ULTRASTRUCTURE, HISTOLOGICAL DISTRIBUTION, AND FREEZE-FRACTURE IMMUNOCYTOCHEMISTRY OF GAP JUNCTIONS IN RAT BRAIN AND SPINAL CORD

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The historical development of concepts of gap junctions as sites for electrical, ionic, and metabolic coupling is reviewed, from the initial discovery of gap junctions linking heart cells, to the current concepts that gap junctions represent ‘electrotonic synapses’ between neurons. The ultrastructure and immunocytochemistry of gap junctions in heart, brain, and spinal cord of adult rats is examined using conventional thin sections, negative staining, grid-mapped freeze-fracture replicas, and immunogold-labeled freeze-fracture replicas. We review evidence for neuronal gap junctions at ‘mixed’ (combined electrical and chemical) synapses throughout adult rat spinal cord. We also show immunogold labeling of connexin43 in astrocyte and ependymocyte gap junctions and of connexin32 in oligodendrocyte gap junctions. Ultrastructural and freeze-fracture immunocytochemical methods have provided for definitive determination of the number, size, histological distribution, and connexin composition of gap junctions between neurons in all regions of the central nervous systems of vertebrate species.

KEYWORDS: connexin; connexon; electron microscopy; immunogold labeling; intramembrane particle (IMP)

HISTORICAL CONFUSION OF GAP JUNCTIONS AND TIGHT JUNCTIONS

The ultrastructural features now known as ‘gap junctions’ were first detected as specialized close appositions of plasma membranes in conventional thin-section electron micrographs of the intercalated disc regions of cardiac myocytes (Sjöstrand et al., 1958; Karrer, 1960). However, the use of fixation and staining techniques derived from light microscopy precluded accurate assessment of the morphology of gap junctions in those early ultrastructural studies. Nevertheless, it has long been recognized the unique nature of the intercellular junctions in heart and proposed the name ‘nexus’ (Latin: ‘connection’) to emphasize their suspected role in intercellular propagation of electrical impulses.

With growing recognition that diverse functions were attributable to the specialized cell contacts, Farquhar and Palade (1963) examined epithelial tissues, where they characterized four primary types of intercellular junctions and proposed formal Latin names for each. At the apical margins of columnar epithelia, where intercellular diffusion barriers were known to be generated, they observed a narrow circumferential band in which the apposed plasma membranes appeared to fuse. For this type of intercellular junction, they suggested the name zonula occludens (ZO; ‘occluding belt’). Although they did not examine cardiac tissue, they reviewed the available literature and listed the cardiac ‘nexus’ as a form of zonula occludens based on its appearance after osmium tetroxide fixation. (For descriptions of the intercellular junctions with more widely spaced membrane appositions, see the original report by Farquhar and Palade, 1963.)
Following the convention proposed for epithelial junctions, many authors designated all close membrane appositions as ‘tight junctions’ (Benedetti and Emmelot, 1968), an unfortunate convention that persisted long after evidence was obtained that the ‘nexus’ was a distinctly different, second type of intercellular junction.

The introduction of glutaraldehyde fixation (Sabatini et al., 1963), in combination with uranyl acetate and lead citrate staining (Kellenberger et al., 1958; Reynolds, 1963), and the use of single-tilt, double-tilt, and tilt-rotation stages allowed the heptilaminar (seven-layered) structure and an extracellular gap within the cardiac nexus to be resolved (Fig. 1A), thereby allowing the nexus to be distinguished from the trilaminar and pentalaminal appositions seen at epithelial tight junctions. However, the first incontrovertible evidence that tight junctions consisted of two fundamentally different types of membrane specializations was obtained using colloidal lanthanum hydroxide as an electron-dense extracellular tracer (Revel and Karnovsky, 1967; Brightman and Reese, 1969; Goodenough and Revel, 1970; Friend and Gilula, 1972). The penetration of colloidal lanthanum was blocked at the apical intercellular permeability barriers of columnar epithelia and capillary endothelia. Where the permeability barrier was damaged (as at the edges of cut tissues), lanthanum penetrated and outlined both sides of the epithelial junction, thereby revealing the linear, zipper-like nature of the intercellular sealing strands. This barrier to lanthanum penetration resulted from direct contact of membrane surfaces according to the original concept of occluding or tight junctions. (For a detailed review of the ultrastructure of intercellular junctions, see Staehelin, 1974).

In contrast to its staining of epithelial tight junctions, colloidal lanthanum (Revel and Karnovsky, 1967), as well as other electron-dense substances (Unwin and Zampighi, 1980; Forbes and Sperelakis, 1985), penetrated throughout the normal 20 nm-wide extracellular spaces of cardiac muscle and liver. Moreover, the extracellular tracers were not blocked at the margins of the cardiac nexus, but instead, penetrated and delineated a 2 nm-wide gap between the closely spaced plasma membranes (Fig. 1B,C). In high-magnification oblique or tangential sections, an hexagonal array of subunits was outlined in negative contrast (Fig. 1C). Moreover, the negative stain penetrated the hexagons and revealed that each possessed a 2-nm central channel (Fig. 1C, arrows) that connected the cytoplasmic trees of the two apposed cells (Perrachia, 1980). Finally, in sections that were tilted appropriately, the aligned rows of hexagons (later called connexons; Goodenough, 1975) appeared to span the extracellular gap (Perrachia, 1973; Unwin and Zampighi, 1980). (See arrow in Fig. 1B for area of aligned connexons.) To emphasize that lanthanum penetrated throughout the junctional plaque and delineated the narrowed extracellular space, Revel and Karnovsky (1967) proposed the name gap junction, the term that is now used by virtually all investigators of gap junction structure, biochemistry, and function.

