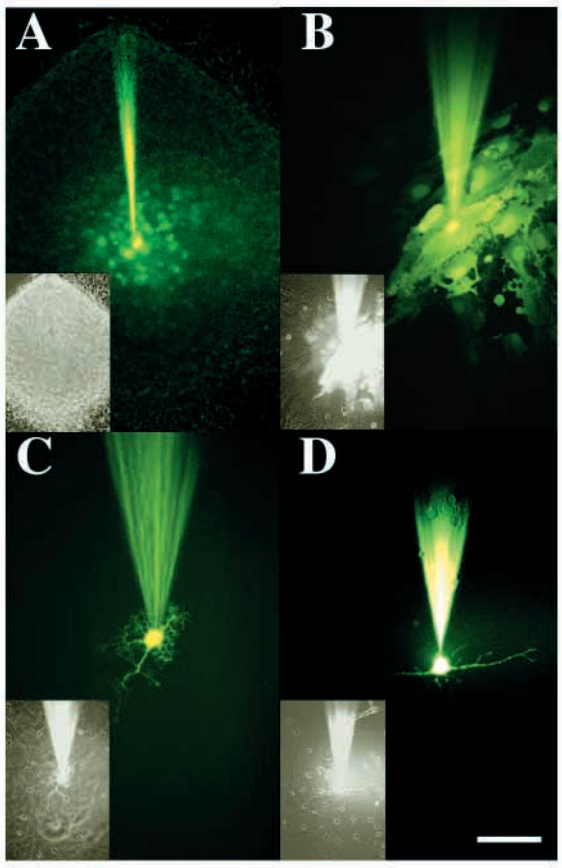

Fig. 2. Dye coupling is present between cells in the neurosphere and between differentiating astrocytes. Lucifer Yellow (LY) was microinjected by iontophoresis into single cells. Fluorescence micrographs were taken 5 minutes after injection; insets show the corresponding phase-contrast views of the same fields. Dye coupling was strong between cells located in the neurosphere center (A) and between cells identified as astrocytes (B). By contrast, no LY diffusion to adjacent cells was observed when an oligodendrocyte (C) or a neuron (D) was microinjected. Bar, 10 μm.

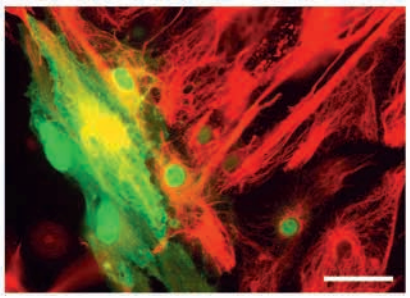

Fig. 3. Identification of coupled astrocytes. After microinjection experiments, neurospheres were fixed and labeled as indicated in the Materials and Methods. A typical cluster of dye-coupled cells (green) showed positive staining for the astrocytic protein GFAP (red). Bar, 20 μm.

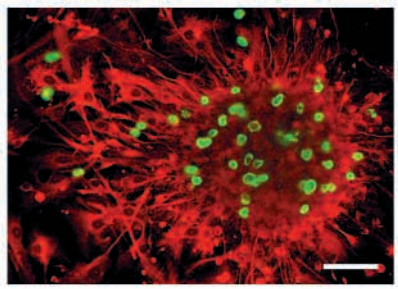

Fig. 4. Cell proliferation in adherent neurospheres. Neurospheres were allowed to adhere for 7 days and then incubated for 24 hours in the presence of 10 μM BrdU. Cells were fixed and processed for double-labeling with anti-BrdU (green) and anti-GFAP (red) antibodies. Note that most BrdU-positive cells were concentrated within the sphere, whereas only a few of them were found in the outgrowth. Bar, 100 μm.

multipolar cells (Fig. 2C) that expressed the antigen recognized by the monoclonal antibody O4, did not exhibit LY diffusion to adjacent cells. Microinjections performed after 16-23 days of adhesion (Table 1), at which time mature oligodendrocytes synthesize myelin basic protein (data not shown), confirmed that these cells were still uncoupled. Similarly, LY remained within injected neurons, which were identified as bipolar cells with a small cell body and thin processes (Fig. 2D), expressing β3-tubulin.

Cell proliferation in neurospheres

To determine whether neurospheres maintained the potential to proliferate following removal of growth factors and adhesion, adherent neurospheres were incubated for 24 hours in the presence of 10 μM BrdU and then processed for immunostaining. After 7 days of adhesion, proliferating cells were concentrated in the neurosphere, whereas only scattered BrdU-positive cells were observed in the outgrowth formed by migrating cells (Fig. 4). Similar observations were made when spheres had been adherent for only 45 minutes and were composed of neural progenitor cells expressing nestin, an intermediate filament protein of neuroepithelial stem cells (data not shown). Thus, cells within the borders of spheres consisted of a mixed population of undifferentiated neural progenitors and neural precursor cells committed to a specific cell type of which they do not yet express the morphological and immunocytochemical markers. For the sake of clarity, we hereafter refer to these two populations of proliferating cells as neural progenitor cells. By contrast, cell layers are composed of differentiated, mainly non-proliferating cells.

Effect of 18-β-glycyrrhetinic acid (βGA) on neural progenitor cells

To assess whether junctional coupling was involved in the growth of neural progenitors, floating neurospheres were incubated in the presence of increasing concentrations of βGA. The size of neurospheres was drastically reduced by βGA (Fig. 5C,D), in comparison with cultures exposed to equal concentrations of the inactive analog glycyrrhyzic acid (GZA) (Fig. 5A,B), solvent (DMSO/ethanol) alone or mock-treated in standard medium (data not shown). To verify the specificity of the βGA effect, we tested its reversibility by incubating floating neurospheres for an additional 4 day

