Published in final edited form as:

Cell Commun Adhes. 2001 ; 8(4-6): 315–320.

Identification of Cells Expressing Cx43, Cx30, Cx26, Cx32 and Cx36 in Gap Junctions of Rat Brain and Spinal Cord

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Abstract

We have identified cells expressing Cx26, Cx30, Cx32, Cx36 and Cx43 in gap junctions of rat central nervous system (CNS) using confocal light microscopic immunocytochemistry and freeze-fracture replica immunogold labeling (FRIL). Confocal microscopy was used to assess general distributions of connexins, whereas the 100-fold higher resolution of FRIL allowed co-localization of several different connexins within individual ultrastructurally-defined gap junction plaques in ultrastructurally and immunologically identified cell types. In >4000 labeled gap junctions found in >370 FRIL replicas of gray matter in adult rats, Cx26, Cx30 and Cx43 were found only in astrocyte gap junctions; Cx32 was only in oligodendrocytes, and Cx36 was only in neurons. Moreover, Cx26, Cx30 and Cx43 were co-localized in most astrocyte gap junctions. Oligodendrocytes shared intercellular gap junctions only with astrocytes, and these heterologous junctions had Cx32 on the oligodendrocyte side and Cx26, Cx30 and Cx43 on the astrocyte side. In 4 and 18 day postnatal rat spinal cord, neuronal gap junctions contained Cx36, whereas Cx26 was present in leptomeningeal gap junctions. Thus, in adult rat CNS, neurons and glia express different connexins, with “permissive” connexin pairing combinations apparently defining separate pathways for neuronal vs. glial gap junctional communication.

Keywords

Astrocyte; connexin; connexon; FRIL; freeze fracture; glia; immunogold; leptomeninges; neuron; oligodendrocyte; pia mater

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Supported by grants from NIH (NS-31027, NS-39040 and NS-38121 to JER; MH-59995 to FED, and from the Canadian Institutes of Health Research to JIN.

INTRODUCTION

Gap junctions are abundant in the mammalian central nervous system (CNS) (Brightman and Reese 1969). However, it is not yet established: 1) whether gap junctions occur between both neurons and glia, with the same connexins expressed in both neurons and glia, or 2) whether neurons share gap junctions only with each other, and if so, whether different connexins are expressed in each cell type. Early studies revealed that glial gap junctions are abundant in mammalian CNS, whereas neuronal gap junctions were reported to be rare, and neuron-to-glial gap junctions were not detected (Brightman and Reese 1969; Sotelo and Korn 1978; Massa and Mugnaini 1982; Wolff et al. 1998). Recently, freeze-fracture revealed abundant “mini” gap junctions in mixed synapses between most neurons throughout adult spinal cord (Rash et al. 1996), where previous studies had found none. Although neuronal gap junctions were more abundant than previously assumed, they nevertheless were outnumbered by glial gap junctions by > 100:1 (Rash et al. 1998).

With the discovery of multiple connexins in CNS (reviewed in Dermietzel and Spray 1993; Nagy and Rash 2000; Dermietzel and Spray 1998; Nagy and Dermietzel 2000) and application of immunocytochemical labeling methods to confocal light microscopy (LM) and thin-section electron microscopy (TEM), several groups proposed that neuronal gap junctions contain Cx26, Cx32 and Cx43 (Micevych and Abelson 1991; Micevych et al. 1996; Nadarajah et al. 1996; Nadarajah et al. 1997; Alvarez-Maubecin et al. 2000). It also was suggested that neuronal (neuron-to-neuron and neuron-to-glia) gap junctions are abundant, constituting 17.9% of all gap junctions in adult and developing cortex (Nadarajah et al. 1996), and 51% of all gap junctions in developing locus coeruleus (Alvarez-Maubecin et al. 2000).

Explanations for these discrepancies in relative numbers of neuronal vs. glial gap junctions and in identification of neuronal coupling partners may derive from the limited resolution of LM and from the low-density of immunogold labels in previous TEM studies (Nadarajah et al. 1996; Alvarez-Maubecin et al. 2000). In contrast to liver, where gap junctions are present in lateral margins of cuboidal cells, CNS gray matter consist of a complex matrix of neurons and neuronal processes ensheathed in astrocyte and oligodendrocyte processes (Figure 1A), with many glial processes smaller than the limits of resolution of light microscopy (ca. $0.3 \times 0.3 \times 0.5 \mu\text{m}$ with red fluorescence and $0.2 \times 0.2 \times 0.4 \mu\text{m}$ with blue fluorescence; Figure 1A, white spot). Thus, in LM when not all cell types are separately visualized, fluorescence images cannot provide unambiguous localization of connexins to any of the three cell types likely to be present near the surface of a neuron labeled with a fluorescent marker. Moreover, TEM localization of Cx32 and Cx26 to putative neuronal gap junctions (Alvarez-Maubecin et al. 2000) is uncertain because labeled membranes did not conform to established criteria for identifying gap junctions (Brightman and Reese 1969; Sloper 1972; Berdan et al. 1987; reviewed in Rash et al. 1998, Nagy et al. 1999), and the level of apparent labeling was not demonstrated to be above nonspecific “background”.