Functional correlations of gap junctions with dye- and electrical-coupling

In functional studies of cells linked by gap junctions, tracer- and dye-coupling experiments revealed that substances up to about a molecular weight of 1000 were able to pass directly from cell to cell without diffusion into the extracellular space (Loewenstein, 1966; reviewed in Perrachia, 1980). Thus, the abundant intercellular bridges in gap junctions, each with a 2-nm central canal, appeared to provide sufficient structural pathways to account for electrotonic coupling of neurons (Bennett et al., 1963), as well as for pacemaker coupling of cardiac myocytes (DeHaan et al., 1971; DeHaan and Sachs, 1972). Likewise, the demonstration of the abrupt formation of gap junctions concurrent with the onset of electrical coupling in pre-fusion skeletal myogenic cells in culture (Rash and Fambrough, 1973) provided additional evidence that gap junctions were the primary ultrastructural correlates for direct electrical- and dye-coupling in a wide variety of vertebrate tissues (reviewed by Perrachia, 1980). Consequently, Palade and co-workers (Simeonescu et al., 1976) proposed a new name, macula communicans (i.e. communicating spot), as a Latin alternative for gap junction. (This suggestion has not been followed by most investigators.)

Tight junctions and gap junctions are biochemically and immunocytochemically distinct

Within the past decade, the tools of biochemistry and molecular biology have revealed that tight junctions and gap junctions are composed of two distinct and unrelated groups of proteins—occludin, ZO-1, ZO-2, and ZO-3 in tight junctions (Stevenson et al., 1986; Anderson et al., 1988; Furuse et al., 1993), vs proteins of the
Fig. 1. Gap junctions in heart (A–C) and spinal cord glial cells (D–F). (A) Thin section containing a gap junction cut perpendicular to the plane of the plasma membrane. The extracellular space is narrowed from the normal separation of 20–30 nm (*), to yield a small gap of 2–3 nm (arrow). Within the narrowed gap, the plasma membranes are precisely parallel and are of increased electron opacity, representing in part, the increased protein content of the junction. In the cytoplasm, periodic projections (A, arrowheads) derive from osmiophilic staining of the hydrophilic amino acids in the cytoplasmic determinants of the connexons. (Originally published in DeHaan and Sachs (1972).) (B) and (C) The narrow extracellular space of an extended cardiac gap junction was negatively stained by precipitation of uranyl phosphate. The stain surrounds and delineates the closely packed connexons (C), as well as a 2-nm central pore or channel within each connexon (arrows). (From M. Forbes and J. Rash, unpublished observations, 1974.) (D–F) Demonstration that most gap junctions cannot be resolved unless goniometric tilting is employed. (D) at +30° tilt (E) at –30° tilt, and (E) at –58° tilt. Only one gap junction or portion of gap junction (numbered 1–3) is resolvable at each angle of tilt. Despite the 60° included angle (D) and (E) may be viewed as stereopairs, whereas severe image X-axis compression at –58° tilt in (F) makes it difficult for most viewers to obtain stereopsis of images (E) and (F). (Versions of (E) and (F), computer-stretched were published in Rash et al., 1997.) In all images, the calibration bar is 0.1 μm unless otherwise specified. Magnifications: (A) × 280,000; (B) × 70,000; (C) × 200,000; (D–E) × 70,000.
connexin† superfamily in gap junctions (Paul, 1986; Beyer et al., 1990; Dermietzel and Spray, 1993). Antibodies have been obtained to purified proteins or synthetic sequences from many of those proteins (Paul, 1986; Furuse et al., 1993), and immunogold-labeling techniques have permitted positive identification and high-resolution localization of the primary tight junctional and gap junctional proteins in a variety of cell types (Zampighi et al., 1989; Furuse et al., 1993; Fujimoto, 1995). Thus, there is now strong ultrastructural, biochemical, and immunocytochemical evidence showing that gap junctions and tight junctions are different structural features with distinctly different biochemistries and functions.

DISCOVERY AND PROPOSED FUNCTIONS OF GAP JUNCTIONS BETWEEN NEURONS IN LOWER VERTEBRATES

In goldfish Mauthner cell club endings, structures later recognized as gap junctions were seen in early electron micrographs (Bennett et al., 1963; Robertson, 1963) and proposed as the structural correlates for the efficient, high-speed electrical communication that underlies the extremely rapid tail-flick escape responses of fish (Bennett et al., 1963).‡ Subsequent thin-section and freeze-fracture electron microscopic analyses of the central nervous systems (CNS) of lower vertebrates revealed abundant gap junctions linking many classes of neurons (Sotelo and Taxi, 1970; Sotelo and Korn, 1978; Tuttle et al., 1986). Electrophysiological analyses of neuron pairs having the most extensive gap junctions consistently revealed strong electrical coupling and extremely rapid impulse propagation from neuron to neuron (Bennett et al., 1963; Sotelo and Korn, 1978). Thus, it was proposed that gap junctions between neurons represent ‘electrotonic synapses’, which provide the intercellular molecular pathways for virtually instantaneous synaptic communication, as well as provide for synchronization of cardiac and neuronal electrical activities and for equilibration of neuronal and cardiac membrane potentials (Bennett et al., 1963; Bennett, 1972; DeHaan and Sachs, 1972; Bennett, 1974; Sotelo and Korn, 1978).

