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Review

Control of neuronal morphology and connectivity: Emerging developmental roles for gap junctional proteins

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ABSTRACT

Recent evidence indicates that gap junction (GJ) proteins can play a critical role in controlling neuronal connectivity as well as cell morphology in the developing nervous system. GJ proteins may function analogously to cell adhesion molecules, mediating cellular recognition and selective neurite adhesion. Moreover, during synaptogenesis electrical synapses often herald the later establishment of chemical synapses, and thus may help facilitate activity-dependent sculpting of synaptic terminals. Recent findings suggest that the morphology and connectivity of embryonic leech neurons are fundamentally organized by the type and perhaps location of the GJ proteins they express. For example, ectopic expression in embryonic leech neurons of certain innexins that define small GJ-linked networks of cells leads to the novel coupling of the expressing cell into that network. Moreover, gap junctions appear to mediate interactions among homologous neurons that modulate process outgrowth and stability. We propose that the selective formation of GJs between developing neurons and perhaps glial cells in the CNS helps orchestrate not only cellular synaptic connectivity but also can have a pronounced effect on the arborization and morphology of those cells involved.

1. Introduction

The unique morphologies and patterns of connections made by neurons during development are thought to arise from an initial cell-type specific period of stereotypic outgrowth, largely under the control of molecular mechanisms that depend on intrinsic developmental programs, which is then followed by an extended period of growth in which cell–cell interactions help to sculpt it's arbor into its final shape, size and participation in different synaptic networks. These cell–cell interactions include (1) cellular recognition, as mediated by molecules such as immunoglobulin superfamily proteins like DSCAMs and Turtle [1,2], (2) selective neurite adhesivity and repulsion, mediated by cell adhesion molecules, particularly proteins of the immunoglobulin, receptor protein tyrosine kinase and phosphatase, cadherin, and leucine-rich repeat families [3–7]; and (3) activity-dependent processes that help select and sculpt the synaptic terminals of the neuron [8,9]. Recent findings from a number of laboratories suggest that gap junction (GJ) proteins may be in a unique position to contribute to all three of these mechanisms. In this review, we highlight the roles played by GJ proteins as adhesion molecules, and as regulators of neuronal circuit formation in the developing nervous system. In the latter role, we suggest, GJ protein arrays may act as surface recognition factors and not simply as conduits that provide for the exchange of electrical and small-molecule signals. Lastly, we present recent evidence that suggests that GJ proteins can have a fundamental role in determining the morphology of particular identified neurons.

Among the key properties supporting a role for GJs in cell–cell recognition is that they (1) belong to large gene families, (2) that most cell types, including neurons express more than one type of GJ protein [10–12], (3) and that they are present at axon-to-axon and dendrite-to-dendrite points of contact between growing neurons [10,11]. There are around twenty-one connexins in mammals and at least thirty-seven in zebrafish [13,14]. Twenty-five innexins have been reported in *Caenorhabditis elegans* [15], and twenty-one different innexin genes in the medicinal leech *Hirudo* [12]. In contrast, only eight innexins appear to be present in *Drosophila*, but multiple splice isoforms (e.g. the *shaking-B* gene gives rise to five possible transcripts [16]), expands that number substantially.

Gap junctions form when two hexamers in closely apposed membranes dock together selectively through their extracellular loops [17,18]. All gap junction proteins share a common topology, with four transmembrane domains connected by two extracellular loops and one cytoplasmic loop, leaving both amino and carboxyl termini in the cytoplasm. Studies using paired *Xenopus* oocytes have revealed that only 'compatible' gap junction proteins can

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form functional gap junctions and that the extracellular loops are critical for this docking process to occur [19–24]. By swapping the extracellular loop domains between different connexins, chimeric gap junctions can be assembled and, at least among some of the connexins, this docking is dependent on key amino acid residues along the second extracellular loop hydrogen bonding with a facing hemichannel [25,26].

2. Gap Junctions as adhesion complexes

There is now considerable evidence that GJs can act as adhesion mediators, providing not only an intercellular bridge for communication but also helping to shape adhesive interactions between cells. Some of this evidence comes from direct experimental results examining the roles of GJ proteins in cellular adhesion assays, and some from indirect evidence, which places GJ proteins in close association with other membrane adhesion proteins, including tight junction proteins and cadherins, scaffold proteins and the cellular cytoskeleton (for review see [27]).

Cellular aggregation assays, in which mono-dispersed cells on a rotary shaker are examined for their ability to aggregate into cell clusters, are a useful measure of cell adhesivity. Cotrina et al. [28] used a short-term aggregation assay with C6-glioma and HeLa cells stably transfected with Cx43 or Cx32. They found that, although these two cell types do not usually adhere to each other, they do so when they both express the same connexin. Furthermore, this aggregation/adhesivity was found to be calcium independent, unlike the classical role documented for cadherins in tissue self-assembly [29–30].

