Review

Update on connexins and gap junctions in neurons and glia in the mammalian nervous system

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Abstract

Among the 20 proposed members of the connexin family of proteins that form gap junctional intercellular communication (GJIC) channels in mammalian tissues, over half are reported to be expressed in the nervous system. There have been conflicting observations, however, concerning the particular connexins expressed by astrocytes, oligodendrocytes, Schwann cells and neurons. Identification of the several connexin proteins at gap junctions between each neuronal and glial cell type is essential for the rational design of investigations into the functions of GJIC between glial cells and into the functional contributions of electrical and “mixed” (chemical plus electrical) synapses to communication between neurons in the mammalian nervous system. In this report, we provide a summary of recent findings regarding the localization of connexins in gap junctions between glial cells and between neurons. Attention is drawn to technical considerations involved in connexin localization by light and electron microscope immunohistochemistry and to limitations of physiological methods and approaches currently used to analyze neuronal and glial coupling. Early physiological studies that provided evidence for the presence of gap junctions and electrical synapses in isolated regions of the mammalian brain and spinal cord are reexamined in light of recent evidence for widely expressed neuron-specific connexins and for the existence of several newly discovered types of gap junctions linking neurons.

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1. Introduction

Gap junctions and their constituent connexin proteins have represented an investigational challenge in all tissues where they occur, but no structure is more complicated or more interconnected than the mammalian central and peripheral nervous systems (CNS and PNS). In the mammalian nervous system, at least six connexins (Cx26, Cx29, Cx30, Cx32, Cx36 and Cx43) have been identified at ultrastructurally defined gap junctions in neurons and glia [71,96,99], and at least five more (Cx31, Cx37, Cx45, Cx47 and Cx57) have been reported to be present in neural tissue. A series of articles summarizing knowledge of connexins and gap junctions in the nervous system appeared in a special issue of Brain Research Reviews 4 years ago [124]. Our contribution to that series gave an overview of the structure, distribution and composition of glial gap junctions, and the differential expression of glial connexins in both adult and developing mammalian CNS [96]. We focused on the organization and connexin composition of gap junctions between astrocytes, as well as between astrocytes and oligodendrocytes. Several reports in that series revealed that the glial syncytium is more complex than previously recognized, and that glial pathways of junctional communication are characterized by the differential connexin composition and, in artificial systems, the conductance regulation of specific combinations of homotypic and heterotypic connexin channels. With the recent proposal that “all” of the mammalian connexins have been identified [162], together with observations that multiple connexins are expressed in CNS and PNS, and that different connexins are expressed by glial cells and neurons [100,118,119], it is clear that gap junctional communication in the nervous system is substantially more complex than was previously appreciated.

With respect to connexin expression and function, the field of neuroscience is undergoing a reawakening regarding concepts of gap junction-mediated electrical communication between neurons in mammalian CNS. Following 30 years of sporadic reports that have either documented the presence of gap junctions or that have provided considerable evidence for electrical or dye coupling between neurons in mammals [10,11,62], a recent surge of papers suggests that gap junctions between neurons, including those at mixed synapses, are both prevalent and of physiological importance throughout the mammalian brain and spinal cord. This hypothesis can now be tested directly. Recent progress has been made possible in part by the discovery of the first gap junction protein established unequivocally to be expressed in mammalian neurons, namely connexin36 (Cx36) [25]. Using newly developed antibodies, we showed that Cx36 immunoreactivity is widely distributed in brain and spinal cord, and in particular, that immunogold labeling with these antibodies was restricted to ultrastructurally identified neuronal gap junctions [118–120]. With the recent development of improved immunocytochemical and electrophysiological approaches, it now is feasible to obtain an understanding of the anatomical organization, local regulation, and contribution of mutations in connexins to the etiology of neurological disease in the mammalian CNS.

As with molecular markers used for identification of chemical transmitter systems (e.g., GABAergic, cholinergic, etc.), it is likely that neurons express multiple connexins, exhibit differential connexin expression among different neuronal populations at different ages and at different morphological loci of coupling (i.e., dendro-dendritic, axo-dendritic synapses), and have different structural configurations of intercellular channels commensurate with different synaptic functions. The increased use of connexin markers to identify electrical and mixed synapses will accelerate the pace of advances in understanding the roles of electrical coupling in “connexinergic” systems.

In this update on data accumulated in the past 4 years, we attempt to clarify several issues concerning connexin composition of glial cells, review evidence for neuronal–neuro-
nal and neuronal–glial GJIC, and provide a glimpse of challenges in the investigations of glial and neuronal gap junctions in the CNS and PNS.

2. Methodological considerations

2.1. Anti-connexin antibodies

Many of the antibodies against Cx26, Cx32 and Cx43 that were used in our early studies of connexins in the CNS (reviewed in Refs. [95,96]) were developed and provided to us by E.L. Hertzberg (Albert Einstein College of Medicine, NY), and several of these remain invaluable as research tools. More recently, a series of monoclonal and polyclonal anti-connexin antibodies have become commercially available, such as those from Zymed Laboratories (South San Francisco, CA; http://www.zymed.com), which we have used extensively. In view of the considerable sequence homology between members of the connexin family of proteins, most antibodies have been raised in mice or rabbits immunized with peptides corresponding to non-conserved, unique sequences within known connexins. Additional anti-connexin antibodies have been developed once cross-reactions with known or newly discovered connexins were recognized [28,97,98,119]. With the apparent identification of the complete set of mammalian connexin genes [162], it should now be possible to screen connexin antibodies against the library of newly discovered connexins for identification of potential antibody cross-reactions, as well as to develop antibodies against particular connexins that avoid antibody cross-reaction with other connexin proteins.

As an example of potential difficulties, over a decade ago when only a few connexins had been identified, we examined Cx32 in brain and particularly hippocampus using a polyclonal anti-Cx32 antibody generated against the entire Cx32 sequence [131,165]. Immunolabeling with that antibody was detected in both neurons and glial cells. Based on the potential for cross-reactions with known connexins or with as yet unidentified connexins, we referred to this immunolabeling as “gap junction protein-like immunoreactivity”. In view of subsequent findings described below, we believe that this polyclonal antibody recognized other glial and/or neuronal connexins. One sequence-specific anti-Cx32 antibody designated 92B produced robust immunolabeling at structures in motoneurons referred to as subsurface cisterns [166–168]. It was uncertain whether this antibody detected Cx32 at subsurface cisterns, or whether it cross-reacted with another protein at subsurface cisterns. We have recently found that labeling of motoneuronal subsurface cisterns with antibody 92B still occurs in Cx32 knockout mice, whereas no Cx32 was seen in liver of Cx32 knockout mice when labeled with this same antibody, confirming that labeling at subsurface cisterns with this antibody is not due to Cx32 detection. In more recent investigations of CNS using non-cross-reacting antibodies, we have found no evidence that Cx32 is expressed by any other cell type than oligodendrocytes [69,119]. Because similar concerns will occur for additional connexin antibodies, it will be essential to test for possible cross-reactivities using connexin KO mice.

2.2. Immunocytochemistry

For LM or EM analysis of connexins in the CNS and PNS, a major aim is to obtain optimal preservation of immunogenicity of connexins while preserving cellular and structural morphology for precise anatomical localization to identifiable structures. Consequently, the connexin antibodies that we use are tested under a variety of fixation conditions. Tissue fixation for immunohistochemistry usually is conducted by transcardial perfusion with aldehyde fixative. A range of fixatives, fixation strengths and post-fixation times are routinely evaluated. This is crucial because, in extreme cases, we have found a near total absence of immunostaining for several connexins in brain that had been post-fixed for 2 h, whereas robust staining was seen for the same connexins in brain that had not been subjected to post-fixation. The post-fixation step was eliminated, not simply by removal of tissue after perfusion with fixative, but rather by transcardial perfusion with a buffered sucrose solution immediately after perfusion with fixative [69,99].

Immunohistochemical control procedures aimed to demonstrate antibody specificity generally involve processing of tissue sections with pre-immune serum, with secondary antibody after omission of primary antibody, or with antibody that has been pre-adsorbed against the peptide used to generate the antibody. While elimination of labeling in controls provides some confidence regarding antigen detection in test specimens, the consensus is that these procedures fall short of a definitive control for validation of antigen detection, especially in situations where the antibody has been affinity-purified. Therefore, additional strategies to increase confidence in labeling specificity may be applied. One of these is to demonstrate identical cellular and subcellular immunolocalization patterns with two different antibodies directed against different sequences within a target protein, as was done for Cx32 localization in the CNS [69]. The likelihood that two antibodies generated against entirely different sequences will have exactly matching anatomical and/or subcellular patterns of labeling arising from cross-reactions of both antibodies with other proteins is small.