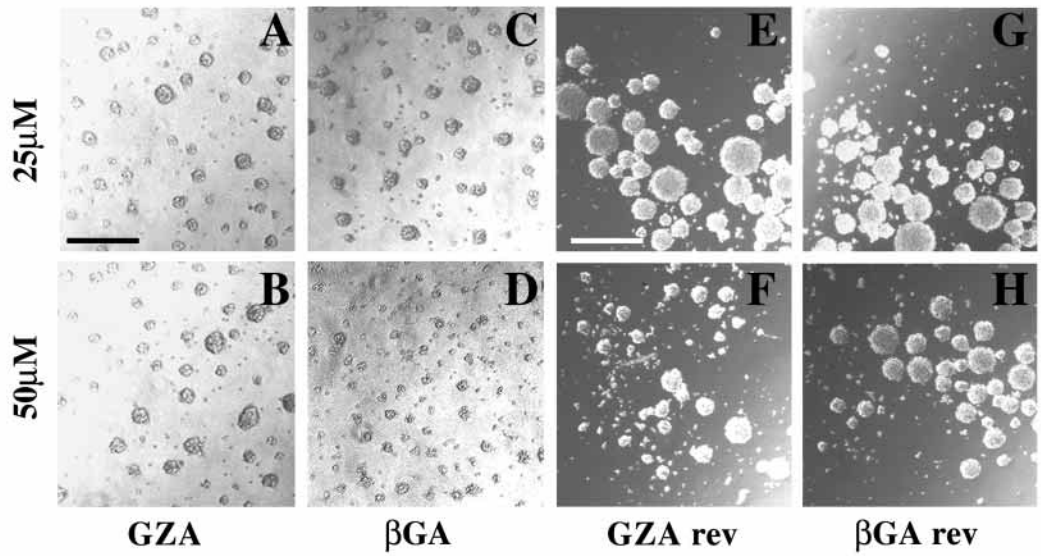

Fig. 5. A blockade of gap junctions decreases the viability of neural progenitors. Single cell suspensions of striatal progenitors were grown for 3 days in the presence of either the uncoupling agent 18-β-glycyrrhetinic acid (βGA) or its inactive analog glycyrrhyzic acid (GZA). The size of neurospheres was reduced by βGA (C,D) in comparison with that of GZA-treated ones (A,B). This effect was reversible (rev), as the size of floating neurospheres pre-treated with βGA (G-H) was similar to that of GZA-treated cultures (E-F) following an additional 4 day recovery period, as described in the Materials and Methods. Bar, 300 μM (A-D); 780 μM (E-H).

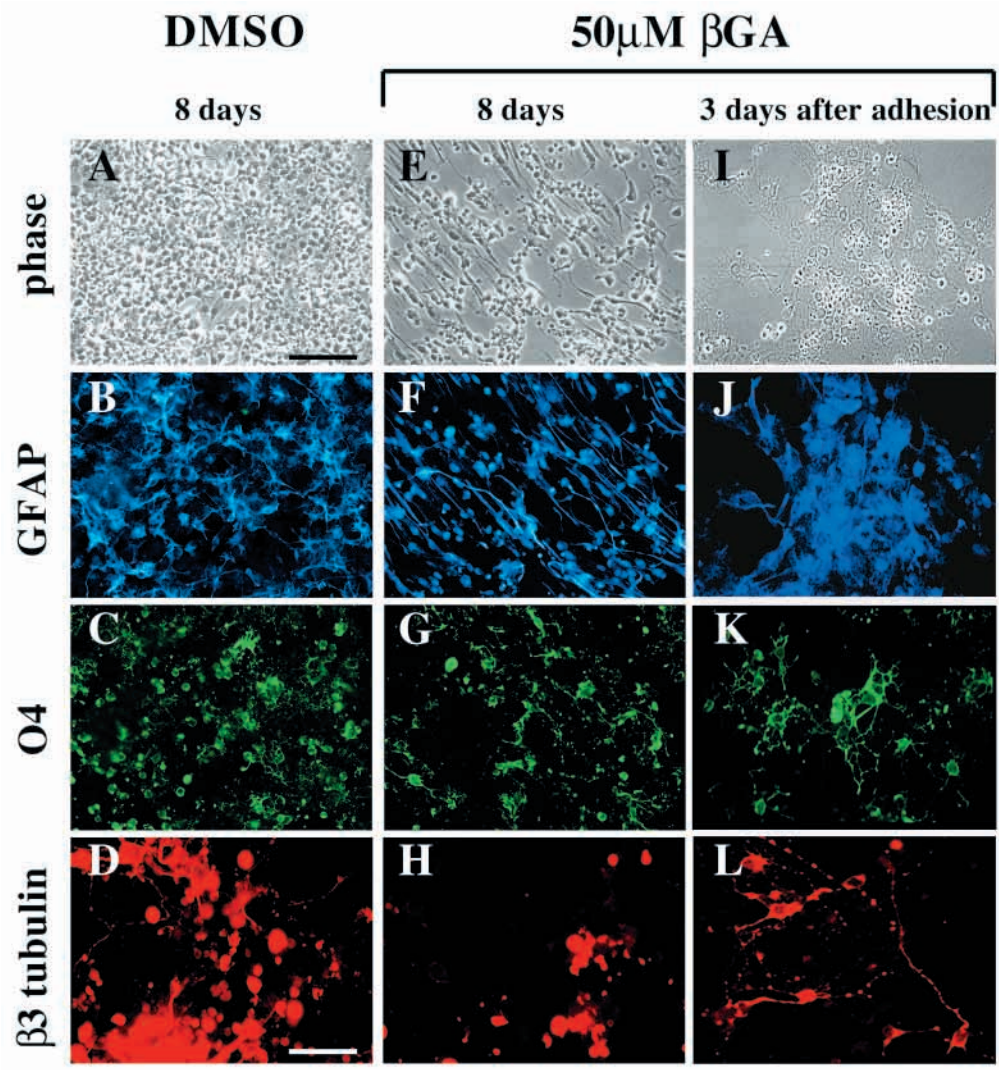

Fig. 6. The uncoupling agent βGA perturbs the morphology of differentiating cells. Floating neurospheres were allowed to differentiate either in the presence of βGA or DMSO, which was used as solvent of the drug. Both the density and morphology of cells migrating out of the spheres were altered by βGA (E-H), whereas DMSO alone (A-D) had no effect. Astrocytes were thinner and elongated (F), and oligodendrocytes (G) exhibited reduced cell arborizations compared with cells grown in the presence of DMSO (B,C, respectively). Neurons (H) were mainly round and devoid of processes. In a series of independent experiments, βGA was added after neurospheres had been allowed to adhere for three days and had formed an outgrowth of differentiated cells(I-L). Under these conditions, βGA did not alter either the morphology or the viability of the three cell types. Bar, 80 μm (A-C, E-G, I-K); 25 μm (D,H,L).

period in standard culture medium without any drugs. At the end of this period, neurospheres pre-treated with 25 μM and 50 μM βGA (Fig. 5G,H) recovered to a size similar to that of spheres incubated with either GZA (Fig. 5E,F) or solvent (data not shown), suggesting that progenitors temporarily exposed to βGA could still proliferate as untreated cells. By contrast, cells incubated in the presence of 100 μM βGA, but not with the same concentration of GZA, were unable to grow and form neurospheres even after removal of the uncoupling drug (data not shown). Thus, in all other experiments, βGA was added at the maximally effective concentration of 50 μM.

