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Organizational Principles of the Connexin-Related Brain Transcriptome

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Abstract We have found that deletion of genes encoding the gap junction proteins Cx43, Cx32 and Cx36 alter the expression levels of large numbers of genes in mouse brain located on all chromosomes and encoding proteins from all major functional categories. Gene regulation in Cx32 and Cx43 null brains was more similar than that in the Cx36 null brain, suggesting the possibility of transcriptomic controls exerted by both genes on both astrocytes and oligodendrocytes. In order to explore the nature of expression linkage among the genes, we examined coordinated expression patterns in wild-type and connexin null brains. Coordination with Cx43 in wild-type brain predicted regulation in Cx43 nulls with considerable accuracy. Moreover, interlinkage within gene networks was greatly perturbed in the Cx43 null brain. These findings suggest several principles regarding regulatory transcriptomic networks involving gap junction genes and raise the issue of the underlying cause of connexin null phenotypes as well as mechanisms of regulation.

Keywords Astrocyte · Cx43 · Cx32 · Cx36 · Gap junction · Glia · Gene expression · Oligodendrocyte · Panglial syncytium

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Introduction

Ever since gap junctions were discovered as structures interconnecting cells, their roles in tissue and organism physiology have been widely speculated. The generation of connexin null mice and the recognition that human disease may be caused by connexin mutations have led to experiments examining whole-animal (or "integrative") physiology in the expectation that aberrations would reflect the function of the missing protein (a strategy we termed "negative physiology" in an early review of such studies: Spray et al., 2000). However, there are reasons to proceed with caution in such interpretations. For example, a recent commentary by Insel & Patel (2007) has questioned whether knockout phenotype reflects the missing function or the animal's compensation for that missing function (as might occur, e.g., in forelimb hypertrophy following hindlimb damage). In addition, gene deletion in individual cells or tissues is often accompanied by compensatory changes in gene expression patterns, the mechanisms of which are not entirely clear.

One thrust of our recent research has been to examine gene expression changes in tissues and cells of mice in which connexins have been deleted through homologous recombination (e.g., Iacobas et al., 2003; Iacobas, Scemes & Spray, 2004; Iacobas et al., 2005a,b; Iacobas, Iacobas & Spray, 2007a,b; Iacobas et al., 2007c). As summarized below, the strategy utilized in these studies has allowed detection of changes in gene expression level, control and coordination that, though small, are significant. Moreover, the analysis of covariance of gene pairs within biological replicas has allowed us to construct preliminary drafts of gene interlinkages in wild-type tissues and cells and to compare these interlinkages to samples where connexin expression is absent. These studies indicate a number of

new principles of organization of the transcriptome, leading to the hypothesis that connexins may be considered to be nodes in gene regulation. Thus, connexin genes not only encode proteins that interconnect cells into functional networks but also coordinate networks of genes that encode both functionally related and unrelated proteins.

We here summarize the apparent principles that our studies of the connexin-dependent transcriptome have revealed. Although we have emphasized the brain in this report, it should be pointed out that the general transcriptomic principles appear to be similar for the heart as well as for cultured astrocytes.

Materials and Methods

At the time we began the array experiments, expression microarrays were very costly; therefore, independent replicas were not commonly compared. However, because our institution provided homemade cDNA and then oligonucleotide arrays at a highly subsidized cost, we were able to undertake such studies rather economically, allowing us to initiate what has become our general protocol of comparing four independent samples of each genotype or treatment condition. Also, we have generally compared each sample against a common reference prepared from several tissues (including embryos) to have a wide diversity of expressed genes, allowing us to establish expression values for each spot relative to the constant reference on every array.