In addition to gap junctions between electrically coupled cells, many non-excitable cells (such as those in liver) were also found to be linked by extensive gap junction plaques, and thus, functions other than electrical coupling were added to the presumed repertoires of gap junctions. For example, the abundance of connexon channels in large gap junctions was suggested as facilitating direct intercellular exchange of ions and small metabolites in a process called ‘metabolic coupling’ (Gilula et al., 1972). Hepatocytes, which are strongly metabolically coupled, were linked by gap junctions consisting (in aggregate) of hundreds of thousands of connexons per cell (Larsen, 1983). Thus, bulk transfer of metabolites and second messenger molecules was proposed to occur between cells having the largest or most extensive gap junctions, whereas ionic and electrical coupling (as in the heart and between Mauthner cells) was proposed to occur where relatively more modest gap junctions were present (i.e. where gap junctions contained a few thousand to a few tens of thousands of connexons). However, much smaller gap junctions containing less than a dozen to a few dozen connexons have been shown to occur between neurons in the mammalian CNS (Raviola and Gilula, 1975; Rash et al., 1997). These neuronal gap junctions may be too small for direct intercellular propagation of nerve action potentials or for bulk transfer of metabolites, and likewise, may be too small to be detected by conventional thin-section electron microscopy or by freeze-fracture. Consequently, analysis of gap junctions consisting of less than 100 connexons required the development of new search strategies and imaging methods.

†Proteins thought to comprise the connexons of liver gap junctions were isolated and named connexins (Goodenough, 1974). Because the isolated proteins did not correspond to the connexon subunits, Goodenough and coworkers subsequently re-assigned the name connexin to the principal class of gap junction proteins in vertebrate tissues (Paul, 1986; Beyer et al., 1990). It should be noted, however, that gap junctions of invertebrates appear to consist of non-homologous proteins called innexins (for invertebrate connexins), apparently reflecting convergent evolution of unrelated channel-forming proteins (named and reviewed in Phelan et al., 1998). In contrast, assertions that still other proteins called ductins constitute the connexon channel of both invertebrates and vertebrates (Finbow et al., 1995) have not yet been supported by immunocytochemical localization to gap junction plaques or by demonstration of intercellular channel activity after expression in an in vitro system (Bruzzone and Goodenough, 1995); also see later section entitled ‘Immunogold tagging to find gap junctions and to identify the connexin isoforms’.

‡The structural basis for intercellular communication via intercellular ion channels was not recognized by Robertson because he interpreted the stained elements according to his ‘unit membrane’ hypothesis (Robertson, 1963), and he continued this interpretation (Robertson, 1981) despite widespread acceptance of the competing ‘fluid mosaic’ model of membranes (Singer and Nicolson, 1972). In Robertson’s ‘unit membrane’ hypothesis, protein molecules were thought to coat the surfaces of membranes but not to penetrate or cross the lipid bilayer. Consequently, Robertson proposed that electrical coupling occurred through areas of decreased membrane resistance where the lipid bilayers of apposed cells were in close apposition—as in tight junctions. In his final review of membrane structure, Robertson maintained many of his original objections to the ‘fluid mosaic model’ and to the existence of transmembrane proteins (Robertson, 1987).
Limitations on analyzing gap junctions using conventional thin-section images

Although published descriptions might lead one to believe that gap junctions are easily detected and identified in conventional thin sections, that is a false impression, at least for nerve tissues. Gap junctions are identifiable only under one of six limited conditions.

1. Gap junction plaques larger than 0.1 μm in diameter may be recognized if the thin-section is obtained precisely perpendicular to the apposed membranes (i.e., so that the electron beam passes parallel to the plane of the membranes; Fig. 1A). In all other orientations, the images of gap junctions appear as indistinct blurring of the apposed membranes (Fig. 1D), thereby resulting in a 75–95% underestimate of the number of gap junctions in any tissue.

2. Obliquely sectioned gap junctions may be tilted in the electron microscope (using a single-tilt, double-tilt goniometer stage, or a tilt-rotation stage) so that the electron beam passes parallel to the apposed membranes (Fig. 1D–F). If a single-axis tilt device were used (Rash and Fambrough, 1973), the investigator would first identify an area of membrane containing a suspected gap junction (Fig. 1E), determine the desired direction(s) of tilt, remove the specimen from the electron microscope, manually rotate the specimen to the appropriate direction, insert the specimen into the electron microscope, re-find the area of interest, obtain the necessary tilt angle(s), and photograph the specimen (Fig. 1D, F). In complex tissues, those steps were so time-consuming that it is not surprising that few neuronal gap junctions were found using conventional thin-sectioning techniques. Moreover, similar time is required when using a double-tilt stage or a tilt-rotation stage, primarily because of excessive specimen drift that occurs during non-eucentric double-axis tilting or during tilt-rotation. In addition, because of tilt-superposition of grid bars and stage components, the maximum usable tilt angle is limited to ±60°. Thus, near-tangentially sectioned gap junctions are not resolvable, even with the highest available tilt angles.