To assess whether junctional coupling was involved in the differentiation of neural progenitors, adherent neurospheres were incubated for 8 days in the presence of 50 μM βGA. Under these conditions, both cell density and morphological features of migrating cells were greatly perturbed (Fig. 6E-H) when compared with DMSO-treated cultures (Fig. 6A-D), which were indistinguishable from mock-treated cells (data not shown). Although a majority of cells were still able to acquire antigenic determinants of either the astrocytic, neuronal or oligodendrocytic phenotype, they also exhibited strikingly different features compared with mock- and DMSO-treated cells. Thus, GFAP-positive cells displayed thin, elongated processes (compare Fig. 6B with F), whereas oligodendrocytes were less branched (compare Fig. 6D with G), and β3-tubulin-positive neurons appeared as round cells with few processes (compare Fig. 6D with 6H). Furthermore, treatment of neurospheres with 50 μM carbenoxolone, another uncoupling agent (Rozental et al., 2001), induced modifications of both cell density and morphology that were comparable to those observed with βGA (data not shown). By contrast, these alterations were not observed when neurospheres were allowed to differentiate before being exposed to the same concentration (50 μM) of either βGA (Fig. 6I-L) or carbenoxolone (data not shown). Taken together, our results suggest that the effects on the growth and differentiation of neural progenitor cells are specifically due to disruption of junctional coupling.

Because βGA reduced cell numbers in both floating and adherent neurospheres, we quantified cell viability following a 3 day incubation in the presence of certain drugs (Fig. 7). βGA caused a dose-dependent and statistically significant decrease in the number of viable cells, in both floating and adherent neurospheres (Fig. 7). By contrast, neither the solvent DMSO/ethanol nor the inactive analog GZA modified

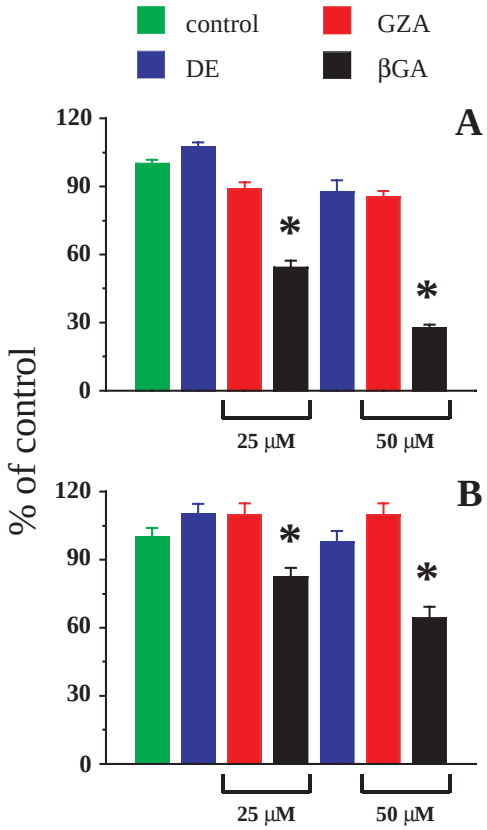

Fig. 7. βGA alters the viability of both neural progenitors and differentiating cells. Viable cells of either floating (A) or adherent (B) neurospheres were quantified colorimetrically by the MTT assay (see Materials and Methods) following three days in the presence of the specified drugs. Data were normalized to the values measured under control conditions. The gap junction inhibitor βGA caused a significant reduction of cell viability at all concentrations tested, whereas neither the inactive analog GZA nor solvent (DMSO/ethanol, DE) affected the total number of viable cells. Results are shown as means±s.e.m. of three independent experiments. Statistical significance was analyzed using the unpaired Student's *t*-test (*P<0.01).

cell viability with respect to that of mock-treated cultures (Fig. 7).

To assess the effect of βGA on coupling, astrocytes grown out of 21-day-old adherent neurospheres, which had been incubated for 6 hours in the presence or absence of 50 μM βGA, were microinjected with LY. As compared to controls, astrocytes treated with βGA were much less coupled (data not shown).

Cx43 expression in neural progenitor cells and differentiating astrocytes

We started our investigation of the molecular identity of connexins in neural progenitors by focusing on Cx43, which is the main component of astrocytic gap junctions. Floating neurospheres grown for 10 days in the presence of EGF were immunolabeled with a monoclonal antibody directed against Cx43. Examination of sections by confocal microscopy revealed an intense Cx43 labeling of the vast majority of

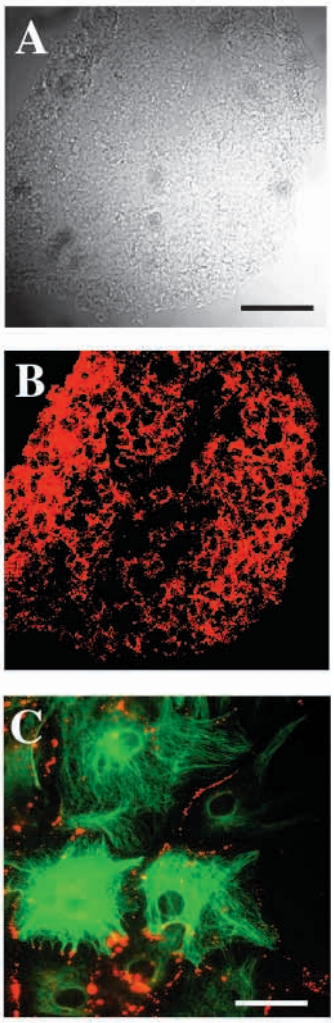

Fig. 8. Neural progenitors and differentiated astrocytes express Cx43. Single cell suspensions of striatal progenitors were expanded for 10 days in the presence of EGF to form floating neurospheres, which were fixed, sectioned and immunolabeled with a monoclonal antibody raised against Cx43 (B). The image corresponds to the merging of 10 planes acquired by confocal microscopy. Virtually all cells in the neurosphere were positive for Cx43. The corresponding Nomarski view is shown in A. Bar, 75 μm. (C) Neurospheres were allowed to differentiate for 8 days and were double-labeled with a polyclonal antibody against Cx43 (red) and a monoclonal antibody anti-GFAP (green). Cx43 was detected in GFAP-positive cells. Bar, 10 μm.

