J Membrane Biol (2007) 217:83–91
DOI 10.1007/s00232-007-9039-7

Gap Junction and Purinergic P2 Receptor Proteins as a Functional Unit: Insights from Transcriptomics

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Received: 27 April 2007 / Accepted: 14 May 2007 / Published online: 31 July 2007

Abstract Gap junctions and purinergic P2 receptors (P2Rs) can be regarded as belonging to a common functional unit, given that they are involved in the transmission of calcium signals between cells. We have previously shown that deletion of the *Gja1* gene alters expression levels of numerous genes encoding proteins with diverse functions, including purinergic receptors (P2Rs), and have found that genes synergistically or antagonistically expressed in wild-type tissues are more prone to be similarly or oppositely regulated in Cx43-nulls. We have now explored the use of coordination analysis of gene expression as a strategy to identify interlinked genes encoding functionally related proteins and pull-downs to evaluate their interlinkage. Our findings indicate that, in brain and in cultured astrocytes, several of these coexpressed genes encode proteins that are components of P2R signal-transduction pathways and/or directly interact with these receptors, including the gap junction protein connexin43 (Cx43) and Cx45 as well as pannexins. It is proposed that coordination analysis of gene expression may provide a novel unbiased strategy for the identification of proteins belonging to supramolecular complexes.

Keywords Pannexin · Connexin · Microarray · Gene expression · Protein interaction · Astrocyte · Calcium signaling

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Introduction

Analysis of the cellular and molecular steps involved in the triggering and transmission of Ca^{2+} waves between astrocytes has led to the identification of two pathways mediating this form of intercellular communication. One of them involves direct communication between the cytosols of two adjoining cells through gap junction channels, while the other depends on the activation of Ca^{2+}-mobilizing receptors on surface membranes of neighboring cells (*for reviews, see* Scemes, 2000; Charles & Giaume, 2002; Scemes & Giaume, 2006). These two mechanisms of Ca^{2+} wave transmission are not mutually exclusive but are likely to work in conjunction to coordinate the activity of cell networks.

Astrocytes *in situ* and *in vitro* express several gap junction proteins, but connexin43 (Cx43) is the most abundant (Dermietzel et al., 2000; Scemes, Suadicani & Spray, 2000). Moreover, these cells express several ionotropic and metabotropic adenosine triphosphate (ATP)-sensitive purinergic P2 receptors (P2Rs), some of which have been implicated in the transmission of calcium signals (Jimenez et al., 2000; Fumagalli et al., 2003; Suadicani et al., 2004). Among the metabotropic P2YRs, the $P2Y_1R$ and $P2Y_2R$ subtypes are likely those predominantly expressed in astrocytes (Ho, Hicks & Salter, 1995; Idestrup & Salter, 1998; Zhu & Kimelberg, 2001, 2004). Although both of these G-coupled P2Rs generate phospholipase C (PLC) and inositol 1,4,5-trisphosphate (IP_3) upon stimulation, and thus contribute to Ca^{2+} elevations, they differ with regard to their sensitivity and selectivity for nucleotides. In addition, some ionotropic P2XRs, specifically the pore-forming $P2X_7R$, may also contribute, under specific circumstances, as gliotransmitter release sites amplifying the extent to which Ca^{2+} signals are

transmitted between astrocytes (Duan et al., 2003; Suadicani, Brosnan & Scemes, 2006).

Over the last 5–6 years, it has become apparent that there is a compensatory mechanism involving gap junction proteins and P2Rs that enables astrocytes to sustain Ca^{2+} signals under circumstances in which the expression/function of one of the components of the intercellular calcium wave is altered. For instance, under inflammatory conditions of the central nervous system, increased expression of $P2Y_2R$ and $P2X_7R$ (John et al., 1999; Narcisse et al., 2005) is likely to compensate for the loss of Cx43, the main gap junction protein in astrocytes. Such a compensatory mechanism leading to changes in the relative contribution of gap junctions and P2Rs for astrocyte Ca^{2+} signaling, also observed in Cx43-null astrocytes (Scemes et al., 2000; Suadicani et al., 2003), suggests that these two groups of proteins are part of the same signaling network, likely involving protein-protein interactions.

Coordination analysis of clusters of genes has been proposed as one strategy to identify interacting networks (Ideker, Galitski & Hood, 2001). In yeast, *Caenorhabditis elegans* and, most recently, cancer cells, genes that are linked by physical interactions within a network have been found to have strongly correlated expression profiles, possibly because two or more genes are under the control of a common transcription factor (Ge et al., 2001; Ideker et al., 2001; Walhout et al., 2002; Wachi, Yoneda & Wu, 2005).