Freeze-fracture replica immunogold labeling (FRIL; Rash and Yasumura 1999; modified from Fujimoto 1995), which overcomes many of the limitations of LM and TEM immunocytochemistry, was chosen to investigate whether five well-characterized CNS connexins (Cx26, Cx30, Cx32, Cx36, and Cx43) are present singly or in combination in ultrastructurally-defined gap junctions in neurons and glia in adult and developing rat CNS. From FRIL data, we also investigated whether astrocytes or oligodendrocytes share gap junctions with neurons.

MATERIALS AND METHODS

Seventeen anti-connexin antibodies were used in this study, including 16 monoclonal and polyclonal antibodies against Cx26, Cx30, Cx32, Cx36 and Cx43 (at least two per connexin), plus one rabbit polyclonal antibody against aquaporin4 (AQP4) (Rash et al. 1998, 2000, 2001; Nagy et al. 2001). Western blots and immunohistochemistry for light microscopy were described previously (Rash et al. 2000, 2001; Nagy et al. 2001). Methods for FRIL immunocytochemistry and TEM, number of adult and early postnatal rats and mice tested, and interpretations of FRIL images are described in previous reports (Rash and Fambrough 1973; Rash and Yasumura 1999; Rash et al. 1997, 2000, 2001; Nagy et al. 2001).

RESULTS AND DISCUSSION

Cx43, Cx30, and Cx26 in Adult Astrocytes

Astrocytes were distinguished from oligodendrocytes and neurons by GFAP filaments in the cytoplasm, high density of IMPs, particularly in E-faces (Rash et al. 1998), and by AQP4 labeling (Figure 1B; modified from Rash et al. 2001). In samples double labeled for Cx43 and AQP4 (Figure 1B and inset), >90% of astrocyte and ependymocyte gap junctions (and >98% of gap junctions with >200 connexons) were immunogold labeled for Cx43 (Rash et al. 1997; Rash et al. 2000; Rash et al. 2001). Likewise, >80% of astrocyte gap junctions were single- or double- labeled for Cx43 and Cx26 (Figure 1C). Cx26 + Cx30 double-labeled astrocyte-to-astrocyte gap junctions were found adjacent to neuronal processes, separated from these processes by less than the limit of resolution of LM (Figure 1D). Such configurations would be particularly problematic for LM. Similarly, in samples double-labeled for Cx43 and Cx30 (Figure 1E), >95% were labeled for at least one of those connexins (Rash et al. 2001), and >80% were labeled for both. Thus, in astrocytes, Cx26, Cx30 and Cx43 are co-localized in >64% of gap junction plaques. Moreover, of > 3500 gap junctions labeled for Cx26, Cx30, and/or Cx43, none (0%) were in the plasma membranes of oligodendrocytes or neurons.

Cx32 in Adult Oligodendrocytes

Of ca 200 immunogold-labeled gap junctions in oligodendrocyte plasma membranes, all were labeled for Cx32 (Figure 1F,G), and none were labeled for Cx26, Cx30, Cx36 or Cx43. In contrast, of >270 immunogold-labeled gap junctions in oligodendrocyte coupling partners (i.e., the cell beneath oligodendrocyte gap junction E-faces, Rash et al. 2001), all were immunogold labeled for Cx43, Cx30 (Figure 1H; Rash et al. 2001) and/or Cx26 (Nagy et al. 2001), but none contained Cx32. Overall, >90% of gap junctions in oligodendrocyte coupling partners were labeled for Cx26, Cx30 and/or Cx43, with the expression of connexins providing further support for the identification of all oligodendrocyte coupling partners as astrocytes (Mugnaini 1986; Rash et al. 1997, 2001).

Cx36 in Adult Neurons

In samples double labeled for Cx36 + Cx43, Cx30 or Cx26, >95% of neuronal gap junctions (>300) were labeled for Cx36 (Figure II), whereas none (0%) were labeled for any of the other connexins tested (Rash et al. 2000; Rash et al. 2001). These data included approximately equal numbers of gap junctions in neuronal plasma membranes (i.e., in neuronal P-faces) and in neuronal coupling partners (i.e., the cell beneath neuronal gap junction E-faces). Thus, in ca. 200 examples, identified neurons were coupled only to other neurons. Moreover, no identified neuron or neuron coupling partner had gap junction plaques containing immunogold-labeled Cx26, Cx30, Cx32, or Cx43. Thus, in ten regions of adult rat retina, brain and spinal cord examined by “grid-mapped” freeze-fracture (Rash et al. 1997) or by FRIL (Rash et al. 2000, 2001), neurons shared gap junctions only with neurons and not detectably with any glial cells.