For the studies described here, Trizol-extracted RNA from each brain of sibling wild-type and connexin null mouse and our in-house-prepared mouse RNA reference was reverse-transcribed in the presence of fluorescent Cy3 and Cy5 deoxyuridine triphosphates (dUTPs) to obtain labeled cDNAs. Slides were hybridized and arrays scanned with invariant photomultiplier gain. After eliminating spots for which data were not of high quality (e.g., local corruption, saturated pixels or hybridization not significantly above background) and performing intra- and interchip normalization, background-subtracted intensity values of all valid spots probing the same gene (redundancy groups) were averaged separately for the experimental and control samples. The raw data corresponding to gene expression experiments in brain discussed in this review have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus database, http://www.ncbi.nlm.nih.gov/geo, series GSE1954 (neonatal wild type, Cx43 null, Cx32 null mice) and GSE6355 (P10 wild type and Cx36 null mice). Averaged normalized spot intensities were used to identify the differentially expressed genes between the two groups of samples with absolute fold change >1.5x and $p < 0.05$. These values were also used to determine the relative expression variability (REV) within control or experimental samples as the midrange of the χ^2 interval estimate of the coefficient of variability of the expression level of each gene. Genes were ordered in decreasing REVs such that gene expression stability (GES) scores were assigned to each gene (for the most stably expressed gene GES = 100, and for the most unstably expressed GES = 1/number of quantified unigenes). Binary logarithms of the normalized, background-subtracted expression levels of each gene across the four replicas were compared to those of every other gene across the same replicas to calculate pairwise Pearson correlation coefficients (ρ_{ij}), where values above 0.9 indicate significant synergistic relations between the two genes, values below –0.9 indicate antagonist relations and values of –0.05 to 0.05 indicate genes whose expression levels are significantly independent. Then, for each quantifiable gene and each type of sample, we determined the *synergome, antagome* and *exclusome* as the sets of all synergistically, antagonistically or independently expressed gene partners, respectively, as well as the *coordination profile* (set of correlation coefficients with expression levels of each other quantified gene in the sampled transcriptome). Since both synergistic and antagonistic expression correlations reflect gene interlinkage, we also calculated the *expressome* as the union of the synergome and antagome. The *exclusome* was determined as a measure of the delimitations of gene networks. In order to obtain a quantifiable parameter with which to compare the interlinkages of two genes with the sampled transcriptome, we calculated the *similarity index*, indicating the degree to which one gene has more or fewer similar synergistic antagonistic or independent relationships than the other gene (Iacobas et al., 2007a), as well as the overlap (OVL) of the coordination profiles (Iacobas et al., 2007b). Both indices take values from –100% to 100%, with high positives indicating similarity, high negatives indicating opposition and close to zero indicating neutrality.

Results

Connexin-Related Regulomes

The *regulome* is defined as the set of significantly differentially expressed genes in experimental samples with respect to controls. We have found that regulomes of connexin-deficient brains, hearts and cultured astrocytes are vast (encompassing 10% or more of the sample transcriptome). Furthermore, the regulomes were found to be quite varied, the regulated genes extending to all chromosomal locations and including all functional categories of encoded proteins. We have also found that brain regulomes are connexin-specific. When brains of Cx43, Cx32 and

Cx36 null mice were compared, we found altered expression of distinct sets of genes. However, these sets overlapped, as illustrated in Figure 1. The OVL was also evaluated by comparing the gene expression levels among the connexin-deficient genotypes.

The largest degree of OVL between the gene expression levels (92%) was found between Cx43 null and Cx32 null brains, whereas OVL of expression profiles was less extensive for either of these connexin nulls compared to the Cx36 null brain (42% with Cx43 null and 48% with Cx32). Thus, for 92%, 42% or 48% of the quantified genes, the absolute fold change of the expression levels in the compared genotypes was <1.5x. A quantitative method by which the regulomes can be compared is through the calculation of Euclidean distances between the profiles of the expression ratios of the two genotypes with respect to the wild type. Applying this method to the data sets on brains of these various connexin null mice indicated that distances between the transcriptomes of the Cx36 null brain and those of Cx32 and Cx43 null brains were significantly larger than the Euclidean distance between the Cx32 and Cx43 null brain transcriptomes (2.4 and 2.2 compared to 1.0: Iacobas et al., 2007b). The similarity between Cx43 null and Cx32 null brain regulomes and their substantial difference when compared to the Cx36 null brain regulome were robust with regard to chromosomal and functional gene cohorts (Iacobas et al., 2007b).