Using pairwise coordination analysis of gene expression levels in a set of biological replicas obtained with Albert Einstein College of Medicine (AECOM) spotted 27k mouse cDNA microarrays (http://www.microarray1k.aecom.yu.edu/), we have identified genes that are coordinately expressed with *Gja1* in brain (Iacobas et al., 2005b, 2007a,b), heart (Iacobas et al., 2005a) and cortical astrocytes (Iacobas et al., 2003; Iacobas, Scemes & Spray, 2004) and indicated that fluctuations in expression levels of correlated genes in the wild-types (WT) can predict the direction of changes of these genes in *Gja1*-nulls. In other words, genes that are synergistically expressed with *Gja1* in WT are more prone to be downregulated in *Gja1*-nulls, genes that are antagonistically expressed with *Gja1* in WT are more prone to be upregulated in *Gja1*-nulls and genes that are independently expressed with *Gja1* in WT are most likely to be unaltered in *Gja1*-nulls.

We have now extended our coordination analysis to data on cortical astrocytes from WT mice obtained using AECOM spotted 32k mouse oligo-arrays to evaluate genes that are coregulated with P2Rs. We found that some of the 1,591 genes were coordinated with P2Rs in astrocytes. Among those coordinated genes were those encoding proteins that are components of P2R signal-transduction pathways (some ion channels, receptors and enzymes), and some of these were shown to directly interact (cytoskeletal and junctional proteins) with P2Rs.

Materials and Methods

Brain Tissues and Cell Cultures

Whole brains of adult WT C57Bl/6 mice were removed from the skulls and, after removal of the meninges, minced in lysis buffer (1 mM $NaHCO_3$, 2 mM phenylmethyl sulfonyl fluoride, 1 mM Na_3VO_4, 5 mM ethylenediaminetetraacetic acid [EDTA], 1% NP-40 and 1 x complete protease inhibitor cocktail [Roche Laboratories, Indianapolis, IN]), sonicated and centrifuged for 10 min at 12,000 rpm at 4°C. The supernatants were used for the antibody array and immunoprecipitation assays (*see below*).

Primary cultures of spinal cord and cortical astrocytes derived from neonatal WT (C57Bl/6 strain) mice were performed as previously described (Scemes, Dermietzel & Spray, 1998; Scemes et al., 2000) and maintained in Dulbecco's modified Eagle medium supplemented with 5% or 10% fetal bovine serum and 1% antibiotics.

Oligonucleotide Arrays

Twenty micrograms of total RNA extracted from four sets of primary cultures of cortical astrocytes derived from neonatal WT and Cx43-null mice were reverse-transcribed into cDNA incorporating fluorescent deoxyuridine triphosphates (dUTPs, Cy3-dUTP). The labeled cDNAs were cohybridized with labeled Cy5-dUTP mouse reference overnight at 50°C on 10 aminosilane-coated Corning (Corning, NY) glass slides, spotted with 32k selected mouse oligonucleotide sequences (70-mer Operon mouse 3.0 series) printed by the Microarray Core Facility of the AECOM. (Cx43 heterozygotes [C57BL/6J-Gja1 strain] are maintained at the AECOM; the AECOM Animal Care and Use Committee approved all experimental procedures used in these studies.) The eight microarrays were scanned with an Axon (Burlingame, CA) GenePix 4000A scanner, and data were acquired through GenePix (Molecular Devices, Sunnyvale, CA) Pro 4.0 software. Spots with substantial local imperfections, foreground signal median values lower than twice the median values of the background signals in both channels and saturated pixels were eliminated from the analysis. The background-subtracted signals were normalized through an in-house-developed iterative algorithm, alternating within-array normalization with interarray normalization until the average corrected ratio differed by <5% from the previous one (Iacobas et al., 2003, 2004). This study was performed according to the standards of the Microarray Gene Expression Data Society, and data

complying with the MIAME (Brazma et al., 2001) have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo; platform GPL5371 and series GSE8105). The expression levels within the sets of four biological replicas were then compared across the quantified genes through the pairwise Pearson correlation coefficient and the *expressome* of each gene (the set of all $p < 0.05$ significantly synergistically or antagonistically expressed partners) and the *exclusome* (the set of all $p < 0.05$ significantly independently expressed partners) determined.

Antibody Arrays

Antibody arrays were prepared on polyvinylidene difluoride (PVDF) membranes as previously described (Duffy et al., 2004b). Briefly, after 15-s immersion in methanol, PVDF membranes were hydrated for 2 min in double distiled water. The membranes were then rinsed three times in phosphate-buffered saline (PBS, pH 7.4) for 10 min and transferred to a Bio-Dot® Apparatus (Bio-Rad, Richmond, CA). One hundred microliters of various selected antibodies diluted in PBS were individually spotted on the membranes through the Bio-Dot wells. After 5-min incubation, the antibody solutions were removed by aspiration using the apparatus vacuum manifold and the membrane spots rinsed with 100 µl of PBS/well. The membranes were then immersed in Tris-buffered solution-Tween-20 (TBST) for 5 min, blocked with 5% skim milk/TBST for 1 h at room temperature (RT) and incubated for 1 h at RT with 20 µg/ml of brain lysates diluted in 5% skim milk/TBST. After three washes in TBST, the membranes were incubated for 1 h at RT with the 181-A Cx43 antibody (from Dr. Eliott Hertzberg, AECOM) or anti-$P2X_7$ antibody (Alomone Labs, Jerusalem, Israel) conjugated to horseradish peroxidase (HRP) and diluted in 5% skim milk/TBST. Antibody conjugation to HRP was performed according to the manufacturer's instructions using the EZ-link™ Plus Activated Peroxidase Kit (Pierce, Rockford, IL). After incubation with the HRP-conjugated antibody, the membranes were washed in TBST (three times, 10 min) and incubated for 2 min in enhanced chemiluminescence (ECL) reagents (Amersham Pharmacia Biotechnology, Piscataway, NJ). HRP-positive spots on the antibody array were then visualized by chemiluminescence detection on X-ray film (Kodak, Rochester, NY).