An additional and more convincing strategy is to demonstrate elimination of immunolabeling of the target protein in knockout animals lacking expression of that protein. Numerous connexin genes have now been deleted in mice [44,52,58,101,161], but these animals have yet to be widely used to confirm immunohistochemical detection of particular connexins. In the case where connexin deletion is found to be lethal at embryonic stages or at birth, constitutive or conditional connexin knockout mice may be used to resolve controversies. Besides providing confirmation for detection of a particular connexin, these mice will be of value in
identifying false-positive immunohistochemical labeling due to antibody cross-reaction with unidentified or unknown proteins.

2.3. Freeze-fracture replica immunogold labeling (FRIL)

FRIL is based on sequential freeze-fracture replication, confocal mapping of replicated tissues, SDS-detergent washing to remove sufficient tissue constituents to permit replica viewing by transmission electron microscopy, followed by immunogold labeling of residual membrane proteins that remain strongly adsorbed to the SDS-washed replica film [37,117,119]. Immunogold-labeled gap junctions and their associated cells are photographed at high magnification, and the TEM images are directly correlated with the initial confocal LM maps, thereby allowing correlation of structure and composition at molecular, cellular, histological, and gross anatomical levels [115,117,119]. FRIL electron microscopy reveals whether the labels are localized to individual gap junctions, whether immunogold beads are present as singlets or as aggregates, whether the primary or the secondary antibody exhibits artifactual aggregation of immunogold beads, and whether artifactual aggregates of label are associated with gap junctions or with areas devoid of gap junctions.

Although freeze-fracture is based on membrane splitting, the fracture plane within vertebrate gap junctions is diverted around each connexon and into the extracellular space, where it separates each gap junction into two hemiplaques (Fig. 1). In regions of membrane splitting, each connexon hemichannels is separated from its partner at the point of contact within the extracellular space. This always results in two hemiplaques, each of which contains the connexins

![Image of Freeze-fracture Replica Immunogold Labeling](image_url)

Fig. 1. Freeze-fracture replica immunogold labeling of neuronal gap junction. (A) Tissue before fracturing. The green line indicates the impending fracture plane. Yellow = presynaptic connexons; orange = postsynaptic connexons; purple = glutamate receptor IMPs forming a postsynaptic density. (B) Connexons/connexins are labeled by rabbit anti-Cx36 antibodies and counter-labeled by goat anti-rabbit IgG bound to 10 nm gold beads. (C) Stereoscopic image of neuronal E-face after labeling for Cx36 using 18 nm immunogold. Immunogold beads frequently are 20–40 nm beyond the margins of gap junctions because SDS washing causes membranes to dissolve into small globular vesicles [117]. Nearby cluster of E-face IMPs represents a postsynaptic density similar to E-face IMPs identified as glutamate receptors [47]. Scale bars: 0.1 μm.
derived from only one cell. Subsequent immunogold labeling allows unambiguous assignment of connexins to only one of the two coupled cells. Usually, the FRIL replicas include portions of membranes and cytoplasm from both cells contributing to a gap junction, thereby allowing positive identification of both cells based on well-established cellular markers (e.g., synaptic vesicles, active zones, postsynaptic densities (Fig. 1B,C). For criteria used in cell identification in freeze-fracture, see Ref. [117]. However, FRIL analysis of gap junctions in CNS tissue is limited because the fracture plane exposes only an extremely small fraction of the gap junctions present in a tissue slice (i.e., a few to a few hundred gap junctions). By way of comparison, each tissue slice examined by LM immunocytochemistry contains hundreds or thousands of times more gap junctions than are present in a FRIL replica, potentially allowing assessment of overall abundance and histological distribution of connexins and gap junctions. However, LM immunocytochemistry also is limited because it can neither reveal whether individual fluorescently labeled puncta correspond to gap junctions or to artifactual “clumps” of label, nor can it provide an accurate measure of the size of most neuronal or glial gap junctions, many of which are less than 0.2 μm in diameter (i.e., smaller than the limit of resolution of LM, Fig. 2). In CNS tissues in particular, the limit of resolution of LM (0.2 μm in the X/Y axes and 0.3 μm in the Z axis) has precluded the use of LM for determining which of the several cell processes that may be present within the smallest resolvable imaging volume may actually contain the several cell processes that may be present within the small precluded the use of LM for determining which of the gap junctions present in a tissue slice (i.e., a few to a few hundred gap junctions). By way of comparison, each tissue slice examined by LM immunocytochemistry contains hundreds or thousands of times more gap junctions than are present in a FRIL replica, potentially allowing assessment of overall abundance and histological distribution of connexins and gap junctions. However, LM immunocytochemistry also is limited because it can neither reveal whether individual fluorescently labeled puncta correspond to gap junctions or to artifactual “clumps” of label, nor can it provide an accurate measure of the size of most neuronal or glial gap junctions, many of which are less than 0.2 μm in diameter (i.e., smaller than the limit of resolution of LM, Fig. 2). In CNS tissues in particular, the limit of resolution of LM (0.2 μm in the X/Y axes and 0.3 μm in the Z axis) has precluded the use of LM for determining which of the several cell processes that may be present within the smallest resolvable imaging volume may actually contain the labeled connexin (Fig. 2, circled areas representing the limit of resolution of LM). Thus, the use of combined LM, TEM and FRIL provides complementary information that reduces uncertainties in identifying and quantifying gap junctions, in determining their connexin composition, and in assigning expression of specific connexins to specific classes of cells.

In our FRIL studies of CNS and PNS connexins, most samples from adult and early postnatal rats were fixed by brief transcardiac perfusion with low concentrations of formaldehyde [114,119]. Formaldehyde-fixed brain and spinal cord slices and segments of sciatic nerve were equilibrated to 30% glycerol (as a cryoprotectant to minimize ice crystal damage during freezing) and ultrarapidly frozen by pneumatically damped contact against a liquid nitrogen-cooled copper mirror [112]. To test the effects of formaldehyde fixation on immunogold “labeling efficiency” as defined in [114], we also froze unfixed, uncryoprotected samples using conventional ultra-rapid freezing, as well as by ultra-high-pressure freezing using Balzers HPF rapid freezing device (courtesy of Prof. L. Andrew Staehelin). For Cx32 labeling (liver and brain) and Cx43 labeling (brain and spinal cord), no systematic differences in labeling efficiency were detected in samples briefly fixed by formaldehyde vs. samples prepared by ultra-rapid freezing. Instead, the primary basis for the relatively low labeling efficiency for any one connexin examined by FRIL (e.g., 1:10 to 1:30) [117] is that only one of at least three connexin isoforms present in a gap junction is labeled [99,100,119]. In astrocyte gap junctions, where at least three connexins are present in most hemiplaques, labeling for a single connexin isoform would allow only about one-third of the connexins to be labeled in each gap junction. Additional reasons for relatively low labeling efficiency in FRIL are the partial removal of connexins during SDS washing in FRIL [114], steric hindrance of immunogold labels, and an inherently slower rate of labeling with progressively larger sizes of immunogold beads (primarily because of increased rotational inertia for larger gold beads; T.J.A. Johnson, personal communication). In support of this notion of lower labeling efficiency for larger immunolabels, we have found that immunogold beads bind with approximate ratios of 1:2:4:8 for 30:20:12:6 nm gold beads that were applied simultaneously for 12 h. Consequently, we label samples for 16–20 h, usually using a combination of sizes of gold beads. For example, in double-labeling experiments, we routinely label one connexin species with 6 and 18 nm gold beads and the second connexin with 12 and 30 nm gold beads, expecting only the smaller beads in each labeling combination to label at maximum efficiency.

Major benefits of this approach are that the use of two or more sizes classes of immunogold beads for each connexin: (1) facilitates detection of gap junctions in low magnification searches based on higher visibility of the larger gold beads (Fig. 3); (2) allows for internal comparison of labeling efficiency for each of several secondary immunogold labels; (3) allows identification of defective secondary immunogold labels based on the absence of one size of gold label and presence of the other(s) (Fig. 3); and (4) provides a method for detection of clumping of each primary antibody. Clumping of two or more sizes of secondary immunogold directed against one primary antibody confirms that the primary
antibody had deteriorated. An additional advantage is that the simultaneous presence of several sizes of gold beads in multiple gap junctions in a single target cell type and absence of labeling of gap junctions in other cell types provides an internal verification of labeling specificity for each primary antibody.