Gap junctions interconnect nervous system cell populations expressing Cx43 and Cx32 (astrocytes and oligodendrocytes), thereby forming what has been termed the "panglial syncytium" (Rash et al., 1997), whereas cells expressing Cx36 (neurons and microglia) are not within this network. Therefore, the similarity in regulomes of Cx43 and Cx32 null brains may represent a panglial transcriptome, which might explain pathological changes in white matter in patients with Cx43 mutations (*see* Loddenkemper et al., 2002) and changes in Cx43 expression in experimental autoimmune encephalomyelitis (EAE) where myelinating cells are affected (Brand-Schieber et al., 2005).

Connexin-Related Variomes

Quantification of the variability in gene expression level within a set of biological replicas allows us to determine whether expression of gene pairs is coordinated in a genotype (*see below*) and whether there are global changes in gene expression variability from one genotype to another. We have found that the overall variability in gene expression in brains of all three connexin null mice examined is significantly lower than that of the wild types: variability was 49%, 29% and 16% less than controls for Cx43 null, Cx32 null and Cx36 null brains, respectively. If we define the variome as the set of genes that are significantly more variable within a genotype than other genes (i.e., low GES scores), we can ask whether these highly variable genes are more or less prone to regulation in connexin null brains. Calculations on the data sets from Cx43 and Cx32 null brains revealed such a positive correlation (as illustrated in Fig. 2): the more variable genes tend to be those with higher fold changes in gene expression (Iacobas et al., 2007b).

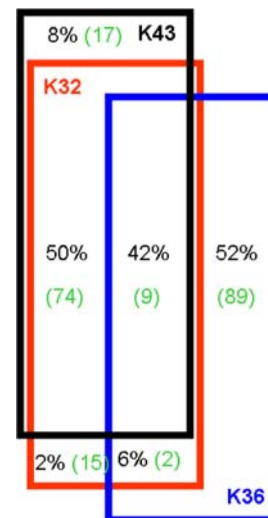

Fig. 1 Comparison of gene expression profiles in Cx32, Cx36 and Cx43 null mouse brains. *Numbers written in black* represent the percentages of quantified genes whose expression levels were not significantly different in the indicated genotypes, while *numbers written in green* represent the percentages of genes regulated similarly in overlapping genotypes. Note that expression of 42% of the genes was similar in all three connexin nulls, while 92% of the genes were similarly expressed when comparing Cx43 null and Cx32 null and 48% when comparing Cx36 null and Cx32 null. In at least one genotype, 9% of the regulated genes were similarly regulated in all three nulls, 83% of the regulated genes were similarly regulated in both Cx43 null and Cx32 null and 11% were similarly regulated in both Cx36 null and Cx32 null. Interestingly, no gene was oppositely regulated in Cx43 null and Cx32 null, while 6% were oppositely regulated in Cx36 null and Cx32 null (3% in Cx36 null and Cx43 null)

This is consistent with our earlier hypothesis that more stable genes tend to encode proteins with general housekeeping cellular function, while more variable genes encode proteins with cell or tissue specificity. Thus, the more tightly controlled and less likely altered genes would be those essential for survival cell processes, while the more highly variant and more likely altered ones would specify more moderate responses.