Coimmunoprecipitation and Western Blots

Precleared lysates were incubated for 3 h with primary antibodies (*see below*) at 4°C prior to the addition of agarose-conjugated immunoglobulin G beads. After overnight incubation at 4°C, immunocomplexes were washed five or six times with lysis buffer solution and bound proteins were eluted with 2 x Laemmli buffer. Samples of immunoprecipitated proteins from whole brain and astrocyte lysates were electrophoresed in 4–20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis minigels (Bio-Rad) and then transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Immunoblots were performed after overnight incubation of membranes with blocking solution (5% dry nonfat milk in 1 x PBS) using primary antibodies (*see below*). After several washes with 1 x PBS containing 0.4% Tween-20 (Sigma, St. Louis, MO), membranes were incubated with HRP-conjugated secondary antibodies (1:2,000; Santa Cruz Biotechnology, Santa Cruz, CA). Detection of bands was performed on X-ray films (Kodak) following incubation with ECL reagents.

Antibodies Used in the Assays

The following antibodies were used in assays: ZO-1 monoclonal and polyclonal (Zymed, San Francisco, CA), monoclonal α-tubulin (Sigma), c-Src monoclonal (Calbiochem, San Diego, CA) and polyclonal (Sigma), Cx43 monoclonal (Zymed) and polyclonal (kindly provided by Dr. Elliot Hertzberg, AECOM), vinculin monoclonal (Chemicon, Temecula, CA) and polyclonal (Santa Cruz Biotechnology), polyclonal caveolin-1 (Santa Cruz Biotechnology), polyclonal α-catenin and polyclonal mitogen-activated protein kinase (MAPK, Sigma), polyclonal $P2X_7R$ (Alomone Labs), monoclonal β-actin (Sigma), polyclonal Cx50 (Santa Cruz Biotechnology), polyclonal Cx45 (Chemicon), polyclonal Cx46 (Santa Cruz Biotechnology), polyclonal APC2 (Zymed), pannexin1 (Pnx1; kindly provided by Dr. Gerhard Dahl, University of Miami Medical School, Miami, FL), polyclonal aquaporin 4 (Santa Cruz Biotechnology), polyclonal Src (Santa Cruz Biotechnology), phospho-extracellular signal-regulated kinases (ERKs) 1/2 (R&D Systems, Minneapolis, MN), polyclonal claudin-1 (Zymed), monoclonal glyceraldehyde-3-phosphate dehydrogenase (GAPDH; R&D systems), monoclonal kinesin (Cytoskeleton Inc., CA), monoclonal synaptophysin (Sigma), monoclonal calmodulin (Upstate Biotechnology, Lake Placid, NY) and monoclonal glial fibrillary acid protein (GFAP, Sigma).

Results

Purinergic Receptor and Gap Junction Genes Are Coordinately Expressed in WT Astrocytes

We have previously shown that deletion of the gap junction protein Cx43 alters the expression levels of numerous unrelated genes in cultured astrocytes (Iacobas et al., 2003), including P2Rs (Scemes et al., 2000; Scemes,

Table 1 Biological function of some of the genes positively coordinated with $P2Y_1Rs$

Biological function	Gene names
Cytoskeleton, motors, adaptors, focal adhesion	**Actins**, **tubulins**, kinesins, **integrins**, ankyrins, **adesintregrin** and **metalloproteinases**
Junctional proteins	**Cadherins**, tight junctions (*ZO3*), gap junctions (*Panx1, Panx2*)
Channels, transporters, exchangers	Aquaporins, **Cl^- channels**, **K^+ channels**, **Na^+ channels**, **Ca^{2+} channels**, Transient Receptor Potential Melastatin (TRPM) channels, ATP Binding Cassette (ABC) transporters, glycine transporter, v-ATPase
Receptors	**Purinergic receptors ($P2Y_{14}$, $P2X_2$), tumor necrosis factors, adrenergic receptors, glutamate receptors, GABA-A receptors, IP_3 receptors**
Enzymes	**Adenylate cyclase, CaM kinase-II, MAP kinases, PLC, PLA_2, PLD, PKC, calpain, protein phosphatases, protein tyrosine phosphatases, serine-threonine kinases**