undifferentiated cells (Fig. 8B). To investigate the expression of Cx43 among differentiating cells, neurospheres were allowed to adhere for 8 days and then double-labeled with antibodies against Cx43 and cell-specific markers. We found that all cells labeled with Cx43 antibodies were also positive for GFAP (Fig. 8C), whereas we never observed a colocalization of Cx43 with either the O4 antigen of oligodendrocytes or β3-tubulin-positive neurons of the cell layers (data not shown).

We next analyzed the time course of Cx43 and GFAP expression in differentiating neurospheres (Fig. 9). Shortly (30 minutes) after adhesion, virtually each cell in the neurosphere expressed Cx43, whereas only a few GFAP-positive processes were detectable (Fig. 9A,B). Three hours after adhesion, Cx43 expression was mainly concentrated within the sphere of the cells, but was also detected in a few cells of the outgrowing layers. At the same time point, Cx43- and GFAP-positive cells were seen at the periphery of the sphere, where the migrating cells were beginning to form the outgrowing layers (Fig. 9C,D). Thus, at early stages of in vitro differentiation, Cx43 was expressed in both proliferating cells and in cells committed to an astrocytic fate. After longer adhesion times (8 days), Cx43 was still detectable within the sphere, as well as in differentiated astrocytes of the migrating cell layers (Fig. 9E,F).

Phosphorylation of Cx43 in adherent neurospheres

The expression pattern of Cx43 suggests that Cx43 is regulated during differentiation of neural progenitor cells. Because Cx43 is post-translationally phosphorylated, we analyzed whether changes in the phosphorylation state of Cx43 occurred during differentiation (Fig. 10). Equal amounts of protein extracted from floating and adherent neurospheres were studied by western blotting using two anti-Cx43 antibodies: a rabbit polyclonal, recognizing both the phosphorylated and nonphosphorylated forms of Cx43, and a mouse monoclonal specific for the latter form of the protein (Nagy et al., 1997). Cell lysates were separated by SDS-gel electrophoresis after no treatment, an incubation with alkaline phosphatase or an incubation with this enzyme in the presence of an excess of phosphatase inhibitors. In protein extracts from floating neurospheres (10 days), both antibodies reacted with a band of similar electrophoretic mobility, which was not perturbed by the alkaline phosphatase treatment (Fig. 10, lanes 1-3). In proteins from adherent neurospheres, the polyclonal antibody detected a broader band, which exhibited a slower electrophoretic mobility (Fig. 10, lane 4). Phosphatase treatment shifted Cx43 mobility towards faster migrating forms, an effect that was reduced in the presence of phosphatase inhibitors (Fig. 10, lanes 5-6). Under these experimental conditions, the monoclonal antibody, which recognizes nonphosphorylated Cx43, did not detect Cx43 in adherent neurospheres, unless protein extracts had been pre-treated with alkaline phosphatase (Fig. 10, compare lanes 4 with 5 in panel B). Consistent with these findings, immunolabeling performed on adherent neurospheres (8 days old) with the polyclonal anti-Cx43 antibody revealed a strong signal at the membrane of both cells located within the sphere, as well as differentiated astrocytes in the outgrowth layers. By contrast, the same two cell populations were weakly labeled when the monoclonal antibody against Cx43 was used. These findings suggest that Cx43 is mainly expressed as a nonphosphorylated form by growing neural progenitors, whereas the phosphorylated species predominates following in vitro differentiation.