3. The characteristic hexagonal array of connexons may be delineated by ‘negative staining’ using lanthanum hydroxide or other electron-opaque tracer (Fig. 1B,C). However, for resolving gap junctions in CNS tissue, lanthanum hydroxide staining has less utility than in other tissues because the extracellular space between neurons is relatively narrower (5–10 nm vs 20–30 nm), thereby making the relative change in electron contrast between non-junctional vs gap junctional extracellular spaces difficult to discern. Once found, however, the distinctive clusters of connexons in neuronal gap junctions would probably be equally well delineated by lanthanum as those in liver or heart.§

4. Connexin molecules may be labeled immunocytochemically with specific tracer molecules—for example, colloidal gold conjugated to appropriate anti-connexin antibodies (Gruijters et al., 1987; Zampighi et al., 1989; Fujimoto, 1995; see later section entitled ‘Immunogold tagging’ to find gap junctions and to identify connexin isoforms).§

5. If the thickness of the plastic section is greater than the diameter of the gap junction, image superposition of the closely spaced and more widely spaced membrane profiles within the same area of contact results in a blurred image that is not positively identifiable as a gap junction, regardless of specimen tilt.

6. Similarly, in thick sections examined by intermediate-voltage or high-voltage electron microscopy (i.e., in electron microscopes operated at 300 keV–3 MeV), the undulating course of the gap junction membranes precludes imaging of the gap, regardless of tilting or stereoscopic viewing. To overcome the limitations imposed by image element superposition, tomographic analysis and computer-simulated image slicing has been proposed (but not yet verified) as a means for visualizing and identifying gap junctions (M. H. Ellisman, personal communication).

GAP JUNCTIONS BETWEEN NEURONS IN THE MAMMALIAN CNS

In contrast to the relatively abundant gap junctions between neurons in lower vertebrates, neurons in most regions of the mammalian CNS have been widely considered to be completely devoid of gap junctions (Shepherd, 1988). Gap junctions were considered to be relatively abundant only in the retina, olfactory bulb, lateral vestibular and mesencephalic trigeminal nuclei, and the inferior olive of the brain stem (Dowling and Boycott, 1966; Brightman and Reese, 1969; Pinching and Powell, 1973).

§Although lanthanum staining has been used to reveal gap junctions between Mauthner neurons in fish (Brightman and Reese, 1969), we are unaware of any descriptions of lanthanum staining of gap junctions between neurons in mammalian CNS.
of 30,000–100,000 membrane profiles from many different neurons, with each profile tediously examined by goniometry. Clearly, either would be a daunting task. Instead, investigators have used methods of essentially random thin-sectioning to establish merely the existence but not the relative abundance of gap junctions on individual neurons.

Based on the repeated failure to find gap junctions linking most neurons in mammalian CNS, many neuroscientists concluded that gap junctions represent a phylogenetically primitive form of synaptic transmission that has been all but abandoned by neurons in the CNS of higher vertebrates (Sotelo and Korn, 1978; Shepherd, 1988). Shepherd (1988), for example, proposed that the apparent absence of gap junctions in most areas of the CNS of higher vertebrates ‘could reflect a mechanism to increase the metabolic and functional independence of neurons, in order to permit more complex information processing’ (emphasis added). This concept appeared to account for the apparent restriction of gap junctions to primitive brain areas, in which synchronized electrical activity was proposed to exist for amplification of responses or for synchronization of nerve activity—as for example, in the tail-flick escape response.

**Gap junctions in spinal cord**

The spinal cord in mammals is, phylogenetically, the most primitive portion of the CNS, and because it has no cognitive functions, it was a logical place to search for gap junctions. As expected, gap junctions were found to be abundant between spinal neurons in lower vertebrates (Bennett et al., 1963; Sotelo and Taxi, 1970). In contrast, the spinal cords of mammals have long been described as being devoid of neuronal gap junctions. The only known exception (Matsumoto et al., 1988, 1989) seemed to be that gap junctions were present in a primitive region of the mammalian spinal cord—that is the sacral segment that contains the motor neurons responsible for the synchronized contractions of male ejaculation. Nevertheless, evidence for both strong and weak electrical coupling between spinal cord neurons in mammals (Nelson, 1966; Gogan et al., 1977; Arasaki et al., 1984) indicated that gap junctions might be a common feature of neurons in spinal cord, but that they were too small to have been detected by conventional thin-sectioning techniques. If so, the predicted small size of the gap junctions and the geometric relationship of the coupled cells might prevent them from acting as efficient electrical transducers.
The IMPs in gap junctions are positively dis-

Freeze-fracture facilitates the search for neuronal gap junctions

In contrast to conventional thin-section electron-
microscopic techniques, freeze-fracture approaches have several distinct advantages for identifying gap junctions.

(1) The fracture plane preferentially splits and exposes large expanses of plasma membrane (Branton, 1966; Pinto da Silva and Branton, 1970), thereby significantly increasing the area that may be searched for gap junctions (Fig. 3A). By comparison, a 0.1-μm thin-section through a 30-μm-diameter neuronal soma would contain 10 μm² of plasma membrane, and a thin section through a 3-μm-diameter dendrite would contain 1 μm² of plasma membrane. In contrast, freeze-fractured neurons frequently contain 40–100 μm² of plasma membrane (Rash et al., 1996).

(2) Freeze-fracture replicas of gap junctions (Fig. 3B–D) yield definitive images of regular hexagonal or polygonal arrays of uniform-diameter 8-nm P-face** intramembrane particles (IMPs) and E-face pits (Kreutziger, 1968; Challcroft and Bullivant, 1970), similar to the thin section images of hexagonal arrays of connexons revealed by negative staining.