The transcript products that have been previously shown by others to either directly interact or to be part of the same signal transduction pathway of purinergic receptors are in bold type

GABA, γ-aminobutyric acid; CaM, calmodulin; PLD, phospholipase D

Duval & Meda, 2003; Suadicani et al., 2003). Coordination analysis revealed that genes belonging to a wide variety of functional categories are correlated within each genotype (Iacobas et al., 2004) and that the expression levels of a particular gene with a significant positive or negative Pearson correlation coefficient with the *Gja1* gene in WT cells can be predicted to be down- or upregulated in *Gja1*-null cells (Iacobas et al., 2004). Correlation analysis of expression levels of the $P2Y_1R$ gene with each of the other quantified genes indicated that $P2Y_1R$ is positively coordinated (Pearson correlation coefficient >0.90) with numerous genes, including those encoding gap junction proteins, cytoskeletal and focal adhesion proteins and PDZ-containing proteins. Table 1 shows some of the genes that were positively coordinated with $P2Y_1Rs$ in WT astrocytes. Note that several of these coordinated genes encode proteins that are components of P2R signal-transduction pathways (some ion channels, receptors and enzymes) and some that have been shown to directly interact (cytoskeletal and junctional proteins) with P2Rs.

To extend this analysis and to evaluate whether other P2Rs and gap junctions are coordinately expressed in astrocytes of WT mice, we calculated the Pearson coefficient between the expression levels of eight P2Rs and seven genes encoding connexins and pannexins (grouped together as gap junction genes in the analysis). Table 2 shows the type of coordination found between the members of these gene families. Thus, six out of eight quantified P2Rs were coordinately expressed with at least one gap junction gene; $P2Y_{14}R$ was positively coordinated with four and $P2Y_1R$ with three gap junction genes. Positive coordination with a single gap junction gene was obtained for $P2X_2R$ and $P2X_4R$, while negative coordination with a single gap junction gene was obtained for $P2X_5R$ and $P2X_7R$ (Table 2). The fact that a large number of P2Rs display a high degree of coordination with members of the gap junction genes likely suggests that they may belong to the same functional network. In order to evaluate whether P2R and gap junction transcripts interact and to gain insight into the nature of this interaction, we used antibody arrays and immunoprecipitation assays.

Table 2 Types of coordination between gap junctions and P2R in WT cultured astrocytes

	$P2Y_1$	$P2Y_2$	$P2Y_{14}$	$P2X_2$	$P2X_4$	$P2X_5$	$P2X_7$
Gja	I	X	Z	X	**A**	X	**A**
Gja7	X	X	Z	X	X	X	X
Gjb1	**S**	X	**S**	X	I	X	X
Gjb5	X	Z	**S**	Z	Z	X	X
Gjc1	**S**	Z	**S**	**S**	Z	X	X
Pnx1	**S**	Z	X	Z	Z	**S**	X
Pnx2	**S**	Z	**S**	Z	Z	X	Z

S synergistic if Pearson >0.90; I, independent if 0.05 > Pearson < –0.05; **A** antagonistic if Pearson >–0.90; X, no coordination if 0.05 < Pearson < 0.90 or –0.90 Pearson > –0.05; Z, not sufficiently quantifiable

Purinergic Receptor and Gap Junction Network

A total of 28 antibodies, both monoclonal and polyclonal, were spotted on the antibody arrays. Antibodies against ZO-1 and GAPDH were included as positive and negative controls, respectively, for the interaction with Cx43. Antibodies against Cx43 and GAPDH were included as positive and negative controls, respectively, for the interaction with $P2X_7R$. Arrays were incubated with adult mouse brain lysate followed by detection of Cx43 (Fig. 1a) and $P2X_7R$ (Fig. 1b) binding using the Cx43-HRP and the $P2X_7R$-HRP conjugated antibodies, respectively. As expected on the arrays probed with the Cx43-HRP, an intense signal for Cx43 was obtained on the spots for ZO-1, while no signal was obtained on GAPDH spots (*see* Fig. 1a). Signals with variable intensities were obtained from other spots, among