Just how strong are connexin-mediated adhesive interactions? Studies using different cell lines suggest that it can be comparable to the well-established role of cadherins in cell and tissue assembly [31]. Carbenoxolone, a general GJ inhibitor, disrupts this self-assembly process, as does application of a blocking antibody recognizing the second extracellular loop of Cx43, both of which reduced cellular aggregate size, and critically, this inhibition is comparable to that produced by N-cadherin blocking antibodies [31]. It has long been recognized that hydrophobic interactions of apposing connexins provide for exceptionally strong binding. Denaturants and chaotropic salts such as urea are required to split established GJ channels into two hexamers on separate membranes [32,33]. Indeed, when cardiac tissue is dissociated by collagenase perfusion, the GJ hexamers are ripped from the plasma membrane of one myocyte and retained by its opposite neighbor rather than being split into their component membranes [34].

Do the innexins share this property of adhesivity? One recent report suggests that they do. *Drosophila* S2 cells are ideal for adhesion interaction assays since untransfected cells are non-adhesive. However, cells transiently transfected with wild-type leech innexins aggregate reliably into multicellular clusters when placed on a rotating shaker [35], suggesting that the innexins, like their vertebrate cousins, share a similar adhesive capacity. Moreover, when two cultures, each expressing a leech innexin, Inx1 or Inx6, are shaken together, they form innexin-specific clusters, indicating that innexin-based adhesivity is selective.

There also exists considerable evidence that GJ proteins can function as part of larger protein complexes at the plasma membrane with roles in cellular adhesion. For example, cadherins can help control the trafficking of connexins to the plasma membrane. Firstly, treatment with antibodies against cadherins inhibits GJ-mediated dye transfer between cells in culture, whereas transgene expression of E-cadherin of poorly coupled cell lines increases GJ coupling [36–38]. Furthermore, the cytoplasmic loop of Cx43 has been shown to be necessary for this localization to occur [39], suggesting that connexon insertion and/or localization in the plasma membrane may be cadherin dependent.

An analogous protein–protein interaction has been described for innexins and cadherin proteins in *Drosophila* epithelial tissues [40]. Fly Inx2 and 3 co-localize to puncta in the membranes of cells in the epidermis. In Inx2 mutants, Inx3 is mislocalized to the cytoplasm and conversely, Inx3 RNAi leads to the mislocalization of Inx2 and, critically, to the mislocalization of *Drosophila* cadherin, causing cell polarity defects in the epidermis [40]. Furthermore, a direct interaction between Inx2 and adherens junction proteins was identified by yeast two-hybrid analysis, and coimmunoprecipitation experiments using embryonic extracts have shown that Inx2, like Cx43, interacts via its cytoplasmic loop domain with the C terminus of *Drosophila* cadherin [41].

A third source of evidence for GJ proteins playing an adhesive role comes from studies implicating connexins in cellular migration and morphology. Perhaps the best studied is Cx43, which has been implicated in helping to control the morphology and migration of cells in a variety of tissues, including cardiac neural crest cells, CNS ventricular neuronal cell migration, wound-healing, epithelial cell and B lymphocyte cell migration and glioma invasivity [42–46]. For example, in the rat CNS, transplanted Cx43-expressing glioma cells disseminated freely throughout the brain parenchyma, whereas Cx43-deficient cells did not [43].

In another example, two GJ proteins, Cx43 and Cx26, have been shown to be expressed at the points of contact between migrating neurons in the mammalian cerebral cortex and radial glia cells, which are thin bipolar cells that extend from the inner ventricular surface to the pial surface of the cortex [47,48]. Acute down-regulation of Cx26 or 43, via electroporation of a short-hairpin RNA plasmid, impairs the migration to the cortical plate of neurons expressing the plasmid [49]. Most striking, cells expressing chimeric connexins capable of docking, but not functional channel formation, showed no change in their ability to migrate [49], suggesting that the role played by these GJ proteins does not require the formation of a functional pore. The role of GJs in this process is not yet fully understood, but at its' simplest, it can be imagined that Cx43 and 26 function in one or more of the following steps in cellular migration; (1) adhesion site formation at the leading edge, (2) adhesion site stabilization or, (3) adhesion site removal at the trailing edge. Lastly, unlike the connexin or innexin's cytoplasmic loop's role with cadherin membrane localization [39], Cx43's function in neuronal, fibroblast and lymphocyte migration appears to be dependent upon the C-terminal domain of the Cx43 [46,48]. In this regard, it may be important to consider that Cx43 has a tubulin-binding domain located in its C-terminus [50,51].

3. Innexons and connexons can act as 'Lock and Key Recognition' factors

In addition to conferring adhesive properties, the second requirement for the hypothesized role of GJ proteins in helping to shape neuronal connectivity and morphology is that they need to function as cell–cell recognition molecules, helping to discriminate potential target cells from non-target cells by conferring a neuronal identity marker and thereby helping to build GJ-defined neuronal circuits.

Considering the case of a cell expressing only two innexins or connexins, which is likely to be a low estimation for most cells including neurons (reviewed in [11]), and assuming that those two proteins are free to associate in a stochastic fashion, then the cell could express two different monomeric hemichannels and up to twelve different combinations of heteromeric hemichannels [52]. Given the numerous possibilities, and the large size of GJ gene families, it can easily be imagined that groups of neurons might display on their surfaces unique and shared hemichannels,