2.4. Electrophysiological methods

The molecular characterization of neuronal connexins has considerable potential to reduce possible errors with single-cell RT-PCR and with light and electron microscopic identification of connexins and gap junctions in individual neurons. Electrophysiological studies of gap junctional coupling among neurons or between glial cells in mammalian CNS allows both independent and functional assessment of gap junctional coupling, but similar to immunolabeling, has the potential for “false positives” and “false negatives”. This potential problem has attracted less attention in regard to studies on glia compared to neurons because there have been fewer studies of electrical and tracer coupling of glia than neurons, and it is well established that glia are extensively coupled [91]. The concerns about artifacts with electrical or tracer coupling methods have also focused more heavily on neurons, because most workers assume neurons are not coupled, and direct ultrastructural evidence for gap junctions between unequivocally identified neurons has been rare. Electrophysiological techniques developed in the last decade provide new approaches to address the methodological and conceptual problems, but do not eliminate the potential for false positives and false negatives. For example, electrical and tracer coupling experiments with micropipets (i.e., either “sharp” or “patch” electrodes) have been criticized because of the potential for artifacts due to cell damage and current/tracer leakage (i.e., the “shish-ka-bob” artifact). However, the use of patch-clamp techniques rather than sharp electrodes replaces one set of problems with another set. Sharp electrodes are more likely to cut through cell membranes than patch electrodes and, thus, potentially lead to artificial dye and electrical coupling. Patch pipets, however, have much larger tip diameters, and when combined with the use of positive pressure when approaching neurons, the possibility for leakage into the extracellular space and internalization by other cells is more of a problem than with sharp electrodes.

Over the last several years, the combined use of whole-cell patch-clamp recording and infrared illumination with differential interference contrast optics (IR-DIC) has allowed paired recordings to be obtained from neurons that were hypothetically coupled through gap junctions. Previous studies with dual intracellular recording using sharp electrodes and “blind” impalement techniques could not ensure, unequivocally, that recordings from neurons that appeared to be electrotonically coupled were not actually dual recordings from a single neuron. Direct IR-DIC visualization of two distinct and clearly separated neurons and two patch pipettes essentially obviates the possibility of dual recordings from a single neuron. It is conceivable, however, that two neurons immediately adjacent to each other could still be artifically coupled because of damage from the two pipettes. The approach of dual whole-cell recording is now used extensively with the brain-slice technique to analyze other questions of synaptic communication, and has obvious potential for future studies of electrotonic coupling. Nevertheless, this approach still has the problem that hundreds of pairs of recordings may be
required if each neuron is coupled only to a few of its neighbors, as has been proposed in hippocampal and neocortical pyramidal cells (e.g., reviewed in Refs. [34–36]).

Several potential artifacts may occur in tracer coupling experiments. Probably the most direct method for eliminating or at least reducing the potential for artifactual coupling involves perforated-patch procedures outlined by Dean et al. [53]. These researchers used amphotericin B at the tip of the patch pipette, which allowed them to approach a neuron and obtain a seal under direct visual observation, and thus to ensure that dye did not leak from the pipette into another neuron. As the perforations in the patched cell membranes formed over the next several minutes following formation of the seal, Dean et al. were able to record the electrophysiological properties of the neuron, including “partial spikes” that represented putative coupling potentials. Next, they obtained whole-cell recordings, and showed that the neurons that had partial spikes had a greater probability of showing tracer coupling than the neurons without the partial spikes. Because tracer coupling was not present in the nearby neurons immediately after the seal was obtained and before the perforations had formed (viewed directly during the experiment), it is unlikely that subsequent movement of dye into other neurons was through an artifactual pathway. The approach of using the perforated-patch technique before achieving the “whole-cell” configuration for dye transfer experiments has numerous advantages, particularly if combined with the use of both large and small molecules in the patch pipette. Unfortunately this method has not been widely used.

3. Connexins and gap junctions in peripheral myelin

3.1. Cx32 in Schwann cells

Cx32 is widely regarded as the primary connexin of Schwann cells in peripheral nerve myelin. Cx32 immunofluorescence labeling is abundant at nodal regions and in Schmidt–Lanterman incisures [127,128]. In these structures, the myelin layers are not compacted and cytoplasm is retained in each successive myelin layer. It has been suggested that gap junctions could serve to link these successive layers of cytoplasm, thereby providing a more direct radial communication pathway from innermost to outermost layers of myelin [9]. The structural organization of gap junctions at paranodes and incisures as revealed by thin-section or freeze-fracture electron microscopy remains uncertain. Distinctions should be made between observations in PNS vs. CNS, as well as in different species. Ultrastructurally identified gap junctions have been observed linking nodal loops in CNS myelin in mammals [126] but they have only recently been detected in incisures in PNS myelin of any mammalian species [82a]. Despite this, the presence of gap junctions at paranodes and incisures has been widely presumed based on Cx32 immunofluorescence localization at these sites.

Gap junctions have been detected in PNS internodal myelin of chickens, and these junctions were reportedly increased many fold during Wallerian degeneration [150]. Similar ultrastructural studies of gap junctions in Wallerian degeneration have not been reported for mammalian PNS. FRIL evidence recently has been obtained for localization of Cx32 at gap junctions in internodal regions of myelin in adult mouse sciatic nerve [82a], with gap junctions observed between the outer tongue and the second layer of partially compacted myelin. However, Cx32 has not been demonstrated in gap junctions between paranodal loops or between apposed membranes at Schmidt–Lanterman incisures, possibly because of technical problems in fracturing through these structures. With the recent development of appropriate immunocytochemical tools and better technical approaches, gap junctions between myelin layers in internodal myelin, at paranodal loops and in incisures are now amenable to more detailed analysis [82a].

3.2. Cx29 in peripheral myelin

Recently, two groups described the cloning of mouse connexin29 (Cx29), whose mRNA is expressed in brain and peripheral nerve but not in other tissues, indicating that it may be a neural-specific connexin [1,138]. In peripheral nerve, it was suggested that Cx29 is produced in Schwann cells, and that a similar cell-specific expression in brain would imply its presence in oligodendrocytes [138]. Consequently, we investigated the localization of Cx29 protein in sciatic nerve using a newly generated antibody [71]. (Cx29 in oligodendrocyte gap junctions is described in Section 5.3.) By LM, Cx29 exhibited a distribution pattern similar to Cx32, as previously described in peripheral nerve [6,9,127,128]. Numerous conical-shaped bands of immunofluorescence labeling appeared intermittently along individual fibers and less frequently at what were likely nodes of Ranvier (Fig. 4). Double immunofluorescence labeling for Cx29 and Cx32 indicated substantial co-localization of these two connexins in sciatic nerve. Despite being widely distributed, including presumptive Schmidt–Lanterman incisures as seen by LM, technical limitations precluded obtaining FRIL images from those areas. However, FRIL images from sciatic nerve revealed Cx29 in the innermost layers of myelin in both normal and Cx32 KO mice but not in the outer layers of internodal myelin [71]. Cx29 was associated with clusters of 8–9 nm IMPs and with ring-shaped clusters of membrane particles called “rosettes” [89]. Whether these rosettes represent specialized junctions with axons, unpaired Cx29 hemichannels or some other novel arrangement of Cx29 has yet to be determined.

Given reports that Cx32 in peripheral nerve is concentrated at nodes of Ranvier and Schmidt–Lanterman inci-
sures [128], it appears that these sites contain an abundance of both Cx29 and Cx32. Thus, as recently suggested [138], the observations that radial dye-transport was only marginally impaired in Cx32 KO mice [9] may be explained by the participation not only of Cx32 in this process but also of Cx29, with Cx29 partially compensating for Cx32 deletion. Acceptance of this interpretation will require ultrastructural localization of Cx32 and Cx29 in gap junctions between successive myelin layers at nodes of Ranvier and at Schmidt–Lanterman incisures in normal animals, and Cx29 but not Cx32 at these structures in Cx32 KO mice. This unresolved issue may be pertinent to the lack of lethality in humans following deletion or alteration of Cx32. However, the resulting peripheral neuropathy, X-linked Charcot–Marie–Tooth disease (XCMT), is characterized by abnormal myelin compaction [15,148] and reduced conduction velocity in the myelinated axons.

4. Cx26, Cx30 and Cx43 in astrocytes

Cx30 and Cx43 have been co-localized by TEM immunocytochemistry and FRIL within astrocyte gap junctional plaques in brain and spinal cord [95,96,98,119]. In our initial FRIL examination of >3000 gap junctions containing Cx43 and >300 containing Cx30 in parenchyma of adult rat brain and spinal cord, all were in astrocyte plasma membranes, and none were in oligodendrocytes or neurons [119]. In addition, Cx43 was found in ependymocyte gap junctions [117] whereas Cx30 was not detected in oligodendrocytes. Thus, in CNS parenchyma of adult animals, Cx30 and Cx43 serve as supporting immunocytochemical markers for identifying astrocytic gap junctions but not gap junctions of other cell types.