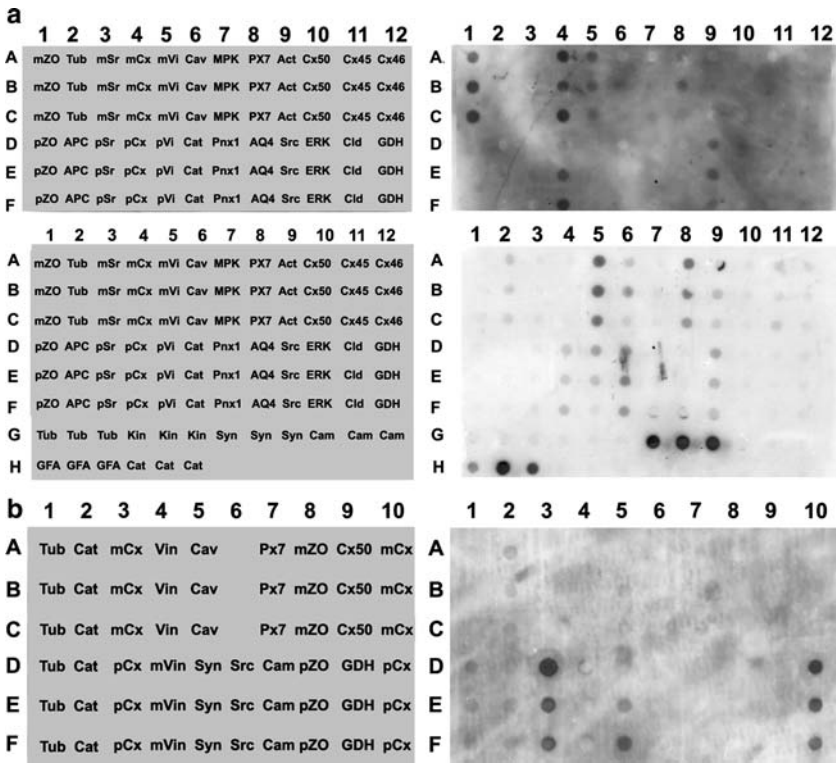

Fig. 1 Antibody array screening for some proteins interacting with Cx43 and $P2X_7R$ in whole-brain lysates. Diagrams of antibody arrays showing the location of the various spotted antibodies (*left panels*) and scanned images of PVDF membranes after detection with Cx43-HRP (**a**) and $P2X_7R$-HRP (**b**) conjugated antibodies (*right panels*). Positive hits for Cx43 or $P2X_7R$ putative binding partners are indicated by the presence of *dark spots* in the array resulting from the chemiluminescence detection of bound HRP-conjugated antibodies. Cx43 binding to ZO-1 (1A-C), Cx43 (4A-C) and Src (9D-F) is shown in the *top right* array in **a** and binding to vinculin (5A-C), Pnx1 (6D-F), $P2X_7R$ (8A-C) and actin (9A-C) is shown in the *lower right* array of **a**. $P2X_7R$ binding to Cx43 (3D-F, 10D-F) and synaptophysin (5D-F) is shown in array of **b**

which are those for catenin, caveolin, $P2X_7R$, actin, vinculin, ERK, Src, Cx45, GFAP and synaptophysin. This result is consistent with reports indicating that Cx43 interacts with some of these proteins (*for reviews, see* Giepmans, 2004, 2006; Herve, Bourmeyster & Sarrouilhe, 2004).

Interestingly, for $P2X_7R$ we found that only Cx43 and synaptophysin were likely interacting proteins (Fig. 1b). Although the number of $P2X_7R$ binding partners is low compared to Cx43 when using similar antibody arrays, this result, besides corroborating the interaction between Cx43 and $P2X_7R$ (Fortes et al., 2004), suggests that $P2X_7R$ and Cx43 networks may overlap to a minor extent.

To validate results obtained with antibody arrays, immunoprecipitation (IP) studies were performed using Cx43 and $P2X_7R$ antibodies followed by Western blotting. As shown in Figure 2a, ZO-1 and vinculin were detected in both the whole-brain lysates (lane a) and the Cx43-IP fraction (lane b), confirming the known interaction of ZO-1 with Cx43 (*see* Giepmans, 2004, 2006; Herve et al., 2004) and the putative interaction of Cx43 and vinculin suggested by our antibody array and by recent studies on cardiac neural crest cells (Xu et al., 2006). Conversely, the detection of Cx43 in the $P2X_7R$-IP fraction of whole-brain lysates (Fig. 2b) also confirmed our antibody array data showing binding of these two proteins. Putative interactions of $P2X_7R$ with other gap junction proteins, such as Pnx-1; with cytoskeletal/cell adhesion proteins, such as vinculin, β-actin and β-tubulin; and with the tyrosine kinase c-Src were also suggested by the presence of these proteins in the $P2X_7R$-IP fraction of whole-brain lysates.

Similar to what was observed in whole-brain lysates, cultured astrocytes are also shown here to display a supramolecular complex involving $P2X_7R$, Pnx1, Cx43, actin and vinculin (Fig. 3a). Interestingly, some of the components of the $P2X_7R$-gap junction complex are also shown to be part of the metabotropic $P2Y_1R$ complex, such as Cx43, vinculin and actin (Fig. 3b).

To evaluate whether genes encoding the identified proteins obtained from pull-down experiments are coregulated with gap junctions and P2R, we performed coordination analysis between vinculin, actin, src, gap junction and P2R genes obtained from oligonucleotide arrays as well as through previously published results using a cDNA array platform (Iacobas et al., 2007a). Based on pairwise Pearson coefficient analysis, we found that indeed vinculin is coordinately expressed with two gap junction genes (*Gja1* and *Pnx1*) and with $P2Y_1R$. Moreover, the $P2X_7R$ gene, which is coordinately expressed with *Gja1*, was also found to be coordinately expressed with actin. Thus, these correlation analyses further support the idea that genes with similar expression profiles are more likely to encode interacting proteins.