Connexin-Related Expressomes

Calculation of Pearson correlation coefficients between expression levels of each gene pair across the four biological replicas indicates extensive linkage among gene expression in all genotypes, with significant synergistic,

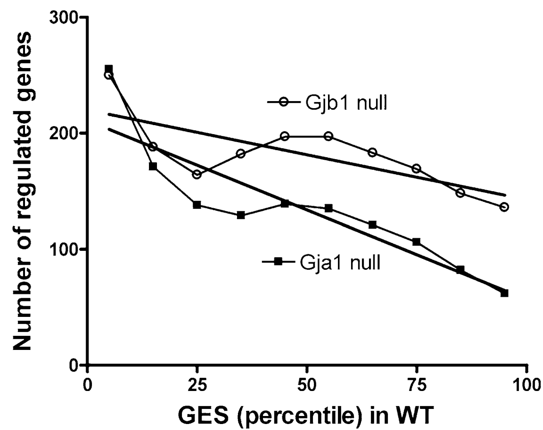

Fig. 2 Number of regulated genes in Cx43 null and Cx32 null brains decreases with their expression stability in the wild-type brain. Linear regression provided significantly nonzero slopes (–1.5 with p = 0.0006 for Cx43 null and –0.8 with p = 0.0131 for Cx32 null brain), indicating that genes with less variability in the wild types tend to be regulated less than highly variable ones in the connexin null brains

antagonistic or independent partners on average extending to 20% of the transcriptome. If such interlinkage indicates tendencies for individual genes to influence the expression of one another, it might be expected that coordination in wild-type brain would predict which genes are regulated in the connexin null brain. As illustrated in Figure 3, there is such a high degree of correspondence in the case of Cx43 null brains, such that genes that are synergistically coordinated with Cx43 in wild-type brain tend to be downregulated in the knockout, antagonistically coordinated genes tend to be upregulated and independent genes tend not to be regulated.

This finding of the expressome predicting the regulome indicates that the changes seen in the connexin null brain are on a continuum with normal expression patterns. If this finding is confirmed for other genes, it could potentially both provide predictions of knockout phenotypes and reduce the necessity for generating them.

When the significant partnerships (i.e., expressomes and exclusomes) and the coordination profiles of individual genes are compared with one another, there is apparent striking likeness or dissimilarity between certain gene pairs. For example, Cx43 and pannexin 1 are very similarly interlinked with all other genes, as presented in Figure 4. Over the entire range of coordination values, even for Pearson coefficients that were not significant for synergistic, antagonistic or independent expression, these two genes have very similar relationships to all others. The striking similarity of Cx43 and Panx1 interlinkages suggests that overexpression of either the *Gja1* or *Panx1* gene may compensate for underexpression of the other. Moreover, some gene pairs show strikingly opposite expression coordination, suggesting that downregulation of either gene might compensate for downregulation of the other. We have termed these highly similar or dissimilar gene pairs as coordination "see-saws," both to denote their appearance in coordination plots and because of the possibility that the underlying controls may push or pull upon network partners.

When coordination see-saws are compared between genotypes, the interlinkages often change. Such alterations in which genes are coordinated with one another appear to indicate that the entire network has been rearranged by disruption of a single gene. Profound network