Although Cx26 is well documented in leptomeningeal tissues of the CNS [31], its expression and cell-type localization in brain parenchyma have been investigated only sporadically over the past decade, with conflicting observations being the rule rather than the exception. Initially, some reports described Cx26 to be present in developing but not adult rodent brain [31], while others reported its expression in mature brain [90,105]. Subsequently, neurons in rat cerebral cortex were reported to express Cx26 during the first postnatal month, but not thereafter [92–94]. Expression of Cx26 in neurons of hippocampus, in neurons and astrocytes in locus coeruleus of both neonatal and adult animals and in neurons of other brainstem structures has also been described [2,139,158]. We also reported that adult rat and mouse brain contain an abundance of Cx26, as demonstrated by Western blotting, but cellular localization using an early set of anti-Cx26 antibodies remained uncertain due to demonstrated cross-reactivity of some of these Cx26 antibodies with Cx30 [97,98]. More recently, an LM study of Cx26 in adult brain using newly developed, non-cross-reacting anti-Cx26 antibodies provided evidence for both its extraparenchymal and parenchymal localization, with far greater abundance in perivascular, sub-pial and subependymal areas [83]. In those regions, Cx26 was found in association with the astrocyte marker glial fibrillary acidic protein (GFAP), suggesting astrocytic expression of Cx26, even though it was rarely found co-localized with Cx43 in brain parenchyma.

Using the same anti-Cx26 antibodies as Mercier and Hatton [83], we also found this connexin to be expressed in brain parenchyma but obtained somewhat different results [99]. Although we observed low or negligible LM labeling for Cx26 in cerebral cortical areas other than at leptomeningeal projections into cortex, Cx26 was widely distributed as typical fluorescent puncta throughout most subcortical areas (Fig. 5). Punctate labeling for Cx26 was found to be extensively co-localized with punctate labeling for both Cx30 and Cx43, not only in perivascular regions, but also in many areas of brain parenchyma. As discussed in the next section, detailed analysis by confocal immunofluorescence microscopy and by FRIL also indicated co-association of Cx26 immunofluorescence with Cx32 at the surface of oligodendrocytes. Cx26 was not detected in neuronal gap junctions in brain or spinal cord of adult rats, nor was it detected in subsequent preliminary studies of early postnatal rat spinal cord in which neuronal gap junctions, nevertheless,
were labeled for Cx36 [120]. These results indicate that Cx26 is a third connexin expressed by astrocytes and targeted to astrocytic gap junctions in widespread subcortical parenchymal structures of adult brain. While we do not exclude the possibility that Cx26 may be expressed by other cell types, particularly in developing brain, more comprehensive LM and EM analyses are required to establish whether Cx26 is incorporated into gap junctions between neurons, particularly at critical developmental stages.

Expression of Cx26, Cx30 and Cx43 by astrocytes is highly heterogeneous throughout the CNS [98,100,167], with some structures such as in those in various subcortical areas containing an abundance of all three connexins, and others such as cerebral cortex containing very low levels of Cx26 and moderate levels of Cx30. Moreover, immunolabeling for Cx30 is not detectable in white matter regions such as anterior commissure and internal capsule, indicating that white matter astrocytes do not express this connexin. It is difficult to imagine that astrocytes in, for example, cerebral cortex have fundamentally different functions from those in subcortical regions. It is more likely that differential levels of connexin expression reflect region-to-region differences in functional requirements for different astrocytic gap junctional coupling states. The presence of several connexins having different permeabilities to ions and molecules and different modes of conductance regulation may confer qualitative and functional differences for gap junctions in different regions of the CNS.

5. Connexins and gap junctions in oligodendrocytes

5.1. Oligodendrocytic Cx32 and coupling partners

For the purposes of this review, connexins and gap junctions at cell bodies and initial processes of oligodendrocytes are considered separately from those in myelinating processes and in compact myelin in the CNS, which are discussed in Section 5.2. Oligodendrocyte–astrocyte (A/A) gap junctions, as well as GJIC with oligodendrocytes (A/O) [79,91,106,116,119,123]. Weak electrical coupling and limited dye transfer between oligodendrocytes have been detected in various systems including preparations of optic nerve and neonatal spinal cord [19,57]. Early freeze-fracture studies found that oligodendrocytes shared gap junctions only with astrocytes, but this conclusion was based on analyses of only a small percentage (<5%) of cell pairs in which an oligodendrocyte and its coupling partner could be identified simultaneously [91,116]. Based on FRIL analyses of cell-specific connexins and on ultrastructural markers for cell identification, the number of cell pairs in which both cells were identified was increased to nearly 100%, thereby allowing for more accurate determination of the relative frequencies of each proposed type of oligodendrocyte coupling partner. Using FRIL, ~97% of intercellular oligodendrocyte gap junctions were identified as oligodendrocyte/astrocyte (O/A) junctions. Thus, the numerous gap junctions which oligodendrocytes share with astrocyte “intermediaries” appear to serve as conduits for indirect coupling between successive oligodendrocytes (O/A/O), thereby permitting otherwise-isolated oligodendrocytes to participate in gap junction intercellular communication within the broader panglial syncytium [91,106,116,119].

A separate issue concerns the connexin constituents of O/A junctions and, in particular, the relationship of the connexins expressed in oligodendrocytes to the three connexins that have been identified in astrocytes (i.e., Cx26, Cx30, Cx43). It is now well documented that oligodendrocytes express Cx32 [30,31,68,69,128]. LM immunofluorescence revealed abundant Cx32-positive puncta along oligodendrocyte cell bodies and their proximal processes [69]. By conventional TEM immunocytochemistry and by FRIL, Cx32 has been documented in gap junction plaques in oligodendrocytes [69,96,119], where Cx32 was localized to the oligodendrocytic side but not to the coupling-partner side of these gap junctions. Cx32 immunogold-labeled
intercellular gap junctions were found exclusively in oligodendrocyte membranes, and these were shared essentially exclusively with astrocytes. LM immunofluorescence analysis of astrocytic connexins Cx26, Cx30 and Cx43 revealed that a small portion of gap junctions (<5%) were distributed along the surface of oligodendrocyte cell bodies (identified by labeling for the oligodendrocyte marker enzyme CNPase) (Refs. [99,118] and Nagy, unpublished observations). By LM, each of these astrocytic connexins was co-associated with Cx32 at the surface of oligodendrocyte cell bodies and proximal processes. By FRIL, Cx26, Cx30 and Cx43 immunogold labeling were found in astrocyte gap junctions shared with oligodendrocytes [99,118,119]. We estimated that >80% of these junctions contain both Cx30 and Cx43 on the astrocyte side, and that >70% of O/A gap junctions contain Cx26 and Cx43, suggesting that the astrocyte side of at least 56% of O/A gap junctions contain all three of these astrocytic connexins. With three connexins in the astrocyte side of these heterologous junctions, it seemed likely that additional connexins must be present in the oligodendrocyte side. Recent experiments bear that out, and evidence for the presence of Cx29 as a second connexin in the oligodendrocyte side of these junctions is described below.

The above observations are consistent with thin-section immunoEM evidence that astrocytic and oligodendrocytic connexins are co-associated [69,97,98,103,167]. All A/A gap junctions that were recognized were labeled for Cx30 or Cx43 on both sides of the junction (symmetrical labeling). In contrast, O/A gap junctions were labeled for Cx30 or Cx43 only on the astrocyte side, whereas they were labeled for Cx32 only on the oligodendrocyte side (asymmetrical labeling). Instances were encountered where neither side of gap junctions formed by an oligodendrocyte were labeled for Cx30 (possible false negatives), and where both sides of gap junctions formed between an oligodendrocyte and an unidentified process were labeled for Cx32. Therefore, a small percentage of A/O junctions may not contain all three astrocytic connexins, and a small percentage of junctions formed by oligodendrocytes may not contain Cx32. Occasionally, however, oligodendrocytes may form homologous or “reflexive” gap junctions between adjacent cytoplasmic processes, some or all of which may arise from the same oligodendrocyte.

5.2. Cx32 in oligodendrocytes and myelin

Initial reports concerning the association of Cx32 with myelinating cells in the CNS emphasized localization of Cx32 in oligodendrocyte cell bodies and the initial processes of these cells, and the absence of Cx32 in compact myelin and along myelin sheaths [128]. Some reports, however, have noted the distribution of Cx32 along internodal region of myelin sheaths in the CNS [31,68,148]. In view of past difficulties in documenting the localization of Cx32 in the CNS, we investigated this connexin in brain and spinal cord using several antibodies against different sequences in the protein [69]. In addition to detection of Cx32 in oligodendrocyte somata and initial processes, as described by others, Cx32 immunofluorescence was found densely distributed along myelin sheaths coursing through gray matter in many brain areas, with more variable levels of Cx32 detected in white matter regions. Similar immunolabeling was observed with several different Cx32 antibodies, providing additional support for Cx32 association with myelin sheaths.

Subsequent investigations of Cx32 in the CNS included examination of neural tissues in Cx32 knockout C57/BL6 mice labeled with a panel of anti-Cx32 antibodies directed at different sequences in Cx32. As reported in detail elsewhere [100,100a], detection of Cx32 by these antibodies in brain and liver of wild-type C57/BL6 mice and absence of Cx32 in liver of knockout mice were confirmed by Western blotting and immunofluorescence. In wild-type mice, intense immunofluorescence was seen around oligodendrocytes (Fig. 6A), which were identified by CNPase immunoreactivity. Cx32 labeling was absent from oligodendrocytes in Cx32 knockout mice (Fig. 6B). Labeling for Cx32 was associated with myelinated fibers in gray matter regions of brain in wild-type mice, but was absent in brain of Cx32 knockout mice (Fig. 6C,D). These results provided further support that Cx32 is present in oligodendrocyte processes throughout brain and spinal cord.