Although further work is needed to determine the nature and the functional consequences of the interaction among

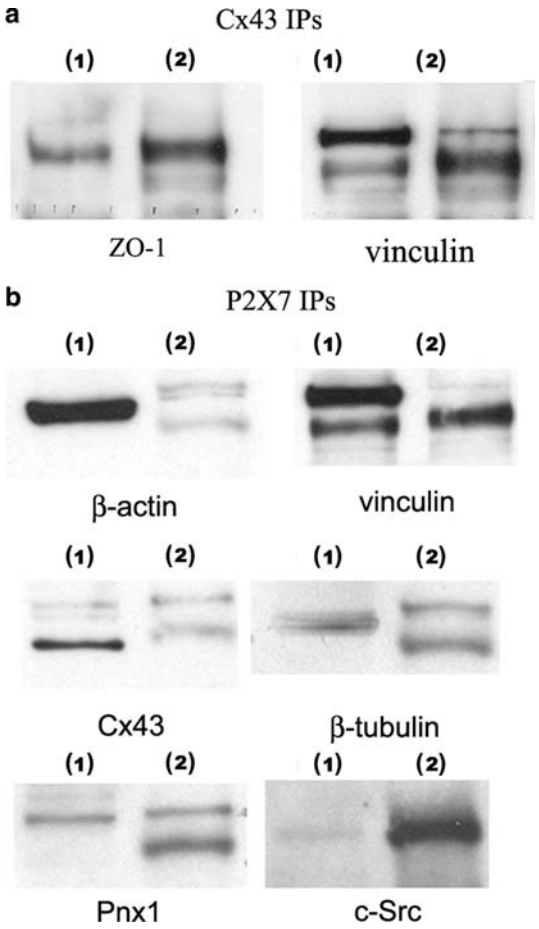

Fig. 2 Components of the Cx43 and the $P2X_7R$ protein complexes in the brain. Western blots of whole mouse brain lysate (**1**) and of brain lysate fractions (**2**) immunoprecipitated with Cx43 (**a**) and $P2X_7R$ (**b**) antibodies. Note the detection of ZO-1 and vinculin in the Cx43 immunoprecipitates (**a**) and of β-actin, vinculin, Cx43, β-tubulin, Pnx1 and c-Src in the $P2X_7R$ immunoprecipitates. The presence of these proteins in the immunoprecipitates demonstrates their interaction with Cx43 and $P2X_7R$ and suggests that these proteins are components of the Cx43 and $P2X_7R$ protein complexes in the brain

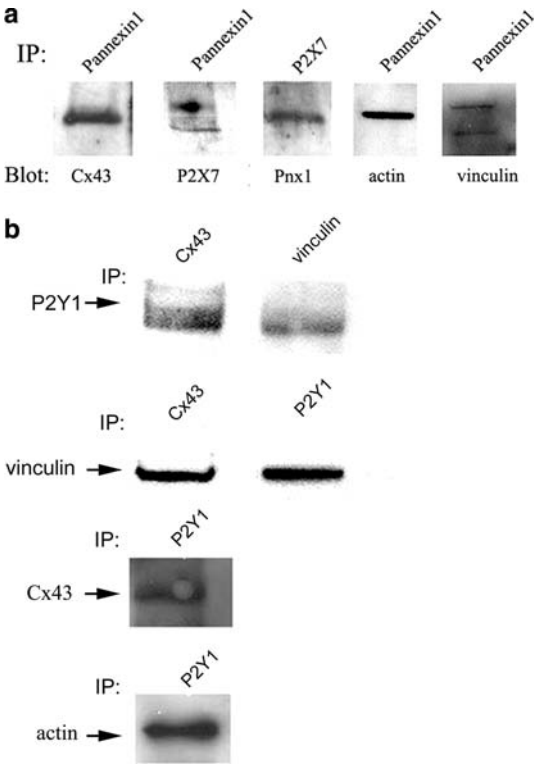

Fig. 3 Components of P2R and gap junction protein complexes in astrocytes. Western blots of lysates from primary cultured astrocytes immunoprecipitated (IP) with different antibodies. **a** Presence of Cx43, $P2X_7R$, actin and vinculin in the IPs using Pnx1 antibodies and of Pnx1 in the IP using the $P2X_7R$ antibody. **b** Detection of $P2Y_1R$ in the IPs with anti-Cx43 and antivinculin antibodies (*first row*). Conversely, vinculin is shown in pull-downs using anti-Cx43 and anti-$P2Y_1R$ antibodies (*second row*), and Cx43 is shown in pull-downs using anti-$P2Y_1R$ antibody (*third row*). *Last row* in **b** shows the presence of actin in the IPs using anti-$P2Y_1R$ antibody. The detection of actin and vinculin in the IPs using P2Rs and gap junction (Cx43 and Pnx1) antibodies and the reciprocal pull-down of P2Rs and gap junctions provide evidence that these proteins are likely to interact and to be members of the same protein complexes

the components of the P2R-gap junction network, these initial studies suggest that focal adhesions are likely to be the sites where these two groups of proteins interact.