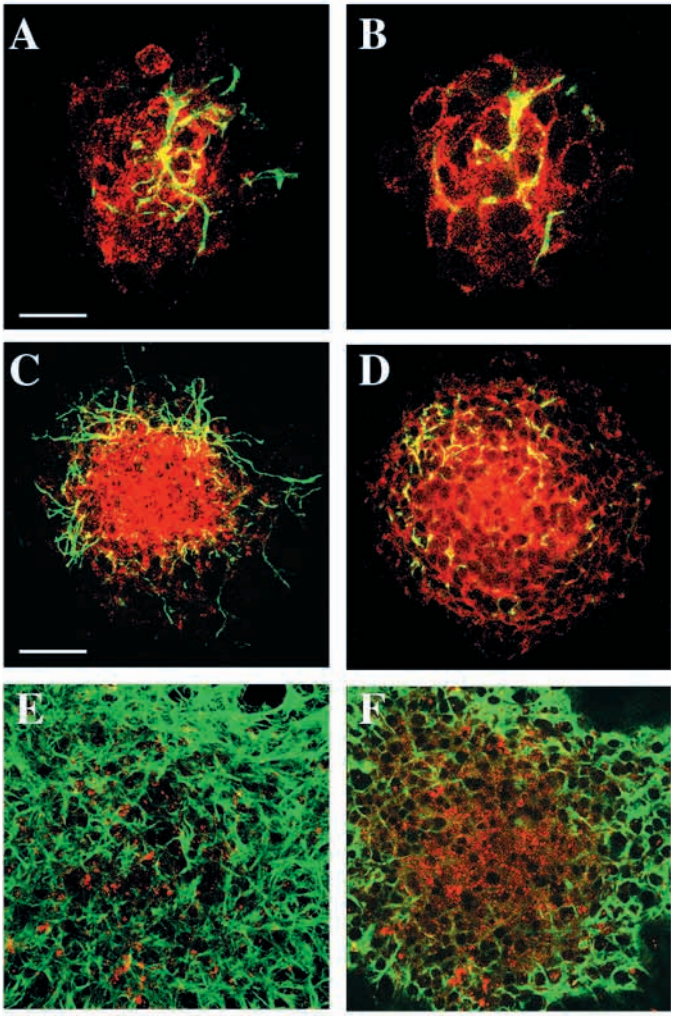

Fig. 9. Time course of Cx43 and GFAP expression in differentiating neurospheres. Floating neurospheres, expanded for 10 days with EGF, were transferred onto coated coverslips and allowed to differentiate for 30 minutes (A,B), 3 hours (C,D) and 8 days (E,F). Double immunostaining was performed using a rabbit polyclonal antibody against Cx43 (red) and a mouse monoclonal against GFAP (green). Pictures were taken from three different neurospheres and are representative of three independent experiments for each time point. Images in A, C and E result from the merging of 14 confocal planes, whereas single plane views of the same neurospheres are shown in B, D and F. At each adhesion time, Cx43 was expressed by cells located within the sphere. Cx43 expression increased with time in cells migrating out of the sphere. GFAP expression was detectable only in a few cells at 30 minutes of adhesion but was strongly enhanced in the outgrowing layers at 3 hours and 8 days of adhesion. Bar, 10 μm in A-B; 30 μm in C-F.

Discussion

Cell-to-cell communication through connexin channels provides a powerful means of intercellular signaling that participates in many aspects of mammalian development (Lo, 1996). To gain insight into the basic features of cell coupling between neural progenitors, we used a neurosphere system as a simplified in vitro model of CNS differentiation. Our data show that progenitor cells were strongly coupled, whereas, after differentiation and migration, intercellular communication was restricted to astrocytes. Furthermore, pharmacological treatment of neurospheres with a drug blocking gap junctions indicates that coupling is crucial for cell viability. The data substantiate the hypothesis for a role for neurospheres in intercellular communication during proliferation and differentiation of multipotent neural progenitors.

Cell coupling between neural progenitors

Microinjection of LY demonstrated that cells located within the neurosphere were always strongly coupled. Although injections were performed on neurospheres that had been adhering for various periods of time, virtually all these cells were nestin positive up to 2 hours after adhesion, confirming that they were undifferentiated progenitor cells (Lendahl et al., 1990). As adhesion time increased, there was a decrease in the number of cells in the neurosphere and a parallel expansion of the outgrowth composed of migrating cells, resulting in an increased proportion of differentiated cells. The population of undifferentiated cells, which was an area of active proliferation

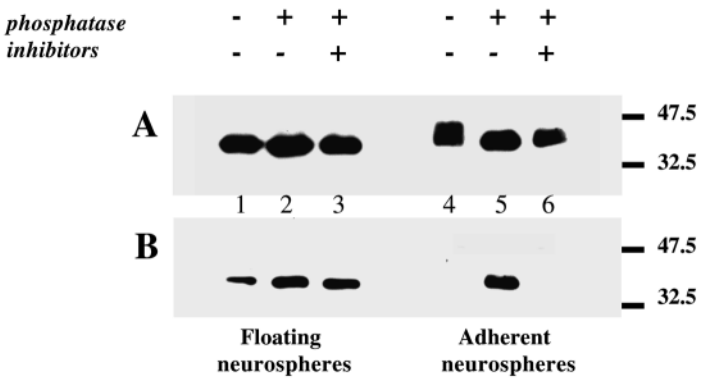

Fig. 10. Cx43 phosphorylation is increased in adherent neurospheres. Cell lysates were prepared from either floating (grown for 10 days) or adherent (8 days) neurospheres. Equal amounts of proteins were incubated overnight at 37°C in the presence of either digestion buffer (lanes 1 and 4), alkaline phosphatase (lanes 2 and 5) or alkaline phosphatase plus an excess of phosphatase inhibitors (lanes 3 and 6). Samples were separated by SDS-gel electrophoresis and immunoblotted with either a rabbit polyclonal (A) or a mouse monoclonal (B) anti-Cx43 antibody. Both antibodies reacted with Cx43 from floating neurospheres, and the apparent electrophoretic mobility was not perturbed by alkaline phosphatase treatment (lanes 1-3). In adherent neurospheres, a broader band was detected when the blot was probed with the polyclonal antibody (panel A, lane 4). Following exposure to phosphatase, Cx43 shifted to faster migrating forms (panel A, lane 5). The monoclonal anti-Cx43 antibody, which recognizes non-phosphorylated Cx43, detected Cx43 species only when the lysates were treated with alkaline phosphatase (panel B, lane 5). It should be noted that twice the amount of protein was loaded in B. This blot is representative of three others from two independent experiments.

compared with the outgrowth layers, remained coupled throughout the adhesion period. To our knowledge, there has been no previous report describing that gap junction communication between cells retains the ability to differentiate into the three main CNS cell types. These findings suggest that cell-cell coupling may be relevant to the maintenance of cells in a proliferative state, which may require synchronization in the center of the sphere. This possibility is in agreement with the observation that neural precursors from the ventricular zone couple and uncouple in a dynamic fashion throughout the cell cycle (Bittman and LoTurco, 1999).

Cell coupling between differentiated cells

As soon as migrating cells acquired an astrocytic morphology and GFAP expression, homocellular coupling was invariably detected. This observation confirms studies performed in cultures and brain slices that have demonstrated that astrocytes establish a functional syncytium through gap junction channels (Giaume and Venance, 1998; Dermietzel and Spray, 1998). By contrast, oligodendrocytes, and neurons did not allow transfer of LY to adjacent cells, in spite of the fact that microinjected cells were in apparent contact with many neighbors. Together, these findings suggest that proliferating cells and differentiated astrocytes form two distinct communication compartments, from which oligodendrocytes and neurons appear to be excluded. Although the occurrence of heterocellular coupling between neurons and astrocytes has been reported (Nedergaard, 1994; Fróes and Campos de Carvalho, 1998; Fróes et al., 1999; Alvarez-Maubecin et al., 2000), no morphological evidence of gap junctions between these two cell types has yet been found (Rash et al., 2001). It is possible that the discrepancy between the functional and the morphological observations reflects the limits of the techniques or, alternatively, that heterocellular coupling occurs during a narrow temporal window (Fróes et al., 1999) and/or in restricted brain areas (Alvarez-Maubecin et al., 2000).