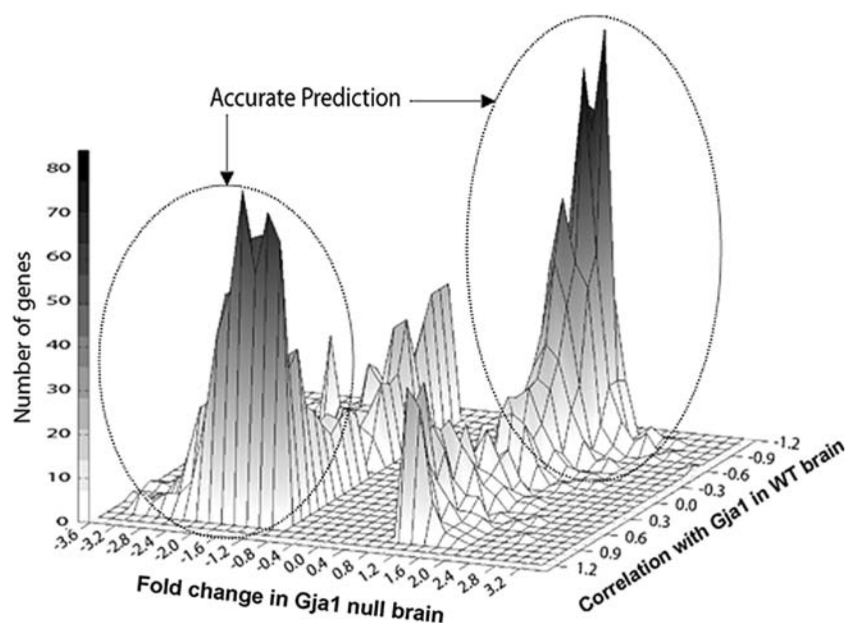

Fig. 3 Expression coordination with *Gja1* in the wild-type brain predicts expression regulation in the *Gja1* null brain. Note the significantly high proportions of genes whose regulation (*circles*) or lack of regulation (peaks on the grid between –0.05 and 0.05 with respect to coordination with *Gja1* in wild type and –1.5 and 1.5 as fold change in the *Gja1* null brain) was accurately predicted from coordination of the genes with Cx43 in wild-type brain

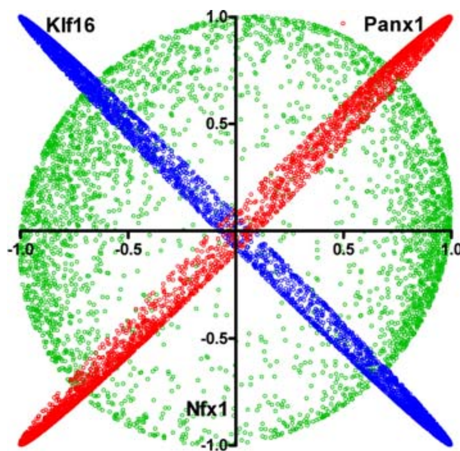

Fig. 4 Examples of genes with striking similarity (*Panx1*, pannexin 1; OVL = 93.2), opposition (*Klf16*, Kruppel-like factor 16; OVL = –94.7) and neutrality (*Nfx1*, nuclear transcription factor X-box binding 1; OVL = –0.4) as coordination profile and expressomes with *Gja1*. Such coordination "see-saws" are in most cases substantially altered in connexin null mice

remodeling as a consequence of downstream ripples from gene deletion presumably indicates the presence of both serial and parallel interconnections within the gene expression networks. Such rearrangements are illustrated in Figure 5 with regard to the relationship among a small group of transcription factors and Cx43. The abundant and complex interlinkages among the genes in the wild-type brain are largely eliminated, with some interactions even changing from synergistic to antagonistic in the Cx43 null.

Discussion

Omics

In this review, we use a number of "omic" terms to designate segments of the brain transcriptome with specific characteristics. Thus, we define the *regulome* as the set of all genes differentially expressed in an experimental group compared to controls, the *variome* and the *stabilome* as the sets of highly variable (GES <5) and the highly invariant (GES >95) genes among the biological replicas. For each individual gene, we define the *expressome* as the set of significantly synergistically and antagonistically expressed gene partners (the synergome and antagome, respectively) and the set of independently expressed partners (exclusome), depending on the values of the pairwise Pearson correlation coefficients between the logarithms of the expression levels among replicas of a single sample.