In Silico Network Involving Purinergic Receptors and Gap Junctions

Using PathwayStudio Software (http://www.ariadnegenomics.com), a program that generates pathways of interaction based on a repository of findings from the literature and other databases, we have started to evaluate the putative pathways and interaction networks that connect P2Rs to gap junction proteins. Figure 4 shows such a network of known interactions involving gap junctions obtained from a list of 34 gene annotations that were coordinated (Pearson coefficient >0.90) with $P2Y_1R$, including gap junctions, P2R subtypes, protein kinase C (PKC) isoforms, zonula occludens isoforms, Src, vinculin and actin.

Discussion

A recurring theme in *The Touchstone of Life* (Loewenstein, 1999) is that redundancies at all levels serve to minimize loss of fidelity of information transfer from nucleic acid to protein synthesis to intercellular communication. In this report, we examine the possibility that networks of transcriptomic control overlap networks of connexin-protein interactions (*interactomes*), converging on similar functional pathways and thereby providing redundancy.

Initial studies of protein-protein interactions were primarily "lucky guesses" that were verified by pairwise pull-down strategies. These were followed by unbiased

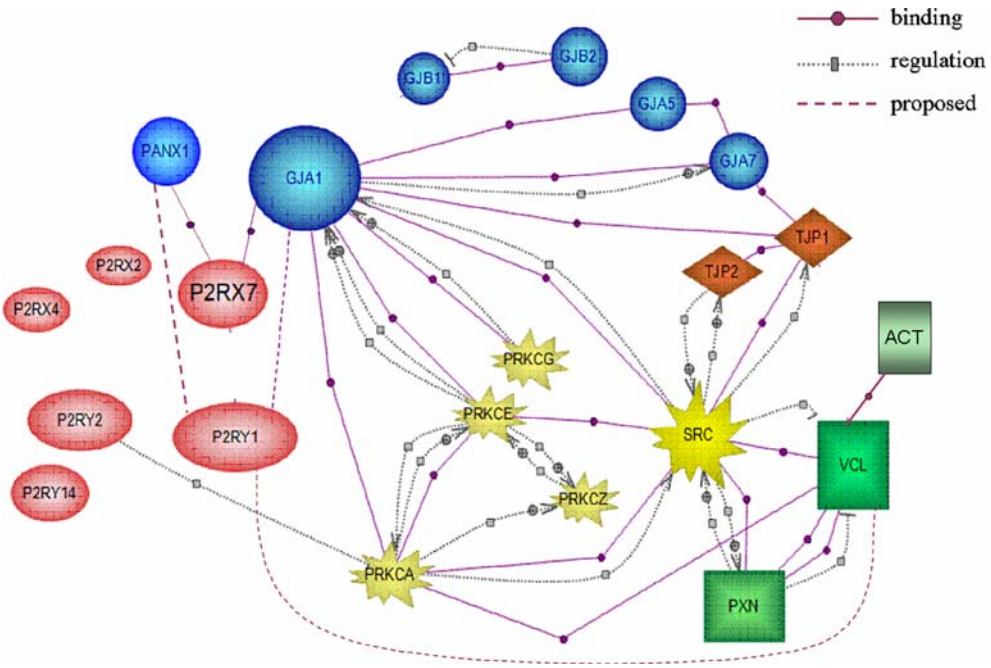

Fig. 4 *In silico* P2R and gap junction network. Network of known interactions involving gap junctions and purinergic receptors obtained from a list of 34 gene annotations that were positively coordinated (Pearson coefficient > 0.90) with $P2Y_1R$ including P2 receptor subtypes (P2Y and P2X), gap junctions (GJ and Panx1), tight junctions (TJ1, TJ2), vinculin (Vcl), paxillin (Pxn), actin (Act), protein kinases (PKC) and src. Network was generated by Pathway-Studio 4.0 (Ariadne Genomics software, http://www.ariadnegenomics.com) based on a repository of findings from the literature and other databases. Genebank annotations for these entities were used to build the pathway, which was constrained to provide only the direct interactions (binding and regulation) known to occur between the selected proteins. *Dashed lines* between $P2Y_1R$, Gja1 and Panx1 indicate findings described in this report

proteomic strategies including yeast two-hybrid screens (in the gap junction field primarily using the connexin carboxyl terminus as bait [*see* Giepmans & Moolenaar, 1998; Jin, Lau & Martyn, 2000]) and mass spectrometry (MS/MS) on coimmunoprecipitated proteins (*see* Singh & Lampe, 2003). An alternative approach described here is to exploit the observation first made in simpler organisms and more recently extended to mammalian tumors that the transcriptomic networks identified through coordination analysis of gene expression data sets may predict to some extent interacting proteins within functional pathways (Ge et al., 2001; Ideker et al., 2001; Walhout et al., 2002; Wachi et al., 2005).