Fig. 6. Immunofluorescence localization of Cx32 in mouse brain. (A) Low magnification showing distribution of Cx32 in the superior colliculus of wild-type mouse (A). Area shown extends from the colliculus surface (upper right) to the central gray (CG). Labeling is seen associated with fibers (arrowheads) and with the cell bodies of oligodendrocytes that are evident throughout the field, but are most prominently seen in superficial layers (small arrows). (B) Similar field of the superior colliculus as in A, but from a Cx32 knockout mouse showing absence of immunolabeling for Cx32. Higher magnification showing immunofluorescence labeling for Cx32 associated with fibers (arrows) and oligodendrocyte cell bodies (arrowheads) in the midbrain ventral tegmental decussation of wild-type mouse (C), and a corresponding field in midbrain of Cx32 knockout mouse showing absence of labeling (D). Scale bars: A, B, 300 μm; C, D, 50 μm.
Fig. 7. Oligodendrocyte gap junctions labeled for Cx32. (A) Three labeled gap junction plaques in oligodendrocyte somatic plasma membrane in adult rat cerebellum. (B) Small gap junction on outer tongue of myelin in rat spinal cord; labeled for Cx32 by one 20 nm gold bead. (C) “Reflexive” gap junction between outer tongue and the second layer of myelin; labeled by four 10 nm gold beads. A slightly larger gap junction (arrow) on the myelin outer tongue is not labeled. Inset shows the labeled gap junction at higher magnification. From adult rat cerebellum. Scale bars: 0.1 μm.
By FRIL, Cx32 was demonstrated in gap junctions in the oligodendrocyte somatic plasma membrane (Fig. 7A), on the outer surface of myelin (Fig. 7B), as well as in gap junctions between the outer tongue and second layer of myelin (Fig. 7C), confirming the association of Cx32 with CNS myelin, as seen by LM. Gap junctions between the myelin surface and closely associated astrocytes represent heterologous O/A gap junctions [79,91,116,119], whereas those between inner layers of myelin represent “autologous” or “reflexive” gap junctions. This hypothesis is supported by stereoscopic images of gap junctions viewed from the perspective of inside the myelin sheath. In the myelin outer surface, connexins in the myelin coupling partner were never labeled for Cx32, but instead, consistently were labeled for Cx43 (Fig. 8A), Cx30 plus Cx43 (Fig. 8B–D) and/or Cx26, which along with other ultrastructural features defined the myelin coupling partners as astrocytes and not oligodendrocytes [118,119].

Cx32 within gap junctions of CNS myelin may serve several distinct functions. Because Cx32 is not present in oligodendrocyte coupling partners, Cx32 in the outer turn of myelin necessarily forms heterotypic junctional communicating channels, linking to one or more of the connexins in the astrocytic processes that couple to this layer of uncompacted myelin [103]. By FRIL, gap junctions in the outer most layer of myelin consisted of <10 to >1000 connexons, and the gap junction plaques shared with the astrocyte coupling partners contained, at the minimum, Cx43, Cx30 and Cx26 [99,118,119]. Unlike the situation in PNS, where supporting cells do not contact

![Fig. 8. Oligodendrocyte coupling partners labeled for Cx43 and Cx30. (A) In addition to a large astrocyte-to-astrocyte gap junction labeled for Cx43 (20 nm gold beads; lower right quadrant), the gap junction in the nearby myelin E-face is also labeled for Cx43 (enlarged in inset), consistent with the suggestion that the outer tongues of myelin are coupled to astrocytes. (Adult rat suprachiasmatic nucleus.) (B–D) Gap junctions in two outer myelin E-faces showing double labeling for Cx43 (10 nm gold beads) and Cx30 (20 nm gold beads). The smaller gap junctions (D) would not have been detectable without immunogold labeling (adult rat supraoptic nucleus). Scale bars: 0.1 μm.](image-url)
Schwann cell myelin in internodal regions, coupling of astrocytes with oligodendrocytes in the CNS would allow the astrocytes to provide metabolic and ionic support to the several myelinating segments formed by each oligodendrocyte [91,118,119].

Cx32-containing gap junctions in oligodendrocyte plasma membranes at nodes of Ranvier, within compact myelin at Schmidt–Lanterman incisures, and within internodal myelin may form autologous or reflexive junctions between paranodal loops and/or adjacent myelin layers, allowing for radial gap junctional communication pathways similar to those proposed in PNS myelin [107,128]. Gap junctions between oligodendrocytic paranodal loops have been documented in mammalian CNS [126], and incisures of the kind seen in peripheral nerve have been described in CNS myelin [16,27,49,50,82]. The specific membrane domains within myelin that contain connexins/gap junctions, as well as the cellular elements linked by heterologous vs. autologous or reflexive gap junctions, remain to be determined.

5.3. Cx29 in oligodendrocytes and myelin

Comparisons of Cx29 mRNA expression in adult mouse tissues indicated Cx29 to be somewhat more highly expressed in sciatic nerve than in brain. However, message levels in brain were greater at postnatal day 14 than in adult animals, suggesting transient up-regulation of Cx29 expression during development [138]. Immunohistochemical investigations have revealed that Cx29 protein in the CNS is localized in oligodendrocytes and their processes [1a,100,100a] (Fig. 9A–C). In particular, immunofluorescence labeling for Cx29 was densely distributed along internodal regions of myelin sheaths of fibers in gray matter throughout brain and spinal cord in both normal and Cx32 KO mice. Although the distribution and cellular localization of Cx29 was similar to that of Cx32 in adult brain, several differences were found. Oligodendrocyte cell bodies and initial processes in adult mice typically displayed dozens of distinct Cx32-immunopositive puncta (Fig. 9D) that, ultrastructurally, corresponded to Cx32 localization on the oligodendrocyte side of gap junctions shared with astrocytes [69,97,98,99,118]. In contrast, lower levels of Cx29 immunofluorescence labeling were seen at these locations in adult animals under tissue processing conditions that yield robust labeling for Cx29 along myelin sheaths. In younger animals 14 and 20 days of age, dense labeling for Cx29 was associated with the cell bodies and initial segments, as well as with the developing arbors of oligodendrocytes. Although requiring further confirmation, preliminary data from FRIL reveals that mature oligodendrocyte somata have gap junctions containing both Cx32 and Cx29, but with a greater abundance of Cx32 than Cx29 (Fig. 10). In these gap junctions, the connexon particles/pits often are in loose aggregates, which we designate as “diffuse” or “meandering” gap junctions.

Fig. 9. Immunofluorescence localization of Cx29 in adult mouse brain. (A,B) Low and high magnifications showing overall distribution of Cx29 in cerebral cortex (A) and association of immunolabeling with myelinated fibers (B). (C,D) Immunolabeling for Cx29 around oligodendrocyte cell bodies (C, arrows) in the ventrolateral nucleus of the thalamus, and labeling for Cx32 around oligodendrocyte soma and their initial processes in the superior colliculus (D, arrows). Punctate immunolabeling for Cx47 associated with oligodendrocyte somata shown at low (E) and high (F) magnification of an area in the hypothalamus. In all areas examined, oligodendrocyte soma exhibit relatively sparse labeling for Cx29, and dense punctate labeling for Cx32 and Cx47. Scale bar: A, 200 μm; B, C, D, F (shown in F), 50 μm; E, 100 μm.

5.4. Cx47 in oligodendrocytes and myelin

A gap junction gene designated connexin47 has recently been identified and characterized in mouse [152], and a corresponding human ortholog designated connexin46.6 has been identified. Expression of Cx47 was found to be high in brain and spinal cord of adult mice, but was not detected in retina or sciatic nerve. Based on in situ hybridization analysis, Cx47 mRNA was reported to be restricted to cells in gray matter, and was absent in white matter areas of brain and spinal cord. Specifically, Cx47 was reported in pyramidal cells in neocortex, in pyramidal and granule cells of the hippocampus, Purkinje cells and granule cells in the cerebellum, and in motoneurons in the brainstem and spinal cord. It was noted that the expression pattern of Cx47 only partially overlapped with that of Cx36 [152]. More recently, this neuronal localization of Cx47 mRNA was recognized to be incorrect [103a], and it was convincingly demonstrated that Cx47 is expressed not by neurons, but by oligoden-
drocytes [82b,103a]. Our results with a newly generated anti-Cx47 antibodies (Zymed Laboratories, South San Francisco, CA) also indicated detection of Cx47 exclusively in oligodendrocytes in mouse brain and spinal cord [71a]. Punctate immunolabeling for Cx47 associated with oligodendrocytes labeled with the oligodendrocyte marker CNPase (not shown) had an appearance very similar to that of Cx32 around oligodendrocytes (Fig. 9E,F), and was quite distinct from appearance of labeling for Cx29. Interestingly, labeling for Cx47 appeared to be more abundant on oligodendrocyte somata, where it was co-localized with Cx32, and co-associated with astrocytic connexins at presumptive A/O gap junctions [71a].