Analysis of the large-scale data sets that microarrays provide have generally concentrated on identifying genes with large fold changes following experimental manipulation, whereas coordination analysis is usually confined to temporal studies of changes in gene expression (e.g., Iacobas et al., 2006). However, as shown here, the inter-animal variability in gene expression allows coordinations to be quantified by comparing gene expression levels for each gene pair across the biological replicas of a single genotype or treatment group. Such coordination analysis reveals complex patterns of gene expression interlinkage that have predictive value with regard to gene expression changes in the Cx43 null brain. The analysis also reveals genes with very similar interlinkages with the transcrip-

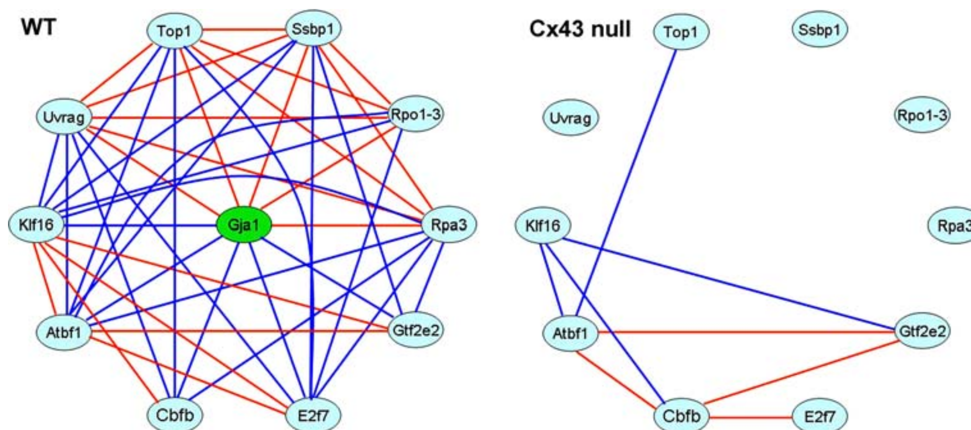

Fig. 5 Interaction networks are markedly rearranged in Cx43 null mouse brain, illustrated using a small portion of the *Gja1* gene cluster with genes encoding transcription factors. *Red lines* indicate synergistic expression and *blue lines*, antagonistic expression. Note that the overall significant interlinkage of the transcription factors was reduced by >77% by ablation of *Gja1*: synergistic pairs from 15 to 4 and antagonistic ones from 20 to 4. Remarkably, in addition to preservation of the synergism of *Atbf1* with *Gtf2e2* and the antagonism with *Top1*, ablation of *Gja1* reversed two interlinkages of *Klf16* from synergistic into antagonistic (with *Atbf1* and *Cbfb*) and created two new significant interlinkages of *Cbfb* (with *Atbf1* and *E2f7*)

tome and those with opposite interlinkages, providing a hypothesis for compensatory gene expression when a gene is deleted.

Coordination analysis of connexin null brains reveals profound rearrangement of interlinkages that exist in the wild-type brain. Presumably, these rearrangements reflect the topology of the gene regulatory networks. Although topological features remain to be fully elucidated, they presumably include *Gja1* and other connexin genes positioned at the interfaces between coordinated gene clusters, as well as being positioned in both feedforward and feedback loops of gene expression regulation. We have previously summarized this key role of the Cx43 gene as that of a "hub" in gene expression regulation (Iacobas et al., 2007a,b). While other genes certainly could and should be regarded as such hubs of modifiable interlinkage, the concept of an intercellular communication gene being central to intertranscriptomic linkages may explain phenotypes of connexin null mice and of pathological conditions where connexin expression is altered.

Examples Where Phenotype May Be Due to Interlinkage of Connexin Expression to That of Other Genes

Connexin null mice display significant changes in expression level of genes encompassing >10% of the transcriptome, including many genes not previously associated with intercellular communication. This raises the major question of whether the expression of regulated genes is normally tied to that of Cx43 in wild types so that their regulation in Cx43 nulls would be a direct consequence of the coordination with Cx43. More generally, if two genes are synergistically or antagonistically expressed in physiological conditions, is there an increased chance for the genes to be similarly or oppositely regulated under pathological conditions? Indeed, coordination analysis revealed that numerous genes belonging to all major, disjoint functional categories and located in all chromosomes are coordinately expressed in wild-type astrocytes (Iacobas et al., 2003, 2004). The coordination occurs with similar frequencies both within each gene cohort and between different cohorts. Moreover, we found that regulation of >80% of the genes whose expression was altered in the knockout was predicted based on coordination with Cx43 in the wild-type brain or astrocyte. This finding indicates that the null phenotype appears to be a simple extension of wild-type coordinated variability.