(3) The IMPs in gap junctions are positively discerned from all other clusters of IMPs by: (a) a distinctive narrowing of the extracellular space, where the fracture plane steps from the E- to the P-face (Fig. 3B,C), (b) precise alignment of the rows of IMPs and pits at the point where the fracture plane steps from the E-face to the P-face (Fig. 3C), (c) a unique, regular hexagonal distribution of IMPs and pits in many (but not all) types of gap junctions (Fig. 3C,E vs. 3B); and (d) the presence of a distinctive central dimple or pore within each P-face IMP (Fig. 3B) and a complementary peg within each E-face pit (Figs 3D,E, 6B). Because the central pegs evaporated (sublimed away) during freeze etching (Rash et al., 1991), we presume that the pegs correspond to the frozen aqueous cores of the connexon ion channels and that the frozen cores were extracted from the tubular IMPs during the fracturing event.

(4) Due to the virtual absence of superposition of image detail in freeze-fracture replicas, time-consuming goniometric tilting is seldom required.

Limitations on previous freeze-fracture methods

Until recently, freeze fracture has been limited in its ability (a) to provide quantitative data regarding the relative number of gap junctions in tissue, (b) by its inability to allow histological mapping of gap junctions, and (c) by the absence of methods to identify the biochemical composition of replicated IMPs. Specifically, conventional freeze-fracture replicas frequently fragmented (Fig. 4A), thereby destroying long-range structural relationships across fragment boundaries. Thus, it was seldom possible to attribute an individual gap junction to any specific cell type, cell layer, or even to determine its histological location. In addition, definitive criteria were not available for positive identification of all of the cell types present in CNS tissue, particularly if only a small portion of a cell’s plasma membrane were exposed in the replica. Finally, immunocytochemical methods for direct labeling of gap junction proteins (i.e. connexins) were either tedious (e.g. the sectioned-labeled replica technique; Rash et al., 1978, 1990; Rash, 1979); were useful for analyzing subcellular preparations or tissue-culture samples (Dinchuk et al., 1987; Zampighi et al., 1989; Rash et al., 1990), but were not compatible with labeling constituents of intact tissue (Pinto da Silva et al., 1981; Rash et al., 1990); or were impractical for quantitative histological analysis due to replica fragmentation (Fujimoto, 1995, 1997). To overcome these limitations, several new techniques were devised and combined to allow visualization and biochemical characterization of gap junctions in CNS tissues.

Grid-mapped freeze fracture and immunogold labeling

To stabilize large replicas, improve resolution of IMPs, provide for direct immunogold labeling of connexins, and to allow histological mapping of gap junctions, grid-mapped freeze fracture (Rash et al., 1995, 1996) has been combined with improved shadowing techniques (Rash and Yasumura, 1992; Rash et al., 1997) and with an SDS-washed freeze-fracture immunogold labeling

(1) Foremost, the entire replica is stabilized in a supporting Lexan plastic film (Steere and Erbe, 1983) on a gold ‘Index’ or ‘Finder’ grid (Rash et al., 1995; Fig. 5, steps 3–5).

(2) After replication and Lexan-stabilization, but before the tissue is removed by detergents or caustic reagents, the sample is thawed and
mapped by confocal microscopy (Fig. 5, step 6) for subsequent correlation with electron micrographs (Fig. 5, step 9).

(3) Improved shadowing methods (Rash and Yasumura, 1992; Rash et al., 1997) produce a two- to four-fold improvement in resolution over previous methods (to c. 1 nm; see Fig. 3D,E), thereby revealing details of connexon ultrastructure within the P-face IMPs and within the complementary E-face pits.

(4) The resulting high-resolution replicas are examined in the electron microscope at magnifications sufficient to detect even the smallest gap junction (i.e. at ×300,000 on the fluorescent viewscreen). Neuronal gap junctions with as few as 6–12 connexons have been found in neurons and glia (Figs 3A and 6C). Those junctions were very small (average: 0.05 μm in diameter; median: 47 connexons; mean: 75 ± 10 connexons) and, thus, 90–95% were too small to have been detected in conventional thin sections, even if examined using a tilt-rotation stage (see Fig. 1D–F).

(5) For immunogold labeling (Fig. 5, step 7 (alt) and Fig. 9, next section, below), the Lexan-stabilized, high-resolution replicas of unfixed or formaldehyde-fixed tissues are washed for 24 hours with 2.5% sodium dodecyl sulfate (SDS) detergent at 37°C. SDS-washing leaves a thin layer of proteins and other macromolecules strongly adsorbed to the platinum-carbon replica (Fujimoto, 1995; see Rash et al., 1990 for rationale). Those adsorbed molecules are then labeled with gold-conjugated antibodies using methods (Fujimoto, 1995) modified for use in grid-mapped freeze fracture (Rash et al., 1998). Note: glutaraldehyde-cross linked tissues cannot be cleaned using SDS detergent.

(6) The delicate (1 nm platinum plus 10–20 nm carbon) replicas are examined by high-magnification transmission electron microscopy.

Spinal cord neurons are coupled by gap junctions at mixed synapses

In our recent grid-mapped freeze-fracture studies of gap junctions linking neurons in rat spinal cord (Rash et al., 1996, 1997), we identified and mapped 99 neuronal gap junctions (Figs 3A and 6A–C). Those junctions were very small (average: 0.05 μm in diameter; median: 47 connexons; mean: 75 ± 10 connexons) and, thus, 90–95% were too small to have been detected in conventional thin sections, even if examined using a tilt-rotation stage (see Fig. 1D–F).