Neuronal coupling via gap junctions is well established both in vitro and in vivo (LoTurco and Kriegstein, 1991; Peinado et al., 1993; Donahue et al., 1998; Rozental et al., 1998; Roerig and Feller, 2000; Venance et al., 2000). In our system, however, neurons expressing the post-mitotic marker β3-tubulin were not coupled among themselves nor to glia, as judged by LY exchange. One explanation is that coupling is transient during cell migration and, therefore, more difficult to detect in cell cultures than in brain slices. Moreover, we cannot exclude the possibility that tracer molecules other than LY may be more permeable to neuronal connexins. However, intercellular communication has been detected between differentiating neurons using LY (LoTurco and Kriegstein, 1991), and in a variety of systems this tracer is as sensitive as neurobiotin to dye coupling (Pastor et al., 1998; Meda, 2000). Our observations are also supported by those made in co-cultures of rat striatal astrocytes and neurons, where coupling was restricted to astrocytes, as demonstrated by both LY microinjection and patch clamp techniques (Rouach et al., 2000).

The presence of coupling between oligodendrocytes is still debated. Thus, oligodendrocytes of grey, but not white, matter exhibited homocellular coupling (Pastor et al., 1998), as did fully differentiated cells in culture (Venance et al., 1995; Dermietzel et al., 1997). Our results support the notion that, during differentiation, oligodendrocytes are not engaged in direct cell-cell communication. Although it has been clearly established that oligodendrocytes express more than one connexin type (Dermietzel et al., 1989; Scherer et al., 1995; Kunzelmann et al., 1997), it is possible that these connexins only form reflexive gap junctions that provide a short-cut pathway for the transfer of signals between different compartments within the same cell (Scherer et al., 1995).

Cell uncoupling and viability

A pharmacological blockade of intercellular communication by βGA produced a striking, dose-dependent inhibition of the viability of precursor cells, as well as a change in cell morphology. Several lines of evidence agree with a specific effect for βGA as the result of its uncoupling properties. First, βGA effects were not mimicked by either the inactive analog GZA or the solvent used to deliver the drug; second, morphological alterations of differentiated cells were similar in the presence of another uncoupler, carbenoxolone. More importantly, removal of 50 μM βGA led floating neurospheres to recover to a size similar to that of the controls. It is intriguing that treatment of adherent neurospheres with βGA affected cell density and the morphology of the uncoupled differentiating

cells: neurons and oligodendrocytes. A possible explanation is that the absence of a well organized astrocytic layer has a profound impact on the survival and differentiation of the other cell types (Hooghe-Peters et al., 1981), which, being uncoupled, should have not been directly affected by bGA. Since neither βGA nor carbenoxolone perturbed the morphology and viability of cells that had been allowed to differentiate (namely, when the drugs were applied after 3 days of adhesion), an alternative hypothesis may take into account the effects observed after continuous treatment with βGA. Uncoupling during the early steps of differentiation may affect the viability, as well as the proper morphological differentiation of surviving progenitors and precursors, thereby leading to alterations in morphology and survival of their progeny. In the presence of βGA, the strong reduction in the size of floating neurospheres and in the density of the layers formed by migrating cells could be accounted for by either a decreased rate of proliferation and/or an increased cell death. Our data cannot distinguish between these two mechanisms but suggest that cell communication is of paramount importance to orchestrate the life cycle of neural progenitors and promote their viability, as suggested in the case of early migratory neural crest cells (Bannerman et al., 2000). Further investigations are needed to specifically address this issue.

Cx43 in neurospheres: expression and post-translational modification

Cx43 protein was expressed by most cells of floating neurospheres and was still present in neural progenitor and precursor cells, even after long adhesion periods under culture conditions promoting differentiation. These results indicate that Cx43 plays a major role in connexin-dependent communication of neural progenitors.

Following migration and differentiation, Cx43 was confined to astrocytes. This segregation prompted us to compare the time course of Cx43 and GFAP expression following adhesion. GFAP was initially detected in the few cells that accumulated at the periphery of neurospheres, and Cx43 was found in these GFAP-positive cells migrating out of the sphere. At later time points, neurospheres contained Cx43-positive and GFAP-negative cells, whereas in the outgrowth, Cx43 was exclusively expressed by GFAP-positive astrocytes. The two areas of Cx43 expression were also those exhibiting dye coupling, suggesting that Cx43 mediates cell communication in these compartments and also contributes to isolating them from cells committed to either a neuronal or an oligodendrocytic fate.

Biochemical analysis demonstrated that a non-phosphorylated form of Cx43 was prevalent in neural progenitors, whereas, following differentiation, there was a massive shift towards phosphorylated species. This was demonstrated by comparing the ability of two anti-Cx43 antibodies, a mouse monoclonal specific for the non-phosphorylated form (Nagy et al., 1997) and a rabbit polyclonal also recognizing the phosphorylated species, to detect Cx43 by western blotting in floating and adherent neurospheres. The differences observed, namely the lack of signal from adherent neurospheres with the monoclonal antibody, unless protein extracts had been pre-treated with alkaline phosphatase, were further corroborated by immunocytochemical analysis of adherent neurospheres. Under these conditions, both antibodies recognized the same cells located within the sphere and in the outgrowth, although a much stronger staining was present with the polyclonal antibody. Phosphorylation of Cx43 has been implicated in several aspects of channel function (Musil et al., 1990b; Moreno et al., 1992; Kwak et al., 1995; Postma et al., 1998; Warn-Cramer et al., 1998) and could be tightly linked to the program of cell differentiation.

In summary, coupling among neural progenitors and the reduced viability of both proliferating and differentiating neural cells observed during pharmacological uncoupling, which presumably affects the channels made by all connexins, supports a central role for intercellular communication during CNS development. In this context, it is puzzling that no major CNS abnormality has been detected in Cx43 knockout mice (Reaume et al., 1995). Other connexins most probably help to define communication compartments during the early steps of differentiation of neural progenitors and provide a compensatory mechanism in the case of Cx43 loss. Our preliminary data show that at least eight other connexins are detectable in neurospheres (N.D., D.G., V.C. et al., unpublished), offering a unique experimental model in which this compensatory possibility can be tested.