There is a long history of the association of gap junction expression with growth control, development and tumorigenesis, much of which was pioneered by W. R. Loewnstein (*for a review of the early literature, see* Loewenstein & Rose, 1992; *for more recent findings*, *see* Kardami et al., 2007). Moreover, studies from connexin null mice have revealed numerous unexpected phenotypic changes, including reduced growth rate and modified expression of purinergic receptors of Cx43 null astrocytes (*for review, see* Iacobas et al., 2007b). The recent discovery that Cx43 mutations are responsible for the human occulodental digital dysplasia syndrome (now numbering more than 30 separate coding region mutations: *see* Debeer et al., 2005) highlights the complex multiple effects that connexin disruption may have at the level of the organism. These mutations, all of which appear to create dysfunctional Cx43 location and function (Gong et al., 2007; Lai et al., 2006; Shibayama et al., 2005), produce structural disturbances of bone, teeth as well as white matter pathology in affected families.

It has recently been reported that Cx43 might play a role in the development of tissue boundaries in developing mesoderm, due to abnormal expression of ephrin's orphan receptors (Davy, Bush & Soriano, 2006). This altered expression pattern resulted in decreased Cx43 expression and function at cell population boundaries and craniofacial defects in these animals, which were largely restored by Cx3 overexpression. A possibly related example of such compensatory genetic interactions involves the demonstration that the decreased cerebellum and brainstem sizes seen in Wnt-1-deficient mice are corrected by overexpression of Cx43 (Melloy et al., 2005). Intriguingly, a recent report on astrocyte-targeted Cx43 deletion reported a strain-dependent susceptibility to a phenotype lacking the cerebellum (Wiencken-Barger et al., 2007). The mechanisms responsible for these interlinkages between phenotypes resulting from deletions and the compensatory replacement of other genes remain to be clarified, but we speculate that they are likely to represent coordinated transcription of the responsible genes. In the case of the WNT-1 pathway, we suspect it may involve the binding to Cx43 of ß-catenin and transcriptional feedback regulating its expression (Ai et al., 2000).

Our comparisons of gene expression levels in Cx32, Cx36 and Cx43 null mouse brains revealed sets of genes that are uniquely regulated in a single genotype, genes that are regulated in two genotypes and genes that are regulated in all three. With regard to overlap between genotypes, Cx32 and Cx43 null brains showed a substantial intersection of regulated genes. We interpret this finding as possibly indicating coordinated gene expression regulation of astrocytes and oligodendrocytes by one another since in the brain Cx43 is largely confined to astrocytes and Cx32 is largely confined to oligodendrocytes. Thus, the panglial syncytium extending throughout the brain (*see* Rash et al., 1997) may have a transcriptomic counterpart.

The issue of the extent to which different connexins perform functions that are not interchangeable has been

addressed through knockin/knockout mice, where the coding region of one connexin gene is replaced with that of another. Thus far, Cx43 has been replaced with Cx32, Cx40, Cx31 and Cx26, in all cases resulting in less neonatal lethality than the Cx43 null, although Cx40 and Cx32 substitutions were more effective in rescuing cardiac conduction (Plum et al., 2000; Zheng-Fischhofer et al., 2006; Alcolea et al., 2004; Winterhager et al., 2007). Moreover, the mice in which Cx43 was replaced by Cx26 showed profoundly disturbed reproductive organs (Winterhager et al., 2007). The finding that each of these Cx43 replacements results in a different cardiac functional phenotype and affects other organs differentially indicates that each connexin must have both overlapping and individual properties with regard to the phenotype of the animal. While the differences in permeability of the channels formed by the connexins has been stressed as a possible cause of such specificity, it remains possible that differences in affinity for Nexus binding partners with transcriptional activity may play a role in such phenotypic differences (*see next section*).