All of the neuronal gap junctions were present in mixed synapses (Fig. 3A), so defined because they contained ultrastructural components of both chemical and electrical synapses (Sotelo and Korn, 1978). Ultrastructural components characteristic of chemical synapses included: (a) the presence of clusters of at least 25 round, 50-nm-diameter presumptive synaptic vesicles in either or both sides of the presumed synapse (Fig. 3A); (b) the presence of distinctive active zones; and (c) evidence of ongoing exocytosis and/or endocytosis at the active zones, presumably representing stages of synaptic vesicle...
fusion and/or invagination of coated pits (Figs 6D and 7A, B). (The unusual active zones at spinal cord mixed synapses were designated ‘synaptic sombreros’ and ‘synaptic mesas’ (Rash et al., 1996) based on their obvious three-dimensional shape as revealed in stereoscopic images; Figs 6 and 7). The spinal cord mixed synapses were present on neuronal somata, dendrites, and axon terminals (Fig. 7A; i.e. synapses were axo-dendritic, axo-somatic, dendro-dendritic, and dendro-somatic; Rash et al., 1996).

**Fig. 6.** Freeze-fracture replicas of gap junctions from mixed synapses in lumbosacral region of rat spinal cord. In each of these examples, the fracture plane steps from the E- to the P-face, thereby revealing the narrowing of the extracellular space within the gap junction. (A) Stereoscopic image of one of three active zones at one mixed synapse. Each active zone was designated a ‘synaptic sombrero’ (SS) based on its topology. A single gap junction (arrow) was seen in the ‘brim’ of most synaptic sombreros. (B) At higher magnification, the same gap junction is seen to consist of c. 90 connexons, including both E-face pits and P-face IMPs. The hexagonal array of pits and particles is maintained where the fracture plane steps from E- to P-face. (C) Very small gap junction from the second of two mixed synapses found on the limited area of replica of the same neuron. The gap junction consists of c. 12 E-face pits and four or five P-face IMPs. (D) Portion of the same mixed synapse shown in (A). Note the gap junction in the brim of the synaptic sombrero. The two mixed synapses shown in (A)–(D) are designated M & N in Fig. 8C. The two mixed synapses had six synaptic sombreros and eight gap junctions. Magnifications: (A) × 60,000; (B) × 140,000; (C) × 75,000; (D) × 115,000.
neurons only in male rats (Matsumoto et al., 1988, 1989), we examined freeze-fracture replicas from all regions of the spinal cord (Fig. 8B). Gap junctions were found at mixed synapses on both interneurons and motor neurons in laminae III–IX (Rexed, 1954) of the dorsal and ventral horn (Fig. 8A–C), and they were found throughout the length of the spinal cord (Fig. 8D) in both male and female rats (Rash et al., 1996).

Almost all of the gap junctions in spinal cord neurons were present in clusters (average: 3 gap junctions per cluster; maximum: 6). Using a high-

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Fig. 7. Mixed synapse at axon terminal in lamina VII in the thoracic spinal cord. (A) In the nerve terminal, six gap junctions are present interspersed with six to eight ‘synaptic mesas’ (SM), the second type of active zone associated with gap junctions. Synaptic vesicle (SV) exocytosis occurs within the mesa-like active zones (see arrowheads in (B)), whereas endocytosis occurs in the surrounding plasma membrane. Note the terminal loops of myelin (arrow) and the longitudinally fractured axon cytoplasm (*). (B) At higher magnification, two gap junctions (arrows) are seen adjacent to cross-fracture images of synaptic vesicle exocytosis (arrowheads), which occurred atop synaptic mesas. Magnifications: (A) × 20,000; (B) × 75,000.
magnification search strategy to photograph every patch of neuronal membrane encountered that was larger than 4 \( \mu \text{m}^2 \), we estimated that, on average, each spinal cord neuron had approximately 1000 gap junctions clustered in approximately 300 mixed synapses. Three hundred mixed synapses per neuron represent only 3–5% of the estimated 10,000 chemical synapses on the typical spinal cord neuron (Kandel et al., 1992). However, if mixed synapses are found only in excitatory synapses, 300 mixed synapses would represent a major fraction of the estimated 500–1000 excitatory chemical synapses on each spinal cord motor neuron (Eccles, 1957; Kandel et al., 1992). Regardless, the unusual active zones (i.e. synaptic sombreros and synaptic mesas) observed in mixed synapses may represent useful markers for immunocytochemical studies designed to determine whether the mixed synapses are excitatory, inhibitory, or both.

Possible roles of small-diameter gap junctions in mixed synapses

Most of the gap junctions found in spinal cord mixed synapses were probably too small to permit direct intercellular propagation of nerve action potentials (Pereda et al., 1994). Thus, gap junctions at mixed synapses may provide for metabolic coupling or ionic coupling (Gilula et al., 1972), either of which may include mechanisms for defining functional groups of neurons (Lo Turco and Kriegstein, 1991; Yuste et al., 1992). Interestingly, small gap junctions also have been proposed to propagate pacemaker potentials during initial synchronization of heart cells in culture (DeHaan et al., 1971; DeHaan and Sachs, 1972) and to equilibrate membrane potentials immediately prior to fusion of skeletal myogenic cells (Rash and Fambrough, 1973).

Gap junctions in hippocampus

In early freeze-fracture examinations of hippocampus, gap junctions were found on cells that were identified as neurons (Schmalbruch and Jahnsen, 1981; MacVicar and Dudek, 1982). Based on recent improvements in the freeze-fracture technique, as well as on improved criteria for identifying neurons and glia, all of the previously published freeze-fracture images of gap junctions in hippocampus were recently re-interpreted as showing gap junctions on glial cells (Rash et al., 1997), or on cells that were not identifiable due to limitations of the techniques available at that time. Nevertheless, we emphasize that definitive gap junctions have been found in hippocampus on identified interneurons in thin sections (Kosaka, 1983; 1985), but they have not yet been identified on principal cells. By analogy to data from spinal cord, where the small size of neuronal gap junctions made them difficult to discern in conventional thin-sections, we hypothesized that small gap junctions/mixed synapses occur in the hippocampus, but that most of them have been overlooked based on their small size and/or their location on dendrites.