Cx36 in Developing Neurons; Cx26 in Developing Astrocytes and Leptomeningeal cells

In samples from 4-day and 18-day postnatal rat spinal cord that had been double-labeled for Cx26 + Cx36, >20 gap junctions in leptomeningeal cells (Figure 1J) were immunogold-labeled for Cx26 (Nagy et al. 2001). In contrast, neuronal gap junctions (6 of 6) were immunogold labeled for Cx36 (Figure 1K), whereas none were labeled for Cx26. Although it may be concluded that gap junctions in developing neurons contain Cx36, and that astrocyte junctions contain Cx43 and Cx26, additional samples labeled for Cx26, Cx30, Cx32, and Cx43 must be examined by FRIL to determine if any of these latter connexins are present in early postnatal neurons.

CONCLUSIONS

By FRIL, Cx32 was found only in gap junctions of oligodendrocyte plasma membranes; Cx30, Cx43 and Cx26 were in astrocyte junctions; Cx43 (but not Cx30) was in ependymocyte junctions; Cx43 and Cx26 (but not Cx36) were in leptomeningeal junctions; and Cx36 (but not Cx26, Cx30, Cx32 or Cx43) was in neuronal gap junctions (Figure 2). Intercellular gap junctions in oligodendrocytes were shared only with astrocytes, with these heterologous gap junctions also being heterotypic, having Cx32 (plus as yet undetermined connexins) in the oligodendrocyte side linking to Cx26, Cx30 and Cx43 in the astrocyte side. The lack of intercellular gap junctions linking oligodendrocytes supports the proposal that oligodendrocytes are isolated from other oligodendrocytes except through astrocyte “intermediaries” (Mugnaini 1986; Rash et al. 1997). Likewise, in >4500 gap junctions in single- and double-labeled FRIL samples, no evidence was found for Cx26, Cx30, Cx32 or Cx43 in adult neuronal gap junctions. Finally, in >300 examples of Cx36-labeled neuronal gap junctions and >4000 labeled glial gap junctions, neurons shared gap junctions only with other neurons and not with glial cells. Thus, gap junctions in the different cell types in the CNS contain distinctive sets of connexins, and the coupling specificities of these connexins appear to define separate pathways for neuronal vs. glial gap junctional communication.

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Fig. 1.

A) Thin section electron micrograph of normal adult rat spinal cord. Four neuronal processes (N) surround three astrocyte processes (A), two of which are linked by a gap junction (arrow and inset). Overlapping the plasma membranes of two neurons and two astrocytes is a white spot corresponding to the limit of resolution of light microscopy (0.2 μm). By LM, photons arising from any of the six plasma membranes of the four cells within the spot would appear to arise from a fluorescently-labeled neuron. **B)** Astrocyte plasma membranes after double labeling for Cx43 and AQP4. The AQP4 array (inset) is labeled by a 10 nm gold bead, whereas the two gap junctions (arrows) are labeled for Cx43 by > 150 20 nm gold beads. **C)** Astrocyte gap junction after double labeling for Cx26 and Cx43. Arrow points to E-face image of an

AQP4 square array, **D**) FRIL image of two neurons (N1 and N2) with two intervening astrocyte processes (A1 and A2) linked by a gap junction that was labeled for Cx26 (10 nm gold) and Cx30 (20 nm gold). Inscribed box represents the volume corresponding to the limit of resolution of LM ($0.2 \times 0.2 \times 0.4 \mu\text{m}$). By conventional LM immunofluorescence, this image would appear as two neuronal processes linked by a gap junction containing Cx26, **E**) Astrocyte-to-astrocyte gap junction double labeled for Cx30 and Cx43. **F**) Gap junction in oligodendrocyte soma labeled for Cx32 by thirty 10 nm gold beads. A small gap junction at lower left is not labeled. **G**) Gap junction in outer layer of oligodendrocyte myelin after labeling for Cx32 (10 nm gold beads). White arrow = tight junction strands. **H**) Four gap junctions in oligodendrocyte E-face, with connexins in the oligodendrocyte coupling partners labeled for Cx30 and Cx43, identifying the coupling partners of astrocytes. **I**) Gap junction in neuron of adult rat retina labeled for Cx36 (white arrows). In retina, gap junctions often consist of distinctive “strings” of connexons. Black arrow = unlabeled gap junction. Other retinal gap junctions are of the conventional plaque type (Rash et al, 2001) . **J**) Gap junction in leptomeningeal cell of 4-day rat spinal cord after double-labeling for Cx26 (four 20-nm gold beads) and Cx36 (10-nm gold beads; none present). TJ = tight junction. **K**) Two gap junctions in neuron of ventral horn of 18-day rat after labeling for Cx36 (10 nm gold beads). DS = dendritic spine. In all micrographs, the calibration bars are $0.1 \mu\text{m}$, unless otherwise indicated.

Cell Coupling Partners and their Connexins, as Identified by FRIL

FIG. 2.

Drawing of cell types in mammalian CNS, with number of FRIL-labeled gap junctions at each intercellular location where gap junctions have been found and the number of gap junctions found at each site.