Substantial structural abnormalities were observed in CNS myelin sheaths of Cx32/Cx47 double knockout mice, indicating essential functions of these connexins in CNS myelin formation and/or maintenance [82b,103a]. The absence or relatively minor deficits in myelination observed in the CNS of either Cx32 or Cx47 knockout mice suggests functional compensation by one or both of the other oligodendrocyte connexins, Cx29 and Cx47, or Cx29 and Cx32, respectively, in these mice. However, it was difficult to imagine how compensation may occur in view of results indicating Cx29 and Cx32 expression by mutually exclusive subpopulations of oligodendrocytes and localization of these connexins to mutually exclusive subpopulations of myelinated fibers [1a]. To address this issue, we have documented the percentage of oligodendrocytes that express each of the above connexins. Among a population of more than 9000 cells examined in various brain regions, we found that 93–98% of oligodendrocytes display immunofluorescence labeling for Cx29, Cx32 and Cx47, indicating that virtually all oligodendrocyte somata contain all three of these connexins. While these observations provide the possibility for compensatory functions of connexins in oligodendrocytes, this possibility may still be an oversimplification that does not take into account the differential, largely complimentary subcellular localization of Cx29 and Cx47 that we have observed in oligodendrocytes [71a,100,100a], and the lack of permissive gap junctional coupling between Cx29 with Cx32, or Cx29 with itself [1a]. If each of the oligodendrocyte connexins is critical for the normal function of myelinating cells, then the occurrence of subtle physiological abnormalities in oligodendrocytes and/or myelin following individual deletion of these connexins cannot be excluded in the absence of further detailed investigations.

6. Cellular expression of other connexins

6.1. Cx45 in the CNS

On the basis of Northern blot analysis, Cx45 mRNA has been detected in adult rodent brain [68,138], indicating its expression in neural tissue. However, conflicting reports have appeared regarding the types of cells that express Cx45 in the CNS. Initially, immunofluorescence labeling for Cx45 in adult rat cerebral cortex and hippocampus was reported to
be associated with oligodendrocytes, but not with neurons or astrocytes [31,68]. In cultured oligodendrocytes, Cx45 mRNA or protein was undetectable; however, the message was detected by RT-PCR, and evidence from whole cell voltage clamp recording from these cells was presumed to indicate the presence of unitary conductances corresponding to channels formed by Cx45 [31]. In contrast, expression of Cx45 mRNA and protein has been reported in motoneurons of developing and adult rat spinal cord [20]. Expression of Cx45 has also been examined in Cx45-deficient mice that had received targeted replacement of the Cx45 coding region by the lacZ reporter gene expressing β-galactosidase (β-gal), which in turn was under control of the Cx45 promoter. Although homozygous Cx45+/− animals died during embryonic development, heterozygous Cx45+/− mice survived to adulthood and exhibited staining for β-gal in many tissues. LacZ expression, presumably reflecting cells that normally produce Cx45, was abundant in neonatal neural tissues and, in adult animals, was observed in neurons in the CA3 stratum pyramidale of the hippocampus, in layers II and VI of the occipital and entorhinal cerebral cortex, in the thalamus, and in the cerebellum where LacZ expression was restricted to basket and stellate cells [67,81c]. In addition, a few NG2-positive oligodendrocyte precursor cells were also found to express LacZ [81a]. Finally, results from a gene micro-array approach involving cDNA prepared from mRNA isolated from rat brain capillaries indicated that Cx45 in brain is expressed exclusively by unidentified cells associated with brain capillaries [70]. Reasons for the differences between these results and, in particular, reports indicating absence of Cx45 expression in neurons are at present unclear.

7. Neuronal gap junctions and electrical synapses

7.1. Neuronal gap junctions

Gap junctions are the morphological substrate of one type of electrical synapse [10–14,62]. Because thin-sectioning methods for TEM were unable to find large numbers of gap junctions between neurons in mammals, many neuroscientists have long dismissed electrical synapses as major contributors to neural function in higher vertebrates. Over the past three decades, however, several lines of ultrastructural evidence for gap junctions and/or coupling between neurons have been obtained in numerous areas of the adult mammalian CNS [4,5,7,8,17,23,42,43,46,48,51,55,63–66,72–77,80–85,110,113,115,121,122,125,135,136,140–146,155,156,163,164,169]; reviewed in [95]. Widespread distribution of neuronal gap junctions is also supported by suggestions that fast prepotentials (also called spikelets, d-spikes or short latency depolarizations) are found in many neuronal systems, including dye-coupled neurons, and these events may represent evidence for gap junctional coupling [102,111,154,157,159]. However, fast prepotentials have also been interpreted as representing dendritic or axonal spikes rather than evidence for gap junctions.

A new concept emerging is the importance of synchronous field potentials (i.e., gamma or 30–80 Hz and high-frequency or ≥200 Hz oscillations) among ensembles of neurons [32,153]. High-frequency oscillations temporally correlate firing patterns in neural networks and may contribute to such cognitive processes as perception and attention [132,133,151]. Gap junctions in electrical and mixed synapses may impart fundamental alterations to the properties of neural networks [13]. For example, electrical synapses may contribute to generation and maintenance of synchronized neuronal “bursting” firing patterns [134]. Moreover, such synapses between GABAergic interneurons in cerebral cortex and hippocampus, striatum and cerebellum [38,56,59,61,147] may facilitate neuronal synchrony [39,78,153] and may be involved in detecting the relative timing of excitatory inputs to interneurons [40]. In hippocampus, gap junctions between pyramidal cells may contribute to synchronous neuronal activity [33]. Another emerging principle is that electrical coupling may occur only between neurons of the same type [24,41,104], such as fast spiking/fast spiking and low-threshold spiking/low-threshold spiking neurons in cortex; aspiny/aspy and spiny/spiny neurons in striatum; and oxytocin/oxytocin and vasopressin/vasopressin neurons in hypothalamus. This type-specific neuronal coupling may arise from expression of different connexins in these cells and from the permissiveness or non-permissiveness with which different connexins form functional gap junction channels [160].

7.2. Cx36 in neuronal gap junctions

Numerous connexins, including Cx26, Cx32, Cx36, Cx43, Cx45 and Cx47, have been reported in neurons of normal adult animals based on detection of these by LM and EM immunohistochemical, in situ hybridization and/or in situ RT-PCR technique. The best characterized of these with respect to neuronal expression and distribution is Cx36 [26] and, to date, only Cx36 has been established by EM methods to be in neuronal gap junctions [71b,118–120,120a]. By FRIL, Cx36 has been detected in six distinct morphologies of neuronal gap junctions in inferior olive, hippocampus, retina and spinal cord. Of course, the gap junctions most easily detected by thin-section TEM and conventional freeze-fracture are the large “plaques” having their connexins either in regular hexagonal array (Fig. 11A,B) or in irregular patches (not shown). Based on preferential membrane splitting and on detection of immunogold labels, many more gap junctions are detectable by FRIL than by conventional TEM or by freeze-fracture. The predominant forms seen by FRIL are small plaques of IMPs/pits, either in irregular clusters (Fig. 11A, lower gap junction) or in regular hexagonal array (Fig. 11B). In retina, unusual “string” gap junctions are detected (Fig. 11C), and despite their abundance (25–50% of all gap junctions...
detected in retina), complex “string” gap junctions have not been reported previously. String gap junctions would be particularly difficult to recognize in conventional thin-section TEM images because they closely resemble tight junctions. However, string gap junctions are distinguished from tight junctions by the discrete separation of their particles/pits; by the presence of ice “pegs” in the E-face pits [116]; by regions in which the strings widen to contain

Fig. 11. Distinctive varieties of neuronal gap junctions in adult rat CNS. (A) Large, regular gap junction and a small, irregular gap junction in retinal neuron. Both gap junctions are labeled for Cx36 (20 nm gold). At the bottom of the figure is a cluster of E-face IMPs forming a distinctive type of postsynaptic density. (B) Small immunogold neuronal gap junction in adult rat inferior olive. Connexons are in regular hexagonal array. (C) Two “string” gap junctions in adult rat retina. The gold beads follow the strings of E-face pits and P-face particles. Some strings are two to four strands wide. (D) “Reticular” gap junction in E-face of adult rat hippocampal neuron found before the development of the FRIL technique (from Ref. [116]). (E) Cx36-labeled “reticular” gap junction in P-face of neuron in adult rat hippocampus. (F) Two (of three) Cx36-labeled neuronal gap junctions in one small portion of a mixed synapse in adult rat hippocampus. The two gap junctions contain ca. 12 and 22 connexons. The gap junction on the left is in regular hexagonal array, and the gap junction on the right is “diffuse” or “meandering”. Scale bars: 0.1 μm.
two or three rows of particles/pits separated by 10 nm, including some areas where the IMPs/pits are in regular hexagonal array; and most importantly, by immunogold labeling for Cx36. In retina, hippocampus and suprachiasmatic nucleus, distinctive “reticular” gap junctions are observed (Fig. 11D,E). These have morphologies intermediate between “plaque” and “string” gap junctions and may represent transitional states between the two. However, in hippocampus and suprachiasmatic nucleus, “reticular” but not “string” gap junctions have been detected, suggesting that each may comprise a specific type of gap junction. A sixth form of neuronal gap junctions in retina and hippocampus consists of loose clusters of “diffuse” or “meandering” connexins (Fig. 11F), similar to one of the types of gap junctions found linking oligodendrocytes with astrocytes (Fig. 10, above).