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References

Alvarez-Maubecin, V., Garcia-Hernandez, F., Williams, J. T. and van Bockstaele, E. J. (2000). Functional coupling between neurons and glia. *J. Neurosci.* **20**, 4091-4098.

Bani-Yaghoub, M., Bechberger, J. F., Underhill, T. M. and Naus, C. C. (1999a). The effects of gap junction blockage on neuronal differentiation of human NTera2/clone D1 cells. *Exp. Neurol.* **156**, 16-32.

Bani-Yaghoub, M., Underhill, T. M. and Naus, C. C. (1999b). Gap junction blockage interferes with neuronal and astroglial differentiation of mouse P19 embryonal carcinoma cells. *Dev. Genet.* **24**, 69-81.

Bannerman, P., Nichols, W., Puhalla, S., Oliver, T., Berman, M. and Pleasure, D. (2000). Early migratory rat neural crest cells express functional gap junctions: evidence that neural crest cell survival requires gap junction function. *J. Neurosci. Res.* **61**, 605-615.

Ben-Hur, T., Rogister, B., Murray, K., Rougon, G. and Dubois-Dalcq, M. (1998). Growth and fate of PSA-NCAM+ precursors of the postnatal brain. *J. Neurosci.* **18**, 5777-5788.

Bittman, K. S. and LoTurco, J. J. (1999). Differential regulation of connexin 26 and 43 in murine neocortical precursors. *Cereb. Cortex* **9**, 188-195.

Bittman, K., Owens, D. F., Kriegstein, A. R. and LoTurco, J. J. (1997). Cell coupling and uncoupling in the ventricular zone of developing neocortex. *J. Neurosci.* **17**, 7037-7044.

Bruzzone, R., White, T. W. and Paul, D. L. (1996). Connections with connexins: the molecular basis of direct intercellular signaling. *Eur. J. Biochem.* **238**, 1-27.

Ciccolini, F. and Svendsen, C. N. (1998). Fibroblast growth factor 2 (FGF-2) promotes acquisition of epidermal growth factor (EGF) responsiveness in mouse striatal precursor cells: identification of neural precursors responding to both EGF and FGF-2. *J. Neurosci.* **18**, 7869-7880.

Davidson, J. S., Baumgarten, I. M. and Harley, E. H. (1986). Reversible inhibition of intercellular junctional communication by glycyrrhetinic acid. *Biochem. Biophys. Res. Commun.* **134**, 29-36.

Dermietzel, R. and Spray, D. C. (1998). From neuro-glue ('Nervenkitt') to glia: a prologue. *Glia* **24**, 1-7.

Dermietzel, R., Traub, O., Hwang, T. K., Beyer, E., Bennett, M. V. L., Spray, D. C. and Willecke, K. (1989). Differential expression of three gap junction proteins in developing and mature brain tissues. *Proc. Natl. Acad. Sci. USA* **86**, 10148-10152.

Dermietzel, R., Farooq, M., Kessler, J. A., Althaus, H., Hertzberg, E. L. and Spray, D. C. (1997). Oligodendrocytes express gap junction proteins connexin32 and connexin45. *Glia* **20**, 101-114.

Donahue, L. M., Webster, D. R., Martinez, I. and Spray, D. C. (1998). Decreased gap-junctional communication associated with segregation of the neuronal phenotype in the RT4 cell-line family. *Cell Tissue Res.* **292**, 27-35.

Fróes, M. M. and Campos de Carvalho, A. C. (1998). Gap junction-mediated loops of neuronal-glial interactions. *Glia* **24**, 97-107.

Fróes, M. M., Correia, A. H., Garcia-Abreu, J., Spray, D. C., Campos de Carvalho, A. C. and Neto, V. M. (1999). Gap-junctional coupling between neurons and astrocytes in primary central nervous system cultures. *Proc. Natl. Acad. Sci. USA* **96**, 7541-7546.

Giaume, C. and Venance, L. (1998). Intercellular calcium signaling and gap junctional communication in astrocytes. *Glia* **24**, 50-64.

Hooghe-Peters, E. L., Meda, P. and Orci, L. (1981). Co-cultures of nerve cells and pancreatic cells. *Dev. Brain Res.* **1**, 287-292.

Kandler, K. and Katz, L. C. (1995). Neuronal coupling and uncoupling in the developing nervous system. *Curr. Opin. Neurobiol.* **5**, 98-105.

Kumar, N. M. and Gilula, N. B. (1996). The gap junction communication channel. *Cell* **84**, 381-388.

Kunzelmann, P., Blumcke, I., Traub, O., Dermietzel, R. and Willecke, K. (1997). Coexpression of connexin45 and –32 in oligodendrocytes of rat brain. *J. Neurocytol.* **26**, 17-22.

Kwak, B. R., Hermans, M. M., de Jonge, H. R., Lohmann, S. M., Jongsma, H. J. and Chanson, M. (1995). Differential regulation of distinct types of gap junction channels by similar phosphorylating conditions. *Mol. Biol. Cell* **6**, 1707-1719.

Lendahl, U., Zimmermann, L. B. and McKay, R. D. G. (1990) CNS stem cells express a new class of intermediate neurofilament protein. *Cell* **60**, 585-595.

Lo, C. W. (1996). The role of gap junction membrane channels in development. *J. Bioenerg. Biomembr.* **28**, 379-385.

LoTurco, J. J. and Kriegstein, A. R. (1991). Clusters of coupled neuroblasts in embryonic cortex. *Science* **252**, 563-566.

McKinnon, R. D., Matsui, T., Dubois-Dalcq, M. and Aaronson, S. A. (1990). FGF modulates the PDGF-driven pathway of oligodendrocyte development. *Neuron* **5**, 603-614.

Meda, P. (2000). Probing the function of connexin channels in primary tissues. *Methods* **20**, 232-244.

Menezes, J. R., Froés, M. M., Moura Neto, V. and Lent, R. (2000). Gap junction-mediated coupling in the postnatal anterior subventricular zone. *Dev. Neurosci.* **22**, 34-43.

Miragall, F., Albiez, P., Bartels, H., de Vries, U. and Dermietzel, R. (1997). Expression of the gap junction protein connexin43 in the subependymal layer and the rostral migratory stream of the mouse: evidence for an inverse correlation between intensity of connexin43 expression and cell proliferation activity. *Cell Tissue Res.* **287**, 243-253.