In preliminary freeze-fracture studies of gap junctions in hippocampus (Rash et al., 1997), we found two unusual anastomosing gap junctions on neuronal dendrites (for images, see Dudek et al., 1998, this issue). However, because the somata of the coupled neurons were not included in the plane of fracture, the histological locations and the
identities of the coupled neurons could not be determined. Nevertheless, these preliminary data from a small number of replicas suggest that gap junctions may yet prove to be a significant component of the hippocampus.

**Do gap junctions couple neurons to glial cells?**

Recent reports have suggested that 17.9% of all gap junctions in rat cerebral cortex link neurons to glial cells (Nadarajah et al., 1996). Another laboratory has suggested that astrocytes in culture are ione-

cally coupled to neurons (Nedergaard, 1994). In contrast, several laboratories have provided evidence that neuron-to-astrocyte and astrocyte-to-neuron signaling may occur by release of the fast excitatory neurotransmitter, glutamate (Charles, 1994; Parpura et al., 1994; Hassinger et al., 1995; Charles et al., 1996). Likewise, using grid-mapped freeze fracture, we recently showed that of 99 neuronal gap junctions found in the spinal cord, none (0%) were shared with glial cells. Moreover, of more than 400 glial gap junctions in which both contributing cells were identified, none (0%) were shared with neurons. Similarly, in hippocampus, suprachiasmatic nucleus, and supraco- optic nucleus, none (0%) of more than one hundred identified glial gap junctions were shared with neurons. This discrepancy (17.9 vs 0%) is unlikely to be due to chance, but rather to previous inadequate criteria for distinguishing neurons from glia in both thin sections and freeze-fracture replicas, as well as to inappropriate designation of some types of cell appositions as gap junctions (see Rash et al., 1997, for specific criteria). Thus, we conclude that either neuron-to-glial cell gap junctions do not occur in the adult mammalian CNS or that they are so rare that they make no significant contribution to neuronal or glial cell biology.

**IMMUNOGOLD ‘TAGGING’ TO FIND GAP JUNCTIONS AND TO IDENTIFY THE CONNEXIN ISOFORMS**

The newest and most promising development in freeze-fracture electron microscopy for the detec-
tion, identification, and immunocytochemical analysis of gap junctions in CNS tissue is based on the SDS detergent-washing and immunogold-labeling technique of Fujimoto (1995). Using high-resolution replication techniques, in combination with grid-mapped freeze-fracture and a modified SDS-washed immunogold labeling technique (Rash et al., 1998), we have labeled and identified multiple connexins in a variety of nervous and non-nervous tissues (Fig. 9). For example, in liver (a control tissue), relatively high levels of anti-Cx32 (aCx32) labeling were found on both E- and P-faces of gap junctions (Fig. 9A), thereby confirming the observations of Fujimoto (1995). In the suprachiasmatic nucleus, aCx43-immunogold labeling was found on the cytoplasmic surface in astrocyte (Fig. 9B) and ependymocyte gap junctions (Fig. 9C), but on no other cells in the CNS.

In confirmation of data from light microscopic and thin-section electron microscopic immunocyto-

chemistry (Li et al., 1997), we detected aCx32 labeling of gap junctions within the oligodendro-

cyte cytoplasm of heterologous astrocyte-to-oligodendrocyte gap junctions (Fig. 9C), but no aCx32-staining of astrocyte gap junctions. Conversely, we observed aCx43-staining but not aCx32 staining in the subjunctional cytoplasm of astrocytes that were coupled to oligodendrocytes. Because virtually all gap junctions on oligodendro-

cytes represent oligodendrocyte-to-astrocyte couplings (Massa and Mognaini, 1982; Mognaini, 1986; Rash et al., 1997), these data confirm that oligodendrocyte Cx32 must couple to Cx43, Cx26, and/or Cx30 (Nagy et al., 1998), thereby simulta-

eously defining astrocyte-to-oligodendrocyte couplings as heterologous (i.e. between two differ-

tent types of cells) and heterotypic (i.e. involving two or more connexin isoforms). Additional electrical- and dye-coupling experiments will be required to confirm whether heterotypic glial couplings are rectifying (Robinson et al., 1993), voltage-gated (Barrio et al., 1991; Moreno et al., 1991), or both (Gutnick et al., 1981; White et al., 1995).