Gap junction, pannexin and purinergic receptor proteins are major contributors to intercellular calcium wave propagation throughout the astrocytic syncytium (*for reviews*, *see* Scemes, 2000; Charles & Giaume, 2002; Scemes & Giaume, 2006). When we compared genes that were coordinately expressed with those encoding these proteins using microarrays to sample a large portion of the transcriptome, we identified a number of prominent interlinkages, both between these proteins and with other cell signaling molecules. These strong coordinations between pairs of channel molecules included *Gja1/$P2X_4R$*, *Gja1/$P2X_7R$*, *Gjb1/$P2Y_1R$*, *Gjb1/$P2Y_{14}R$*, *Gjb5/$P2Y_{14}R$*, *Gjc1/$P2Y_1R$*, *Gjc1/$P2Y_{14}R$*, *Gjc1/$P2X_2R$*, *Pnx1/$P2X_5R$*, *Pnx2/$P2Y_1R$* and *Pnx2/$P2Y_{14}R$*. In addition, highly significant coordinations were noted between Gja1, Pnx1, vinculin, $P2X_7R$ and actin. Using antibody arrays as an alternative technique to identify proteins interacting with the channel contributors to intercellular calcium wave spread, we found binding to previously reported interactions, including that of Cx43 with ZO-1, caveolin, $P2X_7R$, ERK and Src (*see* Table 3 *for references*). In addition, we identified certain interactions not previously reported, including Cx43 with vinculin and $P2X_7R$ with synaptophysin. IP studies verified a number of these interactions, including Cx43 binding to ZO-1, $P2X_7R$, actin and vinculin. Furthermore, the proteins bound to $P2X_7R$ included Pnx1, vinculin, β-actin, β-tubulin and c-Src and proteins bound to $P2Y_1R$ included Cx43, vinculin and β-actin.

Our initial hypothesis was that transcriptomic interlinkage might overlap with protein-protein interactions. In order to compare these data sets, it is necessary to restrict comparison to cases in which isoforms of encoded proteins match those of the gene array probes. Unfortunately, the number of precise correspondences in two data sets is low. However, of the eight significant coordinations of *Gja1* with the genes encoding protein pairs probed with antibodies, five encoded proteins binding to Cx43. On the oligonucleotide array, the correspondence was six Cx43-

Table 3 Proteins reported to interact with Cx43

Proteins	References*
Drebrin	Butkevich et al., 2004
Actin	Giepmans, 2006
α- and β-tubulin	Giepmans, 2006
α- and β-catenin	Ai et al., 2000; Xu et al., 2001
P120 catenin	Xu et al., 2001
n-Cadherin	Xu et al., 2001
c-Src and v-Scrc	Loo et al., 1999; Duffy et al., 2004a
ZO-1 and ZO-2	Giepmans, 2006
Cx46/Cx50/Cx45/Cx37	Herve et al., 2004
pKC, MAPK	Lampe & Lau, 2004
Caveolin 1	Schubert et al., 2002
Ubiquitin	Laird, 2006
NOV (Ccn3)	Fu et al., 2004; Gellhaus et al., 2004
ZONAB	Penes et al., 2005
Rab GAP-like protein (CIP85)	Lan et al., 2005
Rptpu	Li et al., 2005
Erk1/2	Li et al., 2005
EB1, p150	Shaw et al., 2007
$P2X_7R$	Present study; Fortes et al., 2004
Pnx1	Present study
$P2Y_1R$	Present study
Vinculin	Present study; Xu et al., 2006

* In cases of multiple reports, we have provided a review reference

protein interactions out of 11 significant transcriptomic coordinations of *Gja1* with other genes. Overall, therefore, 11 of 19 protein-protein interactions were as predicted (58%) compared to the approximately 20% overall significant antagonistic or synergistic coordinations (Iacobas et al., 2007a). Although these data are consistent with the hypothesis that transcriptomic coordination may predict protein-protein interactions and that transcriptomic and interactomic networks may superimpose, larger data sets will be required for a definitive conclusion.

In summary, we conclude from these studies that genes encoding gap junction and purinergic receptors are coordinately expressed in cortical astrocytes and that these genes are coordinately expressed with a network of additional genes encoding cytoskeletal proteins and kinases. IP and antibody array studies confirm interactions between certain of these participants in intercellular calcium wave propagation, suggesting that there is redundancy in networks of the genes and proteins involved in this intercellular signaling. Such network interactions likely underlie compensatory changes in P2 receptors in Cx43-null mice (Scemes et al., 2000; Suadicani et al., 2003) that maintain calcium wave spread in the absence of a major pathway present in WT astrocytes.

Acknowledgement This work was supported by National Institutes of Health research grants (NS-41023 to E. S., NS-041282 to D. C. S.). The undergraduate students (C. C., M. C.) were supported by the Summer Research Program at AECOM. We are grateful to Ms. Melissa Aleksey for technical assistance.

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