Possible differences in the function and composition of these distinctive morphological types of gap junctions are not known, nor are data available regarding developmental or maturational states of any of the six types of neuronal gap junctions. The smaller neuronal gap junctions would be difficult to detect in conventional thin-sections, and in the absence of serial-section reconstruction combined with immunocytochemistry, it will be difficult if not impossible to identify “string” and “reticular” gap junctions. However, string and reticular gap junctions may account for the diffuse Cx36 immunofluorescence seen in some retinal neurons [118].

With the difficulty in detecting the predominant smaller gap junctions using thin-section TEM (see Ref. [117]), and the probable difficulty in recognizing the “string” and “reticular” configurations, neurons showing electrophysiological and tracer coupling in areas without evidence for gap junctions should be considered from the perspective that previous negative ultrastructural evidence for gap junctions was based on limited assumptions regarding what constitutes a gap junction, and hence, must be interpreted with caution. The above observations on electrical synapses in mammalian CNS are supported by recent studies involving deletion of the gene for Cx36. Cx36 KO mice do not exhibit electrical coupling and spikelets in cortical low-threshold spiking cells show little or no coupling in fast-spiking cells; have a severe impairment in synchronous low-threshold spiking-mediated inhibition; and, in hippocampus, show a loss of electrical synapses between interneurons and a disruption in synchronous gamma oscillations [29,52].

7.3. Gap junctions at mixed synapses

Mixed synapses provide the structural and functional components required for both chemical and electrical transmission within a single synaptic contact on an individual postsynaptic element [115,141,143]. In goldfish brain, Mauthner cell mixed synapses provide for reciprocal modulation of connexin conductances and receptor activation [109]. In the mammalian CNS, the physiological roles and distributions of mixed synapses are more controversial. Mixed synapses have been observed in spinal cord, retina and brain, and identified based on the presence of both gap junctions and specializations for chemical synaptic transmission within a single synaptic contact (Fig. 12). The axon terminal at each mixed synapse was identified by the presence of synaptic vesicles in the cytoplasm and distinctive “active zones” in the nerve terminal plasma membrane, whereas the associated postsynaptic element was confirmed to be neuronal, based on the presence of distinctive clusters of receptor IMPs designated “postsynaptic densities” (PSDs), dendritic spines, multiple axon terminals synapsing on the same postsynaptic element, and other established markers for neurons (criteria in Ref. [116]).

In a semi-quantitative freeze-fracture study of gap junctions in spinal cord neurons [115], 99 gap junctions were found in 36 neuronal contacts, and most of those were identified as mixed synapses. Gap junctions also were present between neurons in configurations that most likely represented purely electrical synapses, but those configurations were relatively rare in spinal cord. Mixed synapses were found on both motoneurons and interneurons, and it was calculated that on average, each motoneuron and interneuron has approximately 300 mixed synapses, with each containing an average of three gap junctions (range = 1–11) [115]. In many of those, the area of nerve terminal contact exceeded 5 μm², and multiple gap junctions linked the expanded nerve terminal with either a large dendrite or with the neuronal soma (identified by the presence of a nucleus, abundant Nissl substance and/or multiple Golgi lamellae). As many as 11 gap junction plaques were interspersed with either of two types of active zones (“synaptic mesas” and “synaptic sombreros”), many of which exhibited evidence for ongoing synaptic vesicle exocytosis [115]. Postsynaptically, gap junctions frequently were within 0.1 μm of distinctive E-face IMPs (Fig. 1B,C) that closely resembled presumptive glutamate receptor clusters [47]. Although multiple ultrastructural criteria were used to document the existence of mixed synapses between neurons in the mammalian CNS, physiological studies have yet to reveal the properties of mixed synapses in mammals.

By FRIL, mixed synapses subsequently have been found in retina, inferior olive, hippocampus and suprachiasmatic nucleus (Refs. [119,120] and Rash et al., unpublished observations), and >90% of these gap junctions were immunogold labeled for Cx36 using Ab298 (Fig. 11) [119], which effectively labels neuronal but not glial connexins. Thus, by FRIL Ab298 is a useful tool for finding and identifying neuronal gap junctions in both electrical synapses and mixed synapses in rats and goldfish (see also Pereda et al., [109b]).

Based on studies in model systems of lower vertebrates, the electrotonic component at mixed synapses in mammals may undergo some forms of activity-dependent potentiation similar to LTP [108,109] and/or synchronization of high-frequency oscillations [33,153]. The physiological effects of
mixed synapses may also spread beyond the confines of the synapse itself. In the lateral vestibular nucleus, for example, electrical coupling occurs between neurons whose cell bodies have not been shown to be linked by gap junctions, but whose dendrites share gap junctions with branches of the same axon [141]. Thus, a single axon linked by gap junctions to two dendrites may mediate electrical coupling between the dendrites. This arrangement may account for reports of dye coupling between, for example, the somata of hippocampal pyramidal cells wherein typical plaque-type gap junctions have not been found [65,66]. Evidence for such an arrangement of several processes “chained” together by gap junctions is provided by the demonstration that oligodendrocytes do not normally share gap junctions with each other, but instead, are coupled through astrocyte “intermediaries” [91,116,119]. Thus, it is essential to identify anatomical sites where dye and electrical coupling could be mediated by small or unusual gap junctions at axo-dendritic and axo-somatic synapses.

7.4. Evidence for electrical and tracer coupling of neurons

7.4.1. Neurons in hippocampus

For over a decade, ultrastructural evidence has suggested that some interneurons and pyramidal cells in hippocampus and cortex possess gap junctions. Several lines of evidence from the 1980s suggested that hippocampal pyramidal cells and dentate granule cells are electrically coupled through gap junctions (for reviews, see Refs. [34–36]), and many of the electrophysiological and tracer coupling results have recently been replicated and extended [170]. However, careful re-examination of the freeze-fracture micrographs reported to indicate neuronal gap junctions between CA3 pyramidal cells [129] and between dentate granule cells [76] suggest that these junctions were on cell types other than neurons (see Ref. [116]). Freeze-fracture evidence for gap junctions between hippocampal neurons has been obtained using improved methods (see Ref. [36] and Fig. 11D–F), but it still has not been determined whether these were on interneurons or on principal cells. Recently, Schmitz et al. [130] have re-examined the hypothesis that hippocampal pyramidal cells are electrotonically coupled using single and dual recordings with patch electrodes and infrared illumination-differential interference contrast optics (IR-DIC) techniques. They have provided additional new evidence that CA1 pyramidal cells are tracer coupled and electrotonically coupled through axonal gap junctions. Local antidromic stimulation evoked spikelets that were reversibly reduced after application of the gap junction blocker carbamolone. They also observed axonal dye coupling. This form of electrotonic coupling may synchronize fast oscillations [33] and electrographic seizure activity [60]. Thus, several laboratories have provided independent evidence for electrotonic and tracer coupling among pyramidal cells, but ultrastructural evidence is needed to confirm the presence of gap junctions at the putative site of axonal coupling.
Ultrastructural evidence for gap junctions between interneurons has been available [64–66], and some electrophysiological or tracer coupling data has also become available (e.g., Refs. [88,170]).

7.4.2. Neurons in neocortex

Until recently, a comparable situation has existed in neocortex as in hippocampus. Experiments from the 1980s suggested coupling among pyramidal cells in the adult [45], but comparatively less electrophysiological data were available from interneurons. Several recent papers from two independent groups [39,41] have provided direct evidence for electrotonic coupling between cortical interneurons. The authors used dual whole-cell recording with IR-DIC to allow direct identification of interneurons in paired recordings. Those studies provided direct evidence that interneurons are electrotonically coupled, and demonstrated that this neuronal population shows some of the properties expected of a coupled network. The experiments showed that individual pairs could have a wide range of coupling coefficients (i.e., from <1% to >50%). In previous studies, the lowest coupling coefficients might not have been detected without patch pipets (i.e., would not have been detected with sharp electrodes), and the highest coupling coefficients could have been interpreted as dual recordings from the same neuron. The properties of coupled networks that have been demonstrated include low-pass filtering as well as enhanced synchronization when interneurons are depolarized close to threshold. The population of cortical interneurons can behave independently when weakly coupled and hyperpolarized below threshold, but show synchronization when depolarized close to threshold. Thus, a fundamental concept to emerge, as seen in studies of lower vertebrate preparations [12–14], is that weakly coupled neuronal networks can operate independently under some conditions, yet become synchronized when the population of neurons become depolarized close to threshold.