What Is the Mechanism by Which Connexin Gene Expression Impacts the Expression of Other Genes?

As indicated above, the phenotypes that are associated with over- and underexpression of functional gap junction channels are varied and in many cases not predicted solely on the basis of intercellular communication. This raises the essential question of the molecular mechanisms responsible for the gene expression changes underlying the phenomena. There are several types of mechanisms that have been proposed, each of which has experimental support; and most likely a combination of such mechanisms participates in achieving the overall phenotype.

One general class of mechanism that may contribute to the impact of gap junction gene expression on expression of other genes is that of the functional gap junction channel itself. Gap junctions have long been known to transfer intracellular signaling molecules conveying information from one cell to another (*see* Loewenstein, 1999). In addition, it has recently been reported that RNAi-sized oligonucleotides may traverse the junctions (Valiunas et al., 2005), permitting exchange of even higher-density information content. The disruption of such uniform second messenger content within a cell population could generate diffusion gradients and boundaries between cells and cell populations and could be important in development and differentiation. Moreover, such gradients could give rise to differential gene expression through such mechanisms as differential phosphorylation of transcription factors (*see* Stains & Civitelli, 2005).

There are also numerous potential mechanisms by which connexin expression might alter the expression of other genes that do not involve the role of gap junctions as intracellular channels. One such mechanism would be direct interaction of the connexin or connexin fragments either with DNA or with transcription factors (*see* Dang, Doble & Kardami, 2003). Another mechanism would involve connexin binding to other proteins with transcription factor activity. It is now well established that connexins interact with other proteins both at the junctional membrane and during their voyage to and from the junctional site, forming a Nexus of interacting proteins (*see* Spray, Duffy & Scemes, 1999). Thus, Cx43 has been shown to interact with β-catenin and, in the absence of Cx43, β-catenin translocates to the nucleus, stimulating Cx43 transcription (Ai et al., 2000). Similarly, CCN3 (NOV) has recently been reported to be trapped at the junctional membrane by its binding to Cx43 and to dysregulate growth in the absence of such binding (Fu et al., 2004; Gellhaus et al., 2004). Likewise, the ZO-1-associated nucleic acid binding protein ZONAB may serve such a role in oligodendrocytes and astrocytes (Penes, Li & Nagy, 2005), and the scaffolding transcription factor HLDG1 may play a similar role in its interaction with Cx32 (Duffy et al., 2007).

Conclusions

As outlined in a recent review (Ge, Walhout & Vidal, 2003), the availability of large-scale data sets dealing with gene regulatory networks as well as analysis of large sets of protein-protein interactions (interactomes) are providing the opportunity to understand linkages in functional pathways encoded by the genes and executed by the proteins. Among the new concepts emerging is that the transcriptome superimposes on the interactome such that interacting proteins are more likely to be encoded by interacting gene networks. Preliminary evidence for such an organization involving Cx43 is provided in a companion article in this special issue of the *Journal of Membrane Biology* (Iacobas et al., 2007c).

A general strategy that now emerges for the evaluation of phenotypes of animals in which connexin genes are altered either directly or as a consequence of pathological changes is to consider the overall phenotype as a set of phenotypes caused by alterations in linkages of the transcriptome and interactome. The critical new challenges that these new large databases now pose are to understand how the subphenotypes arise as downstream ripples of alterations in network interconnections, to determine the extent to which network alterations may be overcome by experimental manipulation of expression levels of network

components and to determine the molecular mechanism by which network conductivity is achieved.

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