Moreno, A. P., Fishman, G. I. and Spray, D. C. (1992). Phosphorylation shifts unitary conductance and modifies voltage dependent kinetics of human connexin43 gap junction channels. *Biophys. J.* **62**, 51-53.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65**, 55-63.

Musil, L. S., Beyer, E. C. and Goodenough, D. A. (1990a). Expression of the gap junction protein connexin43 in embryonic chick lens: molecular cloning, ultrastructural localization, and post-translational phosphorylation. *J. Membr. Biol.* **116**, 163-175.

Musil, L. S., Cunningham, B. A., Edelman, G. M. and Goodenough, D. A. (1990b). Differential phosphorylation of the gap junction protein connexin43 in junctional communication-competent and -deficient cell lines. *J. Cell Biol.* **111**, 2077-2088.

Nadarajah, B., Jones, A. M., Evans, W. H. and Parnavelas, J. G. (1997). Differential expression of connexins during neocortical development and neuronal circuit formation. *J. Neurosci.* **17**, 3096-3111.

Nagy, J. I., Li, W. E., Roy, C., Doble, B. W., Gilchrist, J. S., Kardami, E. and Hertzberg, E. L. (1997). Selective monoclonal antibody recognition and cellular localization of an unphpsphorylated form of Cx43. *Exp. Cell Res.* **236**, 127-136.

Naus, C. C. and Bani-Yaghoub, M. (1998). Gap junctional communication in the developing central nervous system. *Cell Biol. Int.* **22**, 751-763.

Nedergaard, M. (1994). Direct signaling from astrocytes to neurons in cultures of mammalian brain cells. *Science* **263**, 1768-1771.

Pastor, A., Kremer, M., Moller, T., Kettenmann, H. and Dermietzel, R. (1998). Dye coupling between spinal cord oligodendrocytes: differences in coupling efficiency between gray and white matter. *Glia* **24**, 108-120.

Peinado, A., Yuste, R. and Katz, L. C. (1993). Extensive dye coupling between rat neocortical neurons during the period of circuit formation. *Neuron* **10**, 103-114.

Postma, F. R., Hengeveld, T., Alblas, J., Giepmans, B. N., Zondag, G. C., Jalink, K. and Moolenaar, W. H. (1998). Acute loss of cell-cell communication caused by G protein-coupled receptors: a critical role for c-Src. *J. Cell Biol.* **140**, 1199-1209.

Rash, J. E., Yasumura, T., Dudek, F. E. and Nagy, J. I. (2001). Cell-specific expression of connexins and evidence of restricted gap junctional coupling between glial cells and between neurons. *J. Neurosci.* **21**, 1983-2000.

Reaume, A. G., de Sousa, P. A., Kulkarni, S., Langille, B. L., Zhu, D., Davies, T. C., Juneja, S. C., Kidder, G. M. and Rossant, J. (1995). Cardiac malformation in neonatal mice lacking connexin43. *Science* **267**, 1831-1834.

Reynolds, B. A. and Weiss, S. (1992). Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* **255**, 1707-1710.

Reynolds, B. A. and Weiss, S. (1996). Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. *Dev. Biol.* **175**, 1-13.

Reynolds, B. A., Tetzlaff, W. and Weiss, S. (1992). A multipotent EGF-responsive striatal embryonic progenitor cell produces neurons and astrocytes. *J. Neurosci.* **12**, 4565-4574.

Roerig, B. and Feller, M. B. (2000). Neurotransmitters and gap junctions in developing neural circuits. *Brain Res. Rev.* **32**, 86-114.

Rouach, N., Glowinski, J. and Giaume, C. (2000). Activity-dependent neuronal control of gap-junctional communication in astrocytes. *J. Cell Biol.* **149**, 1513-1526.

Rozental, R., Morales, M., Mehler, M. F., Urban, M., Kremer, M., Dermietzel, R., Kessler, J. A. and Spray, D. C. (1998). Changes in the properties of gap junctions during neuronal differentiation of hippocampal progenitor cells. *J. Neurosci.* **18**, 1753-1762.

Rozental, R., Srinivas, M. and Spray, D. C. (2001). How to close a gap junction channel. *Methods Mol. Biol.* **154**, 447-476.

Scherer, S. S., Deschênes, S. M., Xu, Y. T., Grinspan, J. B., Fischbeck, K. H. and Paul, D. L. (1995). Connexin32 is a myelin-related protein in the PNS and CNS. *J. Neurosci.* **15**, 8281-8294.

Svendsen, C. N., Clarke, D. J., Rosser, A. E. and Dunnett, S. B. (1996). Survival and differentiation of rat and human epidermal growth factor-responsive precursor cells following grafting into the lesioned adult central nervous system. *Exp. Neurol.* **137**, 376-388.

Temple, S. (2001). Stem cell plasticity–building the brain of our dreams. *Nat. Rev. Neurosci.* **2**, 513-520.

Venance, L., Cordier, J., Monge, M., Zalc, B., Glowinski, J. and Giaume, C. (1995). Homotypic and heterotypic coupling mediated by gap junctions during glial cell differentiation in vitro. *Eur. J. Neurosci.* **7**, 451-461.

Venance, L., Rozov, A., Blatow, M., Burnashev, N., Feldmeyer, D. and Monyer, H. (2000). Connexin expression in electrically coupled postnatal rat brain neurons. *Proc. Natl. Acad. Sci. USA* **97**, 10260-10265.

Vescovi, A. L., Parati, E. A., Gritti, A., Poulin, P., Ferrario, M., Wanke, E., Frolichsthal-Schoeller, P., Cova, L., Arcellana-Panlilio, M., Colombo, A. and Galli, R. (1999). Isolation and cloning of multipotential stem cells from the embryonic human CNS and establishment of transplantable human neural stem cell lines by epigenetic stimulation. *Exp. Neurol.* **156**, 71-83.

Warn-Cramer, B. J., Cottrell, G. T., Burt, J. M. and Lau, A. F. (1998). Regulation of connexin-43 gap junctional intercellular communication by mitogen-activated protein kinase. *J. Biol. Chem.* **273**, 9188-9196.