**RELATIVE NUMBER OF NEURONAL V/S GLIAL GAP JUNCTIONS**

Several lines of evidence suggest that gap junctions and their constituent connexins are many orders of magnitude more abundant in glia than in neurons. Dye-coupling studies of mammalian brain slices show hundreds or even thousands of coupled glial cells after dye injection into astrocytes Gutnick et al., 1981; reviewed by Dudek et al., this issue). Diffusion of dye within the astrocyte network is so extensive that the only way to visualize the structure of small clusters of coupled astrocytes is to use agents that block or uncouple most of the junctions (Gutnick et al., 1981). Thus, it is likely that most astrocytes are directly coupled to many other
Fig. 9. Immunogold-labeled gap junctions from liver, suprachiasmatic nucleus, and supraoptic nucleus. All primary and secondary antibodies from Chemicon, Inc. (Temecula, CA, U.S.A.). (A) Hepatocyte gap junctions were labeled with mouse monoclonal αCx32 and counter-labeled with 10-nm colloidal gold conjugated to goat anti-mouse IgG. Gold beads are observed solely on the cytoplasmic surface of the gap junction, with no beads beneath other regions of plasma membrane. This demonstrates high labeling specificity, moderate labeling efficiency, and low non-specific background. (B) Two astrocyte gap junctions (from suprachiasmatic nucleus) labeled with mouse αCx43 and counter-labeled with 10-nm gold conjugated to goat anti-mouse IgG. Gold beads are beneath both E- and P-faces. (C) Ependymocyte from third ventricle, between paired suprachiasmatic nuclei. Ependymocyte gap junctions are labeled with mouse αCx43 and counter-labeled with 20-nm gold conjugated to goat anti-mouse IgG. Note the low ‘background’. (D) Oligodendrocyte gap junction (from supraoptic nucleus) labeled with mouse αCx32 and counter-labeled with 20-nm gold conjugated to goat anti-mouse IgG. These images confirm the presence of specific connexins within individual gap junction plaques. Magnifications: (A) × 55,000; (B) × 85,000; (C) × 100,000; (D) × 115,000.
astrocytes, which in turn are coupled to still others. In contrast, dye-coupling studies of neurons show that only one other neuron or, at most, only a few neurons are dye-coupled (Dudek et al., 1983). Thus, even if one accepts that all neuronal dye-coupling is valid in adult brain slices (see Dudek et al., this issue), the available physiological data suggest that the number of gap junctions between neurons in the adult brain is very small—perhaps only a few per cell. If so, finding and quantifying neuronal gap junctions by conventional thin-section electron microscopy will be very difficult (see Fig. 2).

**Neuronal connexins have yet to be identified**

At the time of writing, no neuronal connexins have been identified unambiguously (but see below). However, immunocytochemical studies had reported abundant Cx32 (Micevych and Abelson, 1991; Nadarajah et al., 1996) and abundant Cx43 (Nadarajah et al., 1996) in neurons, both at the plasma membrane and to membrane cisternae within the cytoplasm (Yamamoto et al., 1989, 1991). Nevertheless, we note that the limits of resolution afforded by light microscopical techniques (0.2 μm in X- and Y-axes; 0.4 μm in the Z-axis) precluded definitive assignment of labeled gap junctions to the surface of any specific cell or cell process in complex nervous tissue. By light microscopy, gap junctions on astrocyte or oligodendrocyte processes smaller than 0.2 μm would appear to be co-planar with the neuronal plasma membrane (see Figure 17 in Rash et al., 1997), and thus, would not be identifiable by LM immunocytochemical methods. The limits of resolution of light microscopy are equally problematic when using in situ hybridization or other techniques that rely on light microscopical approaches for obtaining probes or for localizing labels. In addition, suggestions of abundant Cx43 or Cx32 in neurons have not been supported by our immunogold labeling of spinal cord, hippocampus, and suprachiasmatic nucleus. In those flagged preparations, immunogold-labeled glial gap junctions were found with ease—at least 10 for every one that could be found in the same area and in the same amount of time using conventional search strategies. However, no neuronal gap junctions were found by this flagged gap junction search strategy, and cytoplasmic labeling was not above background. Thus, we conclude that neither Cx43 nor Cx32 occurs in abundance in neurons in adult rat spinal cord, hippocampus, or suprachiasmatic nucleus, but the identity of neuronal connexins has not been determined.††

The flagged gap junction search strategy has proven useful for finding glial gap junctions, but the current lack of identified neuronal connexins temporarily precludes the identification of labeled neuronal gap junctions by freeze-fracture immunogold labeling techniques. Nevertheless, progress in the area of connexin identification in a variety of cell types is now very rapid, with 14 families of mammalian connexins now or soon to be commercially available (Chemicon, Molecular Probes, Zymed and others). Additional connexin probes have been reported by individual laboratories. As candidate neuronal connexins are identified, they will be tested by the SDS-washed, immunogold-labeling method using grid-mapped freeze-fracture techniques.

**CONCLUSIONS**

The combination of grid-mapped freeze-fracture, high-resolution replication, and immunogold-labeling techniques have opened the door to direct visualization and detailed histological mapping of glial gap junctions, as well as high-specificity in situ biochemical identification of their biochemical constituents in intact tissues from the brain and spinal cord. These methods are revealing that different classes of glial cells express different combinations of connexins. For example, heterologous astrocyto-oligodendrocyte couplings employ different connexins in the two cell types, thereby forming heterologous, heterotypic gap junctions—at the minimum, Cx43 and Cx30 in astrocytes, which couple to Cx32 (and perhaps Cx45) in oligodendrocytes.

In spinal cord neurons, gap junctions were shown to be much more common than previously believed. Most neuronal gap junctions in spinal cord were very small, and all were present in mixed synapses, with the average neuron in the spinal cord having approximately 300–400 mixed synapses per cell. Grid-mapped freeze-fracture and immunogold-labeling techniques are now being applied to the analysis of neuronal gap junctions in hippocampus, suprachiasmatic nucleus, and other regions of brain in adult and neonatal rats. These and related techniques may soon provide the answer to the longstanding questions: Which neurons in mammalian CNS are linked by gap junctions, and by what type, how many, and for what purpose?

††After submission of this review, a new family of gap junction connexins was identified in mammalian neurons, and the gene sequence has been published (Condorelli et al., 1998).
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