7.4.3. Locus coeruleus

A series of studies [3,21,22,54] has suggested that neurons in the locus coeruleus, as in the cortex, are extensively coupled early in development, and that coupling decreases as the animals mature. In mature animals, treatment of slices with tetrodotoxin, which blocks voltage-dependent sodium channels and fast action potentials, and ionic manipulations that would be expected to augment calcium spiking, lead to synchronous firing of LC neurons. The basis for this type of synchronization is that the prolonged action potentials, compared to short-duration sodium-mediated action potentials, are more easily transmitted through electrotonic junctions because of their low-pass filtering properties. Ishimatsu and Williams [54] showed that gap junction blockers depress synchronization without blocking electrical activity. The latter point is important because studies using gap junction blockers that do not control for changes in membrane excitability are potentially flawed. These studies support the hypothesis that gap junctions and electrotonic coupling between neurons mediate synchronization of calcium spikes without active chemical synaptic transmission under conditions when tracer coupling cannot be detected. The possible presence of electrotonic coupling without tracer coupling or direct evidence of gap junctions between LC neurons suggests that neurons may be electrotonically coupled, even though studies with tracer coupling would otherwise suggest that they are not.

8. Additional controversial issues

8.1. Evidence for absence of Cx32 and Cx26 in neuronal gap junctions

The adage that “absence of proof is not proof of absence” is equally true for FRIL as for other connexin detection methods. That adage was foremost in our thoughts during our initial FRIL studies of Cx32. For more than 3 years, we had attempted to find Cx32 in neuronal gap junctions, as had been proposed by several investigators [86,87,93]. Although immunogold “flags” for Cx32 had led us to several hundred gap junctions (labeled by several thousand immunogold beads), all of those were in oligodendrocyte plasma membranes, and none were present in neuronal gap junctions. To account for the lack of detection of Cx32 in neuronal gap junctions, six possibilities initially were considered: (1) Cx32 is normally present in neuronal gap junctions, but SDS washing preferentially removed connexins from neurons but not from nearby oligodendrocytes; (2) Cx32 is present in neuronal gap junctions but at a density too low to detect by FRIL (i.e., several orders of magnitude less than in oligodendrocyte gap junctions); (3) Cx32 is present in only a tiny fraction of neuronal gap junctions (e.g., in only a small subpopulation of neurons) [2]; (4) Cx32 occurs in neuronal gap junctions only during certain stages of neuronal development [20]; (5) neuronal gap junctions are so rare as to be essentially undetectable by FRIL, regardless of the connexin tested; and/or (6) Cx32 is not present in neuronal gap junctions (but see adage).

The discovery of Cx36 [25,137] has allowed most of the alternative explanations to be tested. In our initial FRIL study using antibodies to Cx36 in retina, inferior olive and spinal cord, we found 40 neuronal gap junctions, 38 of which (95%) were labeled for Cx36 [118]. In subsequent studies, samples of retina, suprachiasmatic nucleus, inferior olive, and spinal cord were simultaneously labeled for Cx36 plus Cx32, Cx26 and/or Cx43. In the cumulative total of >400 immunogold labeled neuronal gap junctions having >2000 discrete immunogold labels, all (100%) were for Cx36, and none (0%) contained immunogold for any of the other connexins. Moreover, in gap junctions that were immunogold-labeled for Cx43, Cx30 and Cx26, all (100%) of >5000 gap junctions (representing >50,000 separate immunogold
labeling events) were astrocyte gap junctions, and none were neuronal or oligodendrocyte gap junction plaques. Although it is true that FRIL cannot exclude the possibility that Cx32 or Cx26 (or any other connexin) is absent from any particular gap junction, the large numbers of labeled gap junctions examined and the much larger number of separate immunogold labels, each restricted to the gap junctions of a single class of cells, represent evidence that is consistent with the following conclusions: (1) Cx36 is present in relative abundance in neuronal gap junctions in 10 selected regions of adult rat CNS [119], as well as in early postnatal rat spinal cord [120]; (2) Cx36 is not present in detectable amounts in gap junctions of astrocytes or oligodendrocytes; (3) Cx43, Cx30 and Cx26 are present in abundance in astrocyte gap junctions; (4) Cx26, Cx30 and Cx43 are not present in detectable amounts in neuronal or oligodendrocyte gap junctions; (5) Cx32 is present in most oligodendrocyte gap junctions; but (6) Cx32 is not present in detectable amounts in neuronal gap junctions that were, however, labeled for Cx36. Thus, the cumulative FRIL data suggest that of the five connexins tested (Cx26, Cx30, Cx32, Cx36 and Cx43), Cx36 is present and abundant in most neuronal gap junctions, whereas Cx26, Cx30, Cx32 and Cx43 were present only in non-neuronal gap junctions.

8.2. The problem of neuronal–glial coupling

A series of ultrastructural and light microscopic immunohistochemical studies in the cortex by Parnavelas et al. [92–94] and in locus coeruleus by Williams et al. [2] have suggested that gap junctions extensively couple neurons with glia. This hypothesis has a long history, but all the evidence has been weak. Recently, this hypothesis has been applied to the locus coeruleus. Alvarez-Maubecin et al. provided electrophysiological evidence in both immature and adult animals that locus coeruleus neurons are weakly coupled to glia, whereas their ultrastructural data suggested that neurons share abundant gap junctions with astrocytes, with the

Fig. 13. Intracellular recording of field potentials from a presumed glial cell in a hippocampal slice during an afterdischarge (from Ref. [149]). This series of differential recordings illustrates how an intracellular recording from a glial cell can be contaminated by the extracellular field potential unless the extracellular recording is subtracted from the single-ended intracellular recording (i.e., intracellular recording of the transmembrane potential requires differential recording, with subtraction of the extracellular field potential from the single-ended intracellular potential). (A) Differential recording (1 minus 2) shows the glial transmembrane potential changes after a single stimulus of the alveus. The upper three traces illustrate the intracellular recording referenced to ground (1), the extracellular recording referenced to ground (2), and the differential recording of the transmembrane voltage (1 minus 2). These recordings are shown at an expanded time scale below (see underlined portion), and show the onset of the slow depolarization more clearly. The single-ended intracellular trace (i.e., first trace) also shows a burst of population spikes on the rising phase of the slow depolarization. A similar burst of population spikes is illustrated in the single-ended extracellular recording (i.e., second trace). The differential recordings (1 minus 2), at slow and fast time scale, show that when the extracellular recording was subtracted from the intracellular recording, the population spikes were no longer present in the intra-glial recording. (B) Control recording for the experiment outlined above in A. After withdrawing the intracellular electrode by approximately 5 μm, both electrodes recorded identical field potentials during a second afterdischarge. This control procedure needs to be done after any intracellular recording from a glial cell to eliminate the possibility that the recordings from the single-ended glial-cell recording were not actually the extracellular field potential recorded from within the glial cell. Future studies concerning the hypothesis of electronic coupling between neurons and glia will need to perform differential recordings during synchronous activity with appropriate controls to exclude the possibility that the potential changes recorded from the glial cells were not actually the extracellular field potential.
putative neuronal glial gap junctions containing both Cx26 and Cx32. However, the ultrastructural evidence has limitations because of the problems with identification of gap junctions in electron micrographs of non-tilted thin-sections, apparent low labeling density (i.e., one gold bead per putative gap junction plaque), and low signal-to-noise ratio of the putative immunogold labeling, raising questions as to whether connexin labeling or gap junctions were demonstrated. Although the authors used dual recordings between neurons to show apparent synchronization of electrical activity, the recordings of glial activity did not control for field potentials that can be recorded from glia during synchronous neuronal activity (Fig. 13) [149]. This is particularly problematic because the authors suggested that >50% of all gap junctions in locus coeruleus were neuronal/neuronal or neuronal/glial, whereas previous studies suggest that astrocytic gap junctions alone represent up to 99% of gap junctions in most brain regions [18,117,119]. If the proposed neuronal–glial gap junctions in locus coeruleus are abundant, and if each gap junction contains sufficiently large numbers of connexins to be detected by immunogold labeling, the number of available connexons is several orders of magnitude larger than their estimate of the number of open gap junctional channels that would result in a 0.25 mV depolarization (i.e., ca. 1 connexon per neuron). Presently, the available data suggesting that neurons are coupled to glia in neocortex and locus coeruleus needs further experimentation.

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