CONNEXIN32 LOCALIZATION IN OLIGODENDROCYTES AND ASSOCIATION WITH MYELINATED FIBERS IN MOUSE AND RAT BRAIN

BY

JIAN LI

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

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II. ABSTRACT

The distribution and cellular localization of connexin32 (Cx32) in brain and spinal cord of mouse and rat was investigated by light (LM) and electron microscope (EM) immunohistochemistry using several different antibodies against Cx32. By double immunofluorescence staining for the oligodendrocyte markers cyclic nucleotide phosphodiesterase (CNPase) or Rip, Cx32 was consistently found in oligodendrocyte cell bodies and proximal processes. Cx32-immunoreactivity was also clearly visualized along CNPase- and Rip-positive myelinated fibers. Both immunopositive cells and fibers were heterogeneously distributed and were often more intensely labeled when dispersed in, or associated with, regions of gray matter than when concentrated in major white matter tracts. Labeling of myelin sheaths along fibers was restricted to subpopulations of myelinated axons. In the cerebellar cortex, for example, it was selectively localized to sheaths around Purkinje cell axons. Punctate staining, distinct from that corresponding to cells or fibers, was evident in olfactory bulb and hippocampus. By EM, oligodendrocytes exhibited cytoplasmic labeling associated with rough endoplasmic reticulum and Golgi apparatus. Their processes were intermittently stained, most intensely when surrounding myelinated fibers and occasionally in paranodal loops. Cx32-immunoreactive gap junctions with symmetric labeling (staining on both junctional membranes) were observed between oligodendrocytic somata and processes as well as between presumptive oligodendrocytic processes. Unidentifiable elements forming asymmetrically labeled gap junctions (staining on only one side of junctional membranes) were less frequently encountered. Western blot analysis confirmed anti-Cx32 antibody detection of Cx32 in whole brain homogenates and an enrichment of the protein in isolated myelin fractions. These results are consistent with earlier ultrastructural studies indicating the occurrence of inter-oligodendrocytic gap junctions, indicate that these may be more prevalent than previously thought and suggest a specialized role of gap junctions composed of Cx32 along myelinated fibers belonging to subpopulations of neurons.

III. INTRODUCTION

III.1. Gap junctions

Gap junctions are specialized plasma membrane regions that contain collections of transmembrane channels which provide a low resistance pathway directly linking adjacent cells. In mammals they are found in virtually every cell type except mature skeletal muscle, spermatozoa and erythrocytes (Dermietzel and Spray, 1993). These channels provide for direct cell-to-cell transfer of ions and small molecules without exposure to the extracellular space (Bennett, 1966; Loewenstein, 1966). In thin-section electron microscopy, the gap junction is characterized by a septalaminar profile comprised of the two apposing plasma membranes separated by a 2 nm "gap" (Robertson, 1963; Revel and Karnovsky, 1967) that can be penetrated by extracellular dense tracers such as lanthanum and ruthenium red. Freeze fracture replicas showed that the structure is a plaque-shaped, differentiated region of the plasma membrane containing a dense array of intramembrane particles (connexons) on the P-fracture face and a complementary array of depressions or pits on the E-fracture face (Goodenough and Revel, 1970). Makowski and colleagues used X-ray diffraction and electron microscopy to develop a low-resolution (25Å) structural model (Makowski et al., 1977; Makowski, 1988). The model shows that a gap junction plaque is composed of a few thousands of channels. Each channel consists of a hexameric structure (connexon) of six integral membrane subunits (connexins) that surround a central pore. Two apposed connexons join in mirror symmetry to form an axial channel connecting the cytoplasm of gap junctionally coupled cells.

Studies using ions or dyes of known molecular size and charge have demonstrated that the intercellular channels between mammalian cells are about 1.5 nm in diameter, with a molecular size exclusion of about 1000 Da (Flagg-Newton and Loewenstein, 1979; Brink and Dewey, 1980; Imanaga et al., 1987). Patch clamp studies have allowed determination of the conductance of single gap junctional channels, which differs in different preparations and ranges from 30 to 160 pS (Veenstra and DeHann, 1986; Burt and Spray, 1988a; Moore et al., 1991). The aqueous channel appears to contain negative charges, which may account for a mild ion-selective preference for cations over anions (Neyton and Trautman, 1985; Brink and Fan, 1989), and excludes passage of macromolecules such as proteins or polysaccharides, but allows the passage of inorganic ions, amino acids, vitamins, low molecular weight trophic factors and other regulatory molecules (Loewenstein,1981). Regulation of gap junctions may occur by connexin production, junction assembly or modulation of channel conductance, and a number of agents or treatments that alter gap junctional communication have been identified including pH, Ca^{2+}, voltage (transjunctional and transmembrane), connexin phosphorylation, neurotransmitters, growth factors, lipophiles and tumor promoters (Piccolino et al., 1982; Spray et al., 1982; Saez et al., 1986; Burt, 1987; Burt and Spray, 1988b; Chanson et al., 1988; Maldonado et al., 1988; Veenatra, 1990; White et al., 1990).

Gap junction mediated intercellular communication has been implicated in many cellular functions. Due to the large size of the intercellular channels formed by connexon pairs, the exchange of molecules between cells is nonspecific, and includes the entire pool of ions and small metabolites in each cell (Simpson et al., 1977; Goodenough et al., 1980). This form of intercellular communication is ideally suited for the role of intercellular buffering of

cytoplasmic ions (Ledbetter and Lubin, 1979). It also allows action potential propagation by passage of current carrying ions in electrically excitable tissues including myocardium (Barr et al., 1965) and smooth muscle (Dewey and Barr, 1962) and at electrotonic synapses in the nervous system (Furshpan and Potter, 1959). In addition, sharing of low-molecular-weight substrates can bypass the deleterious effects of somatic cell mutations of various enzymes (Cox et al., 1970). Metabolic cooperation of cells through gap junctions likely maintains homeostasis in avascular systems such as ovarian follicles (Gilula et al., 1978) and passage of second messengers through gap junctions may regulate glandular and hormonal secretion (Meda et al., 1990). Involvement of gap junction-mediated intercellular communication has also been suggested in growth control, embryonic development and cellular differentiation (Atkinson et al., 1981; Warner et al., 1984; Azarnia et al., 1988).

III.2. Connexins

Gap junctions are formed by members of a family of related proteins for which the generic name *connexin* has been suggested (Beyer et al., 1987). Current nomenclature appends the molecular mass predicted by cloned DNA sequences to the family name connexin (Cx). Each connexin is thought to consist of four transmembrane segments, three cytoplasmic domains, and two extracellular loops (Gilula, 1987; Goodenough et al., 1988). Comparison of the primary sequences of all the known connexins reveals that the two predicted extracellular domains are the most conserved regions, each containing three invariant cysteines. The transmembrane domains are somewhat less well conserved. The cytoplasmic domains, with

the exception of the short N-terminal region, differ markedly between connexins both in sequence and in length (Beyer et al., 1990).

So far, thirteen connexins have been identified in rodents (White et al., 1995a) and homologous DNAs with about 59% sequence homology (Bennett et al., 1991) have been isolated in several other vertebrate species (Harris et al., 1981; Gorin et al., 1984; Kistler et al., 1985,1988; Miller and Goodenough, 1986; Ebihara et al., 1989; Beyer, 1990; Willecke et al., 1990; Kanter et al., 1992; Reed et al., 1993; Rup et al., 1993). The first connexin cDNA was isolated from a rat liver expression library using an antibody raised against isolated junctions (Paul, 1986). Kumar and Gilula (1986) cloned a cDNA for its counterpart by hybridization screening with an amino-terminal oligonucleotide. Both cDNA encode a polypeptide of 32 KDa, termed connexin32 (Cx32). By screening a rat myocardial library with the Cx32 cDNA at reduced stringency, a cDNA has been cloned which codes for a homologous polypeptide of 43 KDa, termed connexin43 (Cx43), which shows 43% overall homology with Cx32, but contains certain regions with many more identical amino acids (Beyer et al., 1987). Using an oligonucleotide probe based on the partial sequence of a second presumptive gap junction protein isolated from hepatocytes, Zhang and Nicholson (1989) found a cDNA encoding an additional connexin. This cDNA codes for a 26 KDa protein and termed connexin26 (Cx26). Hoh et al. (1991) cloned connexin31 from rat genomic DNA and demonstrated that it is most abundantly expressed in placenta. Using low stringency hybridization and PCR amplification with degenerate oligonucleotides, Haefliger et al. (1992) identified four members of the rat connexin gene family, namely are connexin40 (Cx40), connexin37 (Cx37), connexin33 (Cx33), and connexin31.1 (Cx31.1). Paul et al. (1991) cloned

a new member of the connexin family from rat lens cDNA, with a predicted molecular mass of 46 KDa, called connexin46 (Cx46). Genomic cloning and PCR amplification of genomic DNA has led to the identification of further expressed connexins, including connexin30.3 (Cx30.3) (Hennemann et al., 1992a) and connexin45 (Cx45) (Hennemann et al., 1992b). Connexin50 (Cx50) was cloned by White et al. (1992) and Cx50 mRNA was detected only in the mouse lens, among the 12 organs tested. Recently, a thirteenth member of the connexin family, connexin30 (Cx30), has been cloned (White et al., 1995a). The connexins are a multigene family with a complex tissue distribution. It is known that nearly all connexins are expressed in multiple locations (Beyer, 1993). More than one connexin are often present in the same cell type and can even be in the same gap junctional plaque (Nicholson et al., 1987; Traub et al., 1989; Paul et al., 1991). In the lens, there are only two differentiated cell types which express three connexins, making it one of the least complicated systems in which to study intercellular communication (Goodenough, 1992). In contrast, skin is a more representative organ, utilizing at least five different connexins in a complex and developmentally regulated pattern of expression (Risek et al., 1992; Goliger and Paul, 1994).

III.3. Distribution of connexins in central nervous system

Gap junction proteins are expressed by specific cell populations in the brain (Dermietzel and Spray, 1993) and the expression of different connexins changes in developing brain (Dermietzel et al., 1989). Knowledge of the cell-specific distribution of connexins in brain has come from studies using *in situ* immunolabelling techniques and primary cultures of neuronal and non-neuronal cells. To date five connexins have been detected in adult and developing rat

CNS with probes for Cx43, Cx32 and Cx26 (Zhang and Nicholson, 1989; Naus et al., 1990; Dupont et al., 1991). The mRNA of the recently cloned Cx37 and Cx45 have also been detected in whole brain homogenates (Willecke et al., 1991; Hennemann et al., 1992b). In adult brain, the predominant connexin is Cx43, which is abundant in astrocytes (Dermietzel et al., 1989; Yamamoto et al., 1990a; Micevych and Abelson, 1991) and is also expressed in leptomeninges, endothelial cells and ependyma (Dermietzel et al., 1989). The second class of macroglia, the oligodendrocytes, appear to express a different gap junction protein, Cx32, although to a lower extent *in situ* than the level of Cx43 expression exhibited by astrocytes (Dermietzel et al., 1989). The nature of neuronal connexin(s) in adult brain remains unclear, although Cx32 immunoreactivity was found in certain subpopulation neurons (Nagy et al., 1988; Dermietzel et al., 1989; Shiosaka et al., 1989; Yamamoto et al., 1989) and Cx32 mRNA was detected in discrete cell groups of the grey matter that appeared to be neurons by *in situ* hybridization histochemistry (ISHH) (Micevych and Abelson, 1991). In addition, candidates for neuronal connexin(s) also include Cx37 and Cx45 detected in CNS by molecular techniques (Willecke et al., 1991; Hennemann et al., 1992b). In contrast to the preferred parenchymal expression of Cx32 and Cx43, Cx26 does not reach high levels in adult brain, but rather is confined to non-neuronal cells such as leptomeninges (Spray et al., 1991a; Miragall et al., 1992), ependyma (Dermietzel et al., 1989) and pinealocytes (Saez et al., 1991). For Cx37, with levels four times higher in the immature brain as compared to the adult (Willecke et al., 1991), and Cx45 as well, cell-type expression has not yet been established.

The amount and pattern of connexin expression changes during brain development, particularly with regard to the complementary up- and downregulation of Cx26 and Cx32 and

the postpartum peak of Cx43 expression (Dermietzel et al., 1989). By Northern and Western blot analysis, Belliveau et al. (1991) reported that Cx32 protein and mRNA appear during the second postnatal week in developing rat brain, whereas Cx43 is detectable at embryonic day 18 and increases or remains at relatively constant levels during postnatal development. Dermietzel et al. (1989) found that the Cx32 was detectable by the second postpartum week and increased gradually from postnatal day 14 to adulthood, while levels of Cx26 are high at E18 and gradually decreased by postnatal day 6. Cx43 is abundant and widely distributed in the embryonic as well as the adult brain with no detectable developmental change after birth (Dermietzel et al., 1989). Cx32 mRNA was found to be localized in neurons, glial cells and ependymal cells in various regions of rats at 2 days age by ISHH (Matsumoto et al., 1992).

III.4. Gap junctions between neurons

In the mammalian CNS, conventional or freeze-fracture electron microscopy (EM), dye coupling and electrophysiological techniques have revealed gap junctions between neurons in different brain regions including hypothalamus (Andrew et al., 1981; Cobbett and Hatton, 1984), inferior olivary nucleus (Sotelo et al., 1974; Benardo and Foster, 1986), hippocampus (Schmalbruch and Jahnsen, 1981; Andrew et al., 1982; Shiosaka et al., 1989; Yamamoto et al., 1989), main olfactory bulb (Reyher et al., 1991), retina (Vaney, 1994) and cerebellar cortex (Sotelo and Llinas, 1972). In addition, the occurrence of gap junctions in the cortex of higher vertebrates has been known for some time (Sloper, 1972; Smith and Moskovitz, 1979) and Micevych and Abelson (1991) have shown that significant numbers of cells in cortex, which appear to be neurons, contain Cx32 mRNA. Immunohistochemical and *in situ*

hybridization studies have been conducted to determine connexin expression in neurons. By double immunostaining using neuron-specific enolase (nEn) or neurofilament protein antibodies as markers for neurons, Dermietzel et al. (1989) observed that 20% of the cells in the basal ganglia and the thalamus that were positive for Cx32 also exhibited nEn immunoreactivity, whereas neurons elsewhere, such as those in the pyramidal cell layer of the hippocampal formation where neurons have been reported to be electrically coupling, were devoid of Cx32 immunoreactivity. Reyher et al. (1991) conducted light and transmission electron microscopy (TEM) immunohistochemical studies on rat olfactory bulb using affinity-purified antibodies against the Cx32 or Cx43 carboxyl tail fragment and found that gap junction protein (GJP)-like immunoreactivity (ir) was distributed throughout all layers of the rat main olfactory bulb. Both neuronal and glial elements were connexin-ir and both antibodies gave a similar pattern of staining, but no indication was given as to which of the two antibodies were used to label the gap junctions in the micrographs presented. An affinity-purified anti-Cx32 antibody has been used to determine the distribution of connexin-ir and ultrastructural localization of connexins in the rat hippocampus. The antibody labeled neuronal gap junctions, glial gap junctions, and a class of varicose axons whose identity has not yet been determined (Nagy et al., 1988; Shiosaka et al., 1989; Yamamoto et al., 1989). This antibody also labeled intracellular structures that in motoneurons of rat and cat were identified as subsurface cisterns (SSC), which suggests it might recognize other known or yet to be identified members of the connexin family (Yamamoto et al., 1990b). Micevych and Abelson (1991) reported that Cx32 mRNA was prominently associated with neuronal cellular profiles in grey matter, for example, layer 2 of the neocortex, islands of Calleja, pyramidal cell layer of

the hippocampus, olfactory tubercle, lateral thalamic nuclei and the Purkinje cell layer of the cerebellar cortex. According to another ISHH result by Wackym et al. (1991), Cx32 and Cx43 mRNA were detected in unspecified cells in rat vestibular nuclei. In separate LM and EM work on Cx43, the Cx43-ir neuronal gap junctions were never observed (Yamamoto et al., 1990a), whether Cx43 contributes substantially to the connexin complement of neurons *in situ* has been impossible to rule out because of the high degree of expression of Cx43 in surrounding glia (Dermietzel and Spray, 1993). Thus, neuronal connexin(s) in adult brain still remains to be identified. It may be Cx32 or a novel related protein that forms neuronal gap junction. In addition, the possibility that recently identified Cx37 and Cx45 are expressed by certain subpopulations of neurons can not be ruled out.

Little is known about the mechanisms that regulate neuronal gap junctional communication, but studies have identified some factors that may contribute to this process. The role of dopamine in modulating junctional coupling between horizontal cells in retina has been well characterized in lower vertebrates (Dowling, 1991). Hampson et al. (1992) reported reduction of dye coupling between amacrine cells in mammalian retina by dopamine, which appeared to be mediated by a D_1-like receptor that stimulates cAMP production. Cepeda et al. (1989) produced lesions of rat substantia nigra (electrolytically or by 6-OHDA injection) which markedly increased the incidence of dye-coupling in the neostriatum, providing evidence of dopaminergic modulation of gap junction permeability between neurons in brain. Dye coupling and Cx26 expression were observed between cultured pinealocytes from adult rats, and both were increased by norepinephrine (NE) (Saez et al., 1991). The induction in coupling between pinealocytes by NE is likely to be mediated by synthesis of connexin since

the NE effect is paralleled by an increase in detectable Cx26 and it is prevented by blockade of mRNA or protein synthesis, but not through a gating process (Saez et al., 1991) such as direct phosphorylation of formed junctions as demonstrated by Saez et al. (1986) for hepatic Cx32. Membrane permeant cAMP derivatives have also been shown to increase gap junctional conductance between cultured sympathetic neurons dissociated from superior cervical ganglia of neonatal rats (Kessler et al., 1984). Promotion of coupling was also achieved by insulin (Kessler et al., 1984). The expression level of Cx32 mRNA and the number and diameter of the junctional plaques in rat androgen-sensitive motoneurons of the spinal nucleus of the buibocavernosus (SNB) were reduced after removal of androgen by castration which indicate that androgen regulates the degree of coupling between these cells (Matsumoto et al., 1988,1991). Thus, it appears that gap junctional communication between different subpopulation of neurons is under different regulatory mechanism.

Gap junctions between neurons are generally regarded to mediate synchrony among active cells or to relay signals from pre- to postsynaptic elements (Bennett, 1977; Llinas, 1985). For example, it has been suggested that in the case of oxcytocin cells, electrical coupling may serve to coordinate their activation during the periodic high frequency bursts of action potentials that they exhibit in response to suckling and thereby cause sufficient oxcytocin release to evoke milk ejection (Hatton, 1990). Another role for gap junctions between neurons is second messenger exchange. The second messengers Ca^{2+}, cAMP and IP_3 all diffuse between coupled cells, allowing the possibility that levels of these second messengers may be co-regulated in pre- and postsynaptic elements which could lead to modulation of presynaptic neurotransmitter release by postsynaptic second messenger molecules (Dermietzel and Spray,

1993). This mechanism was proposed by Yang et al. (1990) who reported that at mixed synapses between sensory afferents and an identified reticulospinal neuron (Mauthner cell) in gold fish, the electrotonic coupling potential as well as the chemical mediated excitatory postsynaptic potential can be potentiated for a prolonged time period using a stimulation paradigm similar to that which produces long-term potentiation in hippocampus.

The retina, as a part of the central nervous system, owing to its easy accessibility, presents a model system for studying the roles that gap junctions may play in neural networks. Horizontal cells are second order neurons in the outer part of the inner nuclear layer that are involved in the generation of the antagonistic surround of the receptive fields of bipolar and ganglion cells. Coupling among horizontal cells is believed to enlarge the sizes of receptive fields, effectively increasing convergence of the photoreceptor input onto this cell layer (Negishi et al., 1985). Dopamine has been reported to uncouple these cells by regulating the activity of a cyclic AMP-dependent protein kinase (DeVries and Schwartz, 1989), thereby reducing the size of receptive fields. Effects of dopamine are both acute and long-term and may account for changes in acuity in dark adaptation when dopamine levels in the retina are highest (Lasater and Dowling, 1985; DeVries and Schwartz, 1989; Hampson et al., 1992). Extensive neuronal coupling in developing rat neocortex has been reported by Conners et al. (1983) and the high incidence of coupling during development has led to the hypothesis that coupling mediates intercellular communication responsible for differentiation and growth (Bennett et al., 1981; Caveney, 1985). To investigate cellular interactions during early neocortical development, Lo Turco and Kriegstein (1991) made whole-cell patch clamp recordings from neuroblasts in the ventricular zone of fetal rats. They found that neuroblasts

are physiologically coupled by gap junctions into clusters within the ventricular zone during early corticogenesis and the number of cells within the clusters decreases as neuronal migration out of the ventricular zone progresses. Apparently, coupled neuronal compartments occur at the earliest stage of corticogenesis to allow direct cell-to-cell interaction and the authors speculated that neuroblast clusters could form the basis of columnar organization in the mature neocortex, and communication between cells within a cluster could determine the layer-specific fates of cells within a cortical column. Optical recording of brain slices labeled with the fluorescent calcium indicator fura-2 also revealed that the neonatal rat cortex was partitioned into distinct domains of spontaneously co-active neurons coupled by gap junctions, which may provide a mechanism by which groups of neurons exchange developmentally relevant signals and thereby contribute to the differentiation of functionally mature circuits (Yuste et al., 1992). This is consistent with the current concept that the developing brain requires a particular complement of connexins, the functional properties of which may be important for epigenetic events that determine the later fate of the neuroepithelial derivatives (Dermietzel and Spray, 1993).

III.5. Gap junctions between astrocytes

Astrocytes form large networks coupled by gap junctions (Brightman and Reese, 1969; Massa and Mugnaini, 1982; Yamamoto et al., 1990c). Using immunocytochemical, molecular biological and physiological techniques, the properties of astroglial gap junctions and the protein that constitutes the channels were characterized by Dermietzel et al. (1991) who observed that the predominant connexin in cultured astrocytes is Cx43. These astrocytes

expressed Cx43 mRNA without detectable levels of the mRNA encoding Cx32 or Cx26. A site-specific antibody against Cx43 was used to determine immunohistochemically the cellular localization of this protein in rat brain and the results suggested that Cx43 is one of the major gap junctional proteins utilized for junctional coupling between astrocytes (Yamamoto et al., 1990c). It has been found that Cx43 is widely distributed in rat brain and the levels of gap junctions between astrocytes is regionally heterogeneous (Yamamoto et al., 1990a; Micevych and Abelson, 1991; Batter et al., 1992). Cx43 mRNA was reported to be expressed ubiquitously in astrocytes of rat CNS and also exhibits regional heterogeneity (Micevych and Abelson, 1991). The densities of Cx43-immunoreactivity is highly uneven among brain regions which could be attributed to differences in the amounts of Cx43 expressed (Yamamoto et al., 1990a). At the LM level, Cx43 immunostaining consisted of small puncta and numerous annular profiles (Yamamoto et al., 1990a). Astrocytic end-feet around blood vessels were marked by a honeycomb meshwork composed of puncta with a larger diameter than puncta in the neuropil. Dramatic differences in labeling intensity frequently delineated anatomical boundaries between adjacent nuclei (Yamamoto et al., 1990a). By EM, gap junctions of fibrous and protoplasmic astrocytes were intensely stained and these were considered to correspond to puncta seen by LM. In some brain areas, astrocytic processes commonly gave rise to immunoreactive lamellae that partially ensheathed neuronal cell bodies, axon terminals, dendrites and synaptic glomeruli. Such lamellae were considered to correspond to immunoreactive annular profiles seen by LM (Yamamoto et al., 1990a).

Comparisons of results on Cx43 mRNA levels presented by Micevych and Abelson (1991) with illustrations of Cx43-immunolabelling densities presented by Yamamoto et al. (1990a)

reveals some good correspondence and also some striking differences, which suggest that rates of Cx43 gene transcription may be related to rates of translation into Cx43 protein in some brain regions but not others and point to regulatory control at various cellular levels (Nagy and Dermietzel, 1995). Biochemical studies indicate that both Cx43 protein and mRNA are detectable at late embryonic and early postnatal stages in rat brain and the levels of these increase markedly at one or two weeks after birth (Belliveau et al., 1991). Miragall et al. (1992) reported that the levels of Cx43 expression remained low during early embryonic development (as early as embryonic day 9) and gradually increased to reach the highest levels during postnatal stages and adulthood. Dermietzel et al. (1989) have done quantitative studies on Cx43 in developing rat brain and showed that Cx43 was expressed at embryonic day 12 and was abundant and widely distributed in the embryonic as well as the adult brain. The emergence of Cx43-ir structures and the regional patterns of Cx43 expression during postnatal maturation of the rat brain has been immunochemically documented by Yamamoto et al. (1992). On postnatal day 1, Cx43-ir stellate processes were found to be densely stained in rat brain, but the adult staining pattern was reached by postnatal day 10. Most brain regions displayed a characteristic sequence of transient immunoreactive profiles that ultimately gave rise to the uneven distribution of the protein seen in adults. Cx43 staining first appeared in radial glial cells and was then mainly found in astrocytic processes, in which staining changed from whole process labeling to an exclusive punctate pattern. Binmoller and Muller (1992) were not able to demonstrate Lucifer Yellow spread in astrocytes of the rat visual cortex slice before postnatal day 11, so it was proposed that Cx43 may be produced in radial glial cells in

preparation for the formation of gap junctions between mature astrocytes (Yamamoto et al. 1992).

Coupling between astrocytes may be regulated at the transcriptional or translational level involving connexin production, at the post-translational level involving protein trafficking and gap junction assembly or at the gap junction channel level involving short-term modulation of conductance state (Nagy and Dermietzel, 1995). Burt and Spray (1988b) reported that conductance of cardiac myocyte gap junctions which, like those between astrocytes, are composed of Cx43 is enhanced by agents that elevate intracellular cAMP and is decreased by agents that elevate intracellular cGMP. Based on evidence that isolated heart Cx43, like the liver Cx32, is phosphorylated by cAMP-dependent kinase (Saez et al., 1986; Pressler and Hathway, 1987) and the amino acid sequences of the C-terminal domains of junction proteins from both tissues contain probable sites for phosphorylation by kinase A (Paul, 1986; Beyer et al., 1987), the authors proposed that the mechanism by which increased conductance occurs may involve phosphorylation of Cx43 and conductance of the channel may be regulated by cGMP-dependent phosphorylation of the protein. Alternatively, cGMP may regulate a phosphodiesterase that limits the availability of cAMP-dependent protein kinase (Burt and Spray, 1988b). In cultured striatal astrocytes, the permeability of gap junctions was reduced by norepinephrine (NE) via activation of α-adrenergic receptor sites and a close relationship was found between the extent of phospholipase C (PLC) activation and the magnitude of the uncoupling process, which suggests that biochemical events linked to PLC stimulation are likely involved in the NE-induced uncoupling (Giaume et al., 1991). In addition, in the presence of a cAMP phosphodiesterase inhibitor, the stimulation of β-adrenergic receptors by

isoproterenol led to a large increase in cAMP accumulation correlated with increased dye coupling which suggests that junctional permeability could also be controlled by a cAMP-dependent mechanism (Giaume et al., 1991). Homogenates of the brain revealed two forms of Cx43: one dephosphorylated form migrating as a 41 KDa, and a second, phosphorylated form of about 43 kDa (Nagy et al., 1992). It has been suggested that the brain contains a phosphatase that may be involved in the dephosphorylation of Cx43 to Cx41 and therefore in the regulation of intercellular communication (Hossain et al., 1992). Direct evidence for an induction of astroglial gap junctions by neuronal stimuli has been shown by Rohlmann et al. (1993). Following facial nerve transection in adult rats, immunostaining of astrocytic gap junctions changed in the corresponding, ipsilateral motor nucleus of the brainstem. Enhanced immunostaining was confined to astrocytes surrounding the lesioned motoneurons, whereas on the control side staining remained faint , as in intact rats, which indicates that a change in the potential of astrocytic intercellular coupling via gap junctions is among the most rapid responses to axotomy of neighboring motor neurons. Gap junction conductance can be modulated by cellular kinases such as cAMP-dependent protein kinase and protein kinase C (Stagg and Fletcher, 1990). Astrocytes possess a variety of neurotransmitter and neuropeptide receptors that ultimately result in the activation of these kinases (Murphy and Pearce, 1987; Bevan, 1990). Thus, it is possible that neurally released substances dynamically regulate the connectivity of the glial signaling pathway (Finkbeiner, 1992). In support, others have shown that selective neural stimulation produces astrocytic Ca^{2+} waves (Dani et al., 1992) and that astrocyte coupling can be decreased or increased by activation of α- or β-adrenergic receptor subtypes, respectively (Giaume et al., 1991).

During neuronal activity, K^+ concentrations increase in the relatively small extracellular space (Orkand et al., 1966). A sufficient increase in K^+ could cause neuronal depolarization and be detrimental to continued neuronal signaling. It was proposed that astrocytes regulate K^+, since their K^+-selective membrane act as K^+ electrodes, responding with depolarization to small increases in extracellular K^+ (Orkand et al., 1966). The glial depolarization resulting from a local increase in extracellular K^+ would produce a spatial gradient of membrane potential along a glial syncytium that would drive a "spatial buffer" current, which would move K^+ through the glial cell syncytium to a distal region. It has been assumed that astrocytic gap junctions would most likely provide a direct pathway to the site of K^+ disposal into the perivascular compartments (Gardner-Medwin et al., 1981; Newman, 1986), and that the coupling between astrocytes would increase the volume of the buffer sink, so that if a neuron in proximity to a glial cell was particularly active, the astrocytic compartment would share the load (Kettenmann and Ransom, 1988). Yamamoto et al. (1990a) suggested that requirements for intercellular metabolic cooperation via gap junctions between astrocytes may be markedly different among brain regions since heterogeneous expression of Cx43 in rat brain has been observed. Based on the accumulating data that connexin distribution in brain glial cells is regulated regionally and developmentally, the extension of glial networks is probably more restricted and local, its size depending on several regulatory factors, such as anatomical borders between and within nuclei, developmental stages and short- or long-term regulation of junctional permeability (Giaume and Venance, 1995). Cultured hippocampal astrocytes have been reported to respond to glutamate with a prompt, oscillatory elevation of cytoplasmic free calcium which frequently propagate as waves between adjacent astrocytes (Cornell-Bell et al.,

1990) and Dani et al. (1992) observed similar Ca^{2+} waves propagating within and between astrocytes in response to glutamatergic neuronal afferents firing in cultured hippocampal slices. Studies of qualitative and quantitative Ca^{2+} propagation features by Finkbeiner (1992) suggest that astrocytic Ca^{2+} waves are mediated by an intracellular signal that crosses intercellular junctions. Potential mediators of this signal include IP_3, some other IP_3 metabolite, or the Ca^{2+} ion itself (Cornell-Bell et al., 1990). The critical role of astrocytic gap junctions in wave propagation has implications for a putative glial long-range signaling system and it has been proposed that neurally released substances possibly regulate the connectivity of the glial signaling pathway, thereby regulating glial K^+ buffering capacity (Finkbeiner, 1992). Furthermore, neuronally induced astrocytic Ca^{2+} signals may feed back to influence neuronal excitability or regulate synaptic transmission. Such effects might be mediated through changes in extracellular ion concentrations (effects on extracellular K^+ and Ca^{2+} are most likely to be of physiological importance), or through changes in neurotransmitter metabolism (Dani et al., 1992). Three hypothetical models involving changes in intracellular Ca^{2+} and secondary changes in extracellular potassium, arachidonic acid or a metabolite, or calcium have been suggested by van den Pol et al. (1992) which could underlie glial modulation of neural activity.

III.6. Gap junctions between oligodendrocytes

There is evidence for gap junction formation between oligodendrocytes and heterologous oligodendrocyte/astrocyte gap junctional coupling in vitro as well as in vivo (Sotelo and Angaut, 1973; Massa and Mugnaini, 1982; Sandri et al., 1982; Kettenmann et al., 1983;

Kettenmann and Ransom, 1988; Ransom and Kettenmann,1990; Blankenfeld et al., 1993;). In cell cultures derived from rodent central nervous system, Kettenmann et al. showed that cultured oligodendrocytes are clearly dye-coupled and weakly electrically coupled (Kettenmann et al., 1983; Kettenmann and Ransom, 1988). Using antibodies to specific surface markers (04, 01, and 010) that characterize cells of sequential maturity along the oligodendrocyte lineage to determine the developmental stages of the cells, Blankenfeld et al. (1993) studied the development of functional gap junctional communication in cells of oligodendrocyte lineage and observed that more than 40% of more mature, 010-positive oligodendrocytes showed cell-cell coupling detectable with both dye and current injection. From EM and freeze-fracture studies, gap junctions are observed between perikarya of interfascicular oligodendrocytes (Sotelo and Angaut, 1973), and oligodendrocytes also form reflexive gap junctions between cytoplasmic loops in the perinodal regions in vivo (Sandri et al., 1982). Ransom and Kettenmann (1990) documented the presence of weak electrical coupling between astrocytes and oligodendrocytes in cultures of mouse spinal cord, but in the absence of dye (Lucifer Yellow) coupling. Two hypotheses were proposed to explain the absence of permeability to Lucifer Yellow: (i) the dye crosses the junction but fails to reach detectable levels of resolution due to the low number of junctions; (ii) junctional channels allow the passage of ions but not of molecules having the size of Lucifer Yellow (Ransom and Kettenmann, 1990). Recently, the permeability of oligodendrocyte-astrocyte gap junctions was re-evaluated in studies using optimal experimental conditions for the observation of dye-coupling in an heterogeneous cell population (Venance et al., 1995). Under these conditions, dye-coupling from astrocytes to oligodendrocytes was shown to occur frequently, and bi-

directional dye-coupling was also observed when a second dye, sulphorhodamine B, was used (Venance et al., 1995). This is consistent with the results obtained by Massa and Mugnaini (1982) who identified numerous heterologous gap junctions between astrocytes and oligodendrocytic cell bodies, processes and the outer turn of myelin sheaths by a freeze-fracture study.

Evidence has been obtained, although limited at present, indicating that Cx32 is at least one of the connexins expressed by oligodendrocyte (Dermietzel and Spray, 1993). From the study which examined the distribution of Cx32 mRNA in rat CNS by ISHH, a large population of cells in white matter tracts that were labeled with the Cx32 riboprobe appeared to be oligodendrocyte (Micevych and Abelson, 1991). Immunostaining of oligodendrocytic marker (myelin basic protein, MBP) was shown to colocalize with Cx32 ir in rat brain especially in white matter (Dermietzel et al., 1989). Recently, Scherer et al. (1995) examined the localization and expression of Cx32 in Schwann cells and oligodendrocytes and found that Cx32 is localized in oligodendrocyte cell bodies and processes, but not in compact myelin.

So far few studies have examined regulation of the oligodendrocytic gap junctions. Kettenmann et al. (1990) have shown that electrical coupling between cultured mouse oligodendrocytes is very sensitive to intracellular pH. Saez et al. (1986) reported that the gap junctional conductance of cultured rat hepatocytes which express Cx32 is increased rapidly by agents that elevate cAMP and that Cx32 is phosphorylated by cAMP-dependent protein kinase, suggesting that cAMP-dependent phosphorylation of the gap junction channel modulates the conductance of liver gap junctions. In cultured oligodendrocytes, Venance et al. (1995) failed to increase the proportion of dye-coupled oligodendrocytes with preincubation

of the cells with the membrane-permeable analogue 8-bromo-cAMP or the addition of ATP and cAMP to the pipette solution, but showed that oligodendrocytic gap junctions are sensitive to CO_2-induced intracellular acidification and gap junction uncoupler such as alcohol (octanol and heptanol), which is suggested to act through the lipid environment of the junctions or by intercalating into nonpolar regions of the membrane-spanning domains of the connexins (Bennett et al., 1991). In addition, gap junctions formed by Cx32 in paired oocytes or transfected cells exhibit transjunctional voltage dependence (Spray et al., 1991b). The degree of sensitivity is low and does not suggest a physiological function (Bennett et al., 1991).

Based on the observations of extensive heterologous coupling between astrocytes and oligodendrocytes and between ependyma and astrocytes, as well as homologous coupling between ependymal, ependymoglial and astrocytic cell classes, Mugnaini (1986) proposed a hypothesis that ependyma, ependymoglia, astroglia, and oligodendroglia, by virtue of their gap junctions, form a potentially uninterrupted and comprehensive network that can be termed a supporting functional syncytium. This syncytium represents a substantial intracellular pathway for ions and small solutes, especially potassium and KCl that may enter the glia during sustained neural activity, and may provide a means for maintaining ionic equilibrium throughout the nervous tissues. In the myelinated band of the intact rabbit retina, Robinson et al. (1993) found that the low molecular weight dyes Lucifer Yellow and biocytin passed readily from astrocytes into adjacent astrocytes, oligodendrocytes, and Müller cells, but rarely passed from either oligodendrocytes or Müller cells into astrocytes. In contrast, Venance et al. (1995) reported that bi-directional dye coupling occurred between cultured astrocytes and

oligodendrocytes when a second dye, sulphorhodamine B, was injected into the oligodendrocytes. The discrepancy could be due to, as the authors suggested, (i) the amount of gap junction channel expression forming heterotypic junctions could be higher in cultured brain glial cells than in the intact retina, allowing a detectable concentration of dye in receiving cells, (ii) chemical structure and charge, which are critical parameters for gap junction permeability, could be more restrictive for Lucifer Yellow than for sulphorhodamine B, or (iii) besides Cx32, other unidentified connexins could be present in oligodendrocytes, and several subpopulations of heterologous gap junction channels with distinct selectivity could be formed between oligodendrocytes and astrocytes (Venance et al., 1995).

Astrocytes and oligodendrocytes were found to be immunoreactive to Cx43 and Cx32 respectively (Dermietzel et al., 1989; Yamamoto et al., 1990a,c). The selective expression of Cx43 in astrocytes (Dermietzel et al., 1991) and of Cx32 in oligodendrocytes (Giaume and Venance, 1995) was also observed in primary cultures. Since there is no evidence that astrocytes and oligodendrocytes express the same type of connexin, gap junctions between astrocytes and oligodendrocytes are probably constituted of at least two different connexins (Venance et al., 1995). Two different murine connexin cRNAs were injected into oocytes and the function of heterotypic channels was investigated under conditions where each of the two hemichannels consisted of different exogenous connexins (Swenson et al., 1989; Werner et al., 1989). It was concluded that rat Cx43 and Cx32 can couple with each other. But later studies by White et al. (1995b) showed that cells expressing Cx43 failed to become electrically coupled to cells expressing Cx32 when *Xenopus* Cx38 is inhibited by injecting oocytes with antisence oligonucleotides. In addition, Cx32 does not form heterotypic gap junctions,

permeable to Lucifer Yellow, with Cx43 in the Hela system where DNAs coding for murine connexins Cx43 and Cx32 were functionally expressed in human Hela cells that were deficient in gap junctional communication (Elfgang et al., 1995). Therefore, oligodendrocytes could express other unidentified connexins involved in the formation of heterotypic channels with astrocytic Cx43, since by EM, gap junctions between Cx43 immunolabeled astrocytic and unlabelled oligodendrocytic membranes have been frequently encountered (Ochalski et al., 1995). Moreover, single channel activity recorded from bovine oligodendrocytes indicates the presence of unitary conductance smaller than the 120 to 150 pS usually recorded from Cx32 channel which suggests possible co-expression of another connexin or, alternatively, a specific behavior of Cx32 channels when expressed in oligodendrocytes (Dermietzel and Spray, 1993). It is also possible that astrocytes express other novel connexins to form heterotypic gap junctions with oligodendrocytic Cx32, since evidence for detection of a novel second connexin at astrocytic gap junctions in rat brain with a anti-Cx26 antibody was obtained in our lab recently (Nagy et al., 1996).

It is now widely accepted that the oligodendrocyte is the cell responsible for the maintenance of central myelin (Peters et al., 1991), but little is known about the role of the oligodendrocytic gap junctions during neuronal activity, myelination or myelin remodeling after CNS injury. Several clues imply the importance of this coupling. First, Cx32 levels, which were not detected before the second week after birth, reach a plateau at 4 weeks and this is correlated with the development of myelination (Dermietzel et al., 1989). There is also a good correlation between the onset of dye-coupling in cultured oligodendrocytes and the appearance of Cx32 expression in the cell population in vivo (Giaume and Venance, 1995).

Second, it has been reported that up regulation of the Schwann cell gap junction complement occurs during Wallerian degeneration and subsequent regeneration until the onset of myelination (Tetzlaff, 1980). Third, linkage studies placed the X-linked Charcot-Marie-Tooth disease (CMTX) locus near the map location assigned to Cx32 on the X chromosome (Bergoffen et al., 1993). Subsequently, direct sequence analysis of the Cx32 coding region in CMTX patients revealed numerous mutations (Fairweather et al., 1994; Ionasescu et al., 1994; Orth et al., 1994). Immunolocalization of Cx32 reveals that Schwann cells express Cx32 and concentrate it in the uncompacted membranes adjacent to the nodes of Ranvier and at the incisures of Schimdt-Lantermann (Bergoffen et al., 1993), which leads to the hypothesis that Cx32 "reflexive" gap junctions could "short-circuit" the tube of cytoplasm connecting Schwann cell body to its periaxonal cytoplasm, dynamically reducing the path length that nutrient or trophic molecules must travel (Paul, 1995). Fourth, in peripheral nerve, Cx32 is found in the paranodal myelin loops and Schwann cells, and the levels of Cx32 protein and mRNA change in parallel with those of other myelin-related genes during development, Wallerian degeneration, and axonal regeneration. In the central nervous system, Cx32-ir is found in oligodendrocytes and their processes, but not in compact myelin, and the levels of Cx32 protein and mRNA increase during development in parallel with those of the other myelin genes (Scherer et al., 1995). Therefore, Cx32 is expressed as part of the myelinating phenotype of both Schwann cells and oligodendrocytes, suggesting that this gap junction protein plays an important role in the biology of myelin-forming cells (Scherer et al., 1995).

III.7. Rationale for the present studies

Although Furshpan and Potter first demonstrated gap junctions between neurons in the CNS of lower vertebrates in 1959, the extent of their occurrence and the nature of their contribution to intercellular communication in mammalian CNS has still not received a final answer since studies of gap junction function in mammalian CNS are technically difficult. Traditional methods of gap junction visualization by thin-section and freeze-fracture EM allow the examination of only minute samples, making their use in analyses of junction distributions a daunting proposition. The alternative of dye-transfer and electrophysiological methods, which have been used to demonstrate coupling between glial cells in culture and tissue slices (Kettenmann et al., 1983; Kettenmann and Ransom, 1988; Venance et al., 1995), suffer from limitations of sample size, are usually qualitative, do not provide accurate typology of junctions among coupled cells, and are difficult to apply in vivo. With the discovery of thirteen connexins that form gap junctions (White et al., 1995a) and at least five connexins that have been detected in adult and developing CNS (Nagy and Dermietzel, 1995), identification of connexins expressed in different neural cell types is a prerequisite to functional studies of gap junctions in the cellularly heterogeneous CNS. As the cDNAs of different connexin have been cloned and antibodies against different connexin are available, immunohistochemical methods provide powerful approaches for examining the connexin cellular localization and distribution within CNS. Results for this approach could indicate their legitimate contribution to intercellular communication and permit quantitative estimates of the occurrence of connexins in neural tissues which is essential for assessment of the importance of electronic coupling in the CNS.

Molecular cloning of cDNA for rat liver Cx32 and Cx32 mRNA expression in brain was first reported by Paul in 1986, but the localization of Cx32 in oligodendrocytes has received relatively little attention. So far, attempts to elucidate cellular localization of Cx32 in brain have produced disparate and confusing results. Reyher et al. (1991) provided morphological evidence for rat olfactory bulb granule cells interperikaryal electrotonic coupling via gap junctions and found that these neurons are immunopositive for Cx32. Micevych and Abelson (1991) examined Cx32 mRNA distribution in rat CNS by ISHH and reported that Cx32 mRNA was detected in discrete cell groups of gray matter that appeared to be neurons and in a large population of cells in white matter tracts that appeared to be oligodendrocytes. Dermietzel et al. (1989) localized immunoreactivity to specific cell types in frozen sections of adult rodent brain and observed Cx32-ir in oligodendrocytes and also in a few neurons. However, when Miragall et al. (1992) investigated Cx32 expression in the developing and adult olfactory system by immunocytochemical and biochemical methods, immunolabelling was primarily observed on cells associated with myelinated tracts which were presumably oligodendrocytes, and no definite immunolabelling could be observed in neurons. Recently, Cx32-ir was found in the cell bodies and processes of oligodendrocytes, but the myelin sheaths themselves were not stained (Scherer et al., 1995). This contrasts to the report of Spray and Dermietzel (1995) who showed extensive Cx32-ir material being present in the internodal region of the central myelin, but none at the nodes of Ranvier. So far no report has been available for extensive and systemic examination of Cx32 localization in rodent CNS by using immunohistochemical methods. Detection and documentation of Cx32 cellular localization in the CNS immunohistochemically would advance knowledge of the anatomical

organization of gap junctions composed of Cx32 in normal CNS, allow studies of their responses to injury and provide physiological relationships between heterologous junctions between oligodendrocytes and astrocytes.

IV. MATERIAL AND METHODS

IV.1. Antibodies

Among 11 different anti-Cx32 antibodies that were tested, 7 are listed in Table 1 together with their sequence specificity and the dilution employed for immunohistochemistry. Five of these were chosen for detailed studies including one affinity-purified polyclonal antibody (designated 7B), two monoclonal antibodies (designated 7C7 and 92B) that were raised as previously described (Hertzberg et al., 1988) against different peptides corresponding to regions within the sequence of Cx32 (Paul, 1986) and prepared as previously described (Hertzberg et al., 1988), one monoclonal antibody (designated 72F) that was raised against the whole protein, and one commercially available monoclonal antibody designated 2C2 (Zymed Laboratories Inc.). The location of these regions according to the proposed orientation of the extracellular, transmembrane and cytoplasmic domains of Cx32 (Zimmer et al., 1987; Hertzberg et al., 1988) is illustrated in figure 1. All peptides, except that used to produce 2C2, were conjugated to bovine serum albumin (BSA), injected into mice or rabbits and polyclonal antibody 7B, generated in the latter, was affinity-purified. As a positive control procedure, sections of liver, containing abundant Cx32, were processed with each of the antibodies using standard immunohistochemical methods. Sections of brain and liver were also processed by these methods after primary antibody omission, with 7B preimmune serum, or with the 7B, 7C7, 92B and 2C2 antibodies preabsorbed with the synthetic peptide against which each was raised. For preabsorption of 7B, 7C7 and 2C2, these antibodies were diluted 1:50 in 0.1 M sodium phosphate buffer containing 0.9% saline (PBS) and 100 ml was

incubated for 2 hr at room temperature with 100 μg of peptide. The preabsorbed antibodies were used at the same dilution as non-absorbed antibody.

IV.2. Tissue preparation

A total of 41 male Sprague-Dawley rats weighing 250-350 g and 32 CD_1 wild type mice weighing 30-35 g were used in this study. In order to optimize detection of these anti-Cx32 antibodies, animals were perfused with various combinations of prefixative and fixative solutions (Table 2). We found that the optimal fixation condition for rats is pH change protocol (containing 2% paraformaldehyde) without postfixation and for mice is pH change protocol (containing 3% paraformaldehyde) without postfixation (Table 2, number 7). The rats were deeply anesthetized with equithesins and perfused transcardially on ice. The thoracic cavity and diaphragm were opened to expose the heart. The left ventricle was pierced by a needle attached to the tube of a perfusion pump. The right atrium was then cut to allow blood and perfusion fluid outflow. Prefixative solution consisted of 100 ml of 10 mM sodium phosphate buffer (PB) containing 0.8% saline, 0.1% sodium nitrite and heparin (1 unit/ml). This was followed by perfusion with a pH change protocol where the first fixative solution (200 ml) contained 2% paraformaldehyde in 0.1 M PB, pH 7.5, and the second (200 ml) contained 2% paraformaldehyde in 50 mM sodium borate buffer, pH 9.0, at room temperature. Following fixative the animal was further perfused with 300 ml of 0.1 M PB containing 10% sucrose. The perfusion protocol for mice is similar to that for rats, except that the mice were given 10 ml of perfusion buffer, 25 ml of pH 7.5 fixative solution containing 3% paraformaldehyde, 25 ml of pH 9.0 fixative solution containing 3% paraformaldehyde, and

30 ml of 0.1 M PB containing 10% sucrose. The brain was removed and stored directly in 50 mM PB containing 25% sucrose and 10% glycerol for cryoprotection without postfixation.

IV.3. Immunohistochemistry

All brains were sectioned at 20-30 μm on a sliding microtome and sections were washed for 16 hr at 4°C in PB containing 0.9% saline and 0.3% Triton X-100 (PBST) prior to processing. Sections were processed by the PAP method and double immunofluorescence.

IV.3.1. *Peroxidase anti-peroxidase (PAP) method.* Sections were incubated for 48 hr at 4°C with primary antibody diluted in PBST containing 2% normal goat serum (NGS). For the immuno-staining of those polyclonal anti-Cx32 antibodies, sections were received a 1 hr wash and then incubated for 1.5 hr at room temperature with goat anti-rabbit IgG (Sternberger Monoclonal Inc.) diluted 1:100 in PBST with 2% NGS. This was followed by a 1 hr wash in PBST, and then by a 1.5 hr incubation at room temperature with rabbit PAP (Sternberger Monoclonals Inc.) diluted 1:500 in PBST with 2% NGS; For the immunostaining of those monoclonal anti-Cx32 antibodies, sections were washed in PBST for 1 hr and then incubated for 1.5 hr at room temperature with goat anti-mouse IgG (Sternberger Monoclonal Inc.) diluted 1: 100 in PBST, washed for 1 hr in PBST, and incubated for 1.5 hr with mouse clono PAP (Sternberger Monoclonal Inc.) diluted 1:500 in PBST with 2% NGS. The sections were then washed sequentially for 30 min. in PBST and for 30 min. in 50 mM Tris-HCl buffer, pH 7.4, and then incubated for 10-15 min. in the same Tris-HCl buffer containing 0.02% 3,3'-diaminobenzidine and 0.005% hydrogen peroxide and then washed in Tris-HCl buffer. Free-floating sections were mounted onto slides from a 0.5% gelatin/30% alcohol solution. All

sections were dried overnight, dehydrated in alcohol, cleared in xylene and coverslipped with Lipshaw mounting medium.

IV.3.2. *Double immunofluorescence*. Sections were incubated for 48 hr at 4 ^{o}C with anti-Cx32 antibody 7B diluted 1:250 in PBST and simultaneously with a mouse monoclonal antibody against the oligodendrocyte and myelin marker 2,'3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) (Sternberger Monoclonals Inc.) diluted in 1:1,000. Alternatively, sections were incubated with 7B and a monoclonal antibody (diluted 1:1 in PBST) against the oligodendrocyte marker Rip, which was kindly provided by Dr. S. Hockfield (Friedman et al., 1989). Sections were washed for 1 hr in PBST, incubated for 1.5 hr at room temperature in PBST containing a mixture of Cy3-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch Laboratories, Inc.) diluted 1:100 and FITC-conjugated horse anti-mouse antibody (Vector) diluted 1:100. Sections were then washed for 1 hr in PBST, 1 hr in 50 mM Tris-HCl buffer, pH 7.4, mounted onto slides from Tris-HCl buffer and coverslipped with antifade medium (Valnes and Brandtzaeg, 1985).

V. RESULTS

V.1. General Observations

Positive staining for Cx32 in rat and mouse liver, which contains high levels of this protein, was obtained with five of the antibodies listed in Table 1. As illustrated by examples of 7B (Fig. 2A), 7C7 (Fig. 2C) and 2C2 (Fig. 2E), punctate immunolabeling was seen around hepatocytes. Similar patterns were obtained with 72F in both rat and mouse liver (not shown) as well as with 92B as previously demonstrated (Yamamoto et al., 1991). Immunostaining was abolished in liver sections after preadsorption of antibodies 7B, 7C7 and 2C2 (Fig. 2B,D,F) as well as 92B (not shown) with the synthetic peptides against which each was raised. Immunolabeling in rodent brain was obtained with all but two (48E and 6B10) of the antibodies listed in Table 1. Under optimal fixation conditions, two predominant labeling patterns were obtained with the remaining 5 antibodies. These were Cx32-immunoreactive (Cx32-ir) puncta and diffuse cytoplasmic staining associated with cellular profiles, identified as oligodendrocytes, and immunopositive fibers that corresponded to myelin sheaths. Four of the 5 antibodies each produced labeling of cells and/or fibers of varying intensity as shown in cerebellum of rat or mouse (Fig. 3A-F). The exception was 92B which labeled only fibers in rat. As shown in cerebellum, immunostaining was abolished after preadsorption of antibodies 7B, 7C7 and 92B (Fig. 3G-I) as well as 2C2 (not shown) with appropriate synthetic peptide. No immunoreactivity was observed in sections processed with 7B preimmune serum or in those processed after primary antibody omission (not shown).

Detection of Cx32 in both oligodendrocytes and along myelinated fibers by anti-Cx32 antibodies was highly sensitive to fixation and required weak fixative without post-fixation. A small increase in fixation strength diminished staining of oligodendrocytes cell bodies and an equally small reduction in strength gave poor staining of myelinated fibers. In an attempt to utilize stronger fixation conditions allowing adequate ultrastructural preservation for EM analysis, several antigen retrieval methods were tested (Table 3). While most of these proved unsuccessful, some labeling of fibers was obtained after STUF treatment for 10 min. at 90 ^{o}C (not shown), but this caused severe ultrastructural deterioration. As a result, satisfactory compromise between interpretable ultrastructure and sufficient immunostaining was difficult to achieve.

V.2. Anatomical observations

In both rat and mouse brain, Cx32-ir cells were widely, but heterogeneously distributed (Table 4.). In the granule cell layer of the cerebellum, double immunofluorescence staining revealed cellular co-localization of Cx32 with the oligodendrocyte marker CNPase (Fig. 4A,B). Dispersed among double-labeled cells were some CNPase-ir oligodendrocytes that were devoid of staining for Cx32 (not shown), although a false negative result could not be excluded in these instances. Similar results were obtained in other brain areas, but CNPase-ir cells were generally obscured by very dense CNPase-ir fibers. Most, if not all, Cx32-ir cells had either round or oval somata with diameters ranging from 5 to 8 μm and many, though not all, extended several, short labeled processes. Cx32-ir puncta of about 0.5 μm were often distributed around the periphery of labeled cells. Staining along cellular processes, when

evident, was invariably punctate (Fig. 4C) and these labeled processes could be seen extending to nearby Cx32-ir fibers (Fig. 4D). Cx32-ir cells in some brain regions were found only among very weakly labeled fibers as in hypothalamus (Fig. 4E), while others had few labeled processes and occupied areas nearly devoid of labeled fibers as in the lateral mammillary nucleus (Fig. 4F). Cx32-ir oligodendrocytes were usually sparse and weakly labeled in large fiber tracts such as corpus callosum, internal capsule and fiber bundles traversing the striatum. When present in white matter such as in the hippocampal stratum lacunosum-moleculare (Fig. 4G,H), immunostained cells resembled those in cerebellum.

Cx32 heterogeneity was best revealed in sagittal sections. Immunoreactivity was weak in forebrain structures including striatum and lateral septal nuclei, but concentrated in many areas of the thalamus, midbrain and medulla (Fig. 5A,B). In the latter regions, labeled fibers were often co-distributed with Cx32-ir cells, although dense fiber staining tended to obscure these cells. Conversely, immunopositive cells were clearly evident in areas containing sparse fiber labeling. In superior colliculus, numerous uniformly distributed cells were intermixed with a moderate density of weakly stained fibers in all but the sparsely labeled superficial layer (Fig. 5C). In the dorsal tegmental area, the central gray exhibited only a few labeled oligodendrocytes, while the posterodorsal tegmental nucleus and lateral regions (Probst's bundle) contained numerous stained cells and fibers (Fig. 5D). The central nucleus of the inferior colliculus displayed many Cx32-ir cells (Fig. 5E), while both cells and fibers were present in external inferior colliculus. The midbrain periaquaductal gray was largely devoid of fibers and contained labeled cells that increased in number towards the periphery (Fig. 5C,F). In the facial nucleus, Cx32-ir cells and fibers were moderately distributed (Fig. 5G).

Oligodendrocyte labeling in most brain regions was punctate and distributed around somata and along short processes (Fig. 5E,F).

Cx32-ir fibers were observed both in white and grey matter. Although individual labeled fibers could rarely be distinguished in the former, they were more loosely arranged and thus more clearly evident in grey matter. In the cerebellar granule cell layer as elsewhere, double immunofluorescence staining demonstrated that Cx32-ir fibers were also immunopositive for the oligodendrocyte/myelin marker CNPase (Fig. 6A,B) indicating Cx32 localization to myelinated fibers. Similar results were obtained after double labeling for Cx32 and the oligodendrocyte marker Rip (not shown). Cx32-ir fibers emanating from the Purkinje cell layer and entering central white matter (Fig. 6C) appeared to be exclusively myelinated axons of Purkinje cells. Although labeling often ended abruptly close to Purkinje cells (Fig. 3 and 6C), it could occasionally be followed to the soma of these cells (not shown). Moreover, double-staining for Cx32 and neurofilament protein revealed continuity between Purkinje cell somata and the initial portion of their Cx32-ir myelinated axons (not shown). This selective labeling was further evident in double-labeled sections showing the absence of Cx32 staining in the molecular layer (Fig. 6C), but the presence of CNPase-ir fibers in this layer as well as their higher concentration in the granule cell layer (Fig. 6D).

Labeling for Cx32 along subpopulations of myelinated fibers was also evident in other brain regions. In cerebral cortex, layers I and II were nearly devoid of Cx32-ir fibers and contained only a few scattered labeled cells (Fig. 7A), but did exhibit some CNPase and Rip labeling (not shown). Stained oligodendrocytes were more numerous in layer III than in deeper layers. More striking was the widespread distribution of both CNPase-ir and Rip-ir

fibers in the hippocampus (not shown), while only a few weakly labeled fibers were seen in most hippocampal areas except the pyramidal cell layer of the CA2 and CA3 regions which contained staining of short, tortuous fibers (Fig. 7B). Double-labeling for Cx32 and Rip in cerebral cortex revealed staining of sparsely distributed, vertically arranged fibers (Fig. 6E), whereas staining for Rip was more concentrated and present in both vertically and horizontally oriented fibers (Fig. 6F). Similarly, in the substantia nigra, Cx32-ir fibers were more sparse (Fig. 6G) than those in the same field labeled with Rip (Fig. 6H). Although less evident at the magnifications shown, Cx32-ir fibers in these areas were also Rip-positive, but not all Rip-positive fibers were stained for Cx32. It should be noted that some fibers were very weakly stained for Cx32 suggesting heterogeneity in protein levels associated with different fibers.

The appearance of Cx32-ir myelinated fibers in some distinct areas of sagittal sections is further illustrated in figure 8. Intensely labeled fibers occupied islands of Calleja (Fig. 8A) and ventral pallidum (Fig. 8B), some emerging anteriorly from both structures into surrounding regions largely devoid of staining, and individual immunopositive fibers were seen oriented parallel to the long axis of the claustrum (Fig. 8C). As shown at the border between the striatum and globus pallidus (Fig. 8D), staining for Cx32 in the latter was similar to that in the ventral pallidum, but was sparse in striatal grey matter which contained few labeled cells and fibers. Fiber bundles traversing these two regions were lightly to moderately stained, while those coursing through the reticular thalamic nucleus and ventralateral thalamic nucleus were intensely labeled (Fig. 8E). Immunoreactivity along individual fibers such as those in cerebellar folia (Fig. 6A) or the claustrum (Fig. 8C), was continuous and consisted of fine puncta or evenly dispersed granular profiles. Alternatively, as in ventral pallidum, labeling was

intermittent, consisting of either large puncta (Fig. 8F,G) of about 0.5 μm, elongated segments or spirals around the fiber (Fig. 8F).

Cx32-ir elements at all levels in the spinal cord were similar to those described in brain. Immunopositive oligodendrocytes and myelinated fibers were detected in both grey and white matter as shown in sagittal sections through the dorsal horn at lumbar levels (Fig. 9A,B). Cx32-ir cells had a relatively uniform distribution in both dorsal (Fig. 9A) and ventral (Fig. 9C) horn, although very few were seen in the substantia gelatinosa (Fig. 9A), They were generally more densely labeled in white than in grey matter (Fig. 9C) and had fewer immunopositive processes in the latter (Fig. 9D). Myelinated fibers coursing through grey matter, such as those emerging from the dorsal column (Fig. 9B), were usually well labeled compared with sparse labeling of fibers in white matter of spinal funiculi.

In addition to Cx32-ir cells and fibers, a third rare pattern of immunoreactivity was observed with antibody 7B. This staining consisted of puncta that did not appear to be associated with cells or fibers and did not correlate with either CNPase- or Rip-immunopositive elements (not shown). Such puncta were seen in the olfactory bulb where they ranged from 0.4 to 1.0 μm and surrounded glomeruli or were distributed along the olfactory nerve layer (Fig. 10A). These were also found in hippocampus where they were arrayed in roughly circular patches with diameters of 20 to 40 μm in the strata radiatum and oriens and in the molecular layer of the dentate gyrus (Fig. 10B).

VI. DISCUSSION

The present results demonstrate: 1. A heterogeneous distribution of Cx32 in rat CNS and localization of the protein within oligodendrocytic cell bodies and processes; 2. Presence of Cx32 along subpopulations of myelinated fibers. Furthermore, symmetric labeling of homologous gap junctions between oligodendrocyte elements and asymmetric labeling on the oligodendrocyte side of heterologous gap junctions between oligodendrocytic and presumptive astrocytic elements were observed by our EM analysis (Li et al., 1996). These findings are in agreement with observations of Cx32 localization in oligodendrocytes of rodent CNS (Dermietzel et al., 1989; Scherer et al., 1995; Spray and Dermietzel, 1995) and are consistent with the well documented formation of gap junctions by oligodendrocytes with each other as well as with astrocytes (Dermeitzel et al., 1978; Massa and Mugnaini, 1982; Waxman and Black, 1984; Mugnaini, 1986; Venance et al., 1995).

Association of Cx32 with oligodendrocytic rather than axonal components of myelinated fibers is indicated by our EM results (Li et al., 1996) and suggested by LM observations of overlapping localization of immunolabeling for Cx32 and myelin marker proteins. Moreover, no labeling with the present set of antibodies was detected in neuronal somata or axons by EM or by double immunostaining for Cx32 and neurofilament protein (not shown). Further, we biochemically confirmed an enrichment of Cx32 in isolated myelin compared with microsomal membrane subfractions of brain (Sherer et al., 1995). Interestingly, while our Western blot results indicate Cx32 mobility in myelin to be similar to that in liver, Cx32 in whole brain homogenate exhibited a slightly faster mobility (Li et al., 1996). Although not examined, we

suspect that the bulk of Cx32 represented in whole brain homogenates is contained in a subfraction corresponding to a cytoplasmic and/or membrane form of the protein that has the faster mobility we have also observed in cultured oligodendrocytes (unpublished observations). Our results bear on several issues as well as some questions that remain to be resolved.

VI.1. Oligodendrocytic gap junctions

Widespread astrocytic gap junctional coupling creates a functional syncytium which, among other proposed functions such as long range calcium signaling (Finkbeiner, 1992; Dani et al., 1992; Cornell-Bell et al., 1990), is thought to contribute to ionic homeostasis by providing a means for rapid dispersal of potassium following its accumulation by astrocytes in areas of neuronal activity (Orkand et al., 1966; Gardner-Medwin, 1986; Newman, 1986). Although less frequently encountered than inter-astrocytic junctions, gap junctions between astrocytes and oligodendrocytes are sufficiently numerous to warrant inclusion of the latter within this syncytium (Mugnaini, 1986). Indeed, we provide evidence elsewhere that myelin-associated oligodendrocytic processes in spinal cord often form gap junctions with astrocytic processes where the latter side of such junctions is labeled for Cx43, while the former side is not (Ochalski et al., 1996). Unfortunately, tissue destruction in the present material prevented unequivocal identification of unlabeled astrocytic elements in gap junctional contact with Cx32-ir oligodendrocyte somata or processes. Nevertheless, the common occurrence of gap junctions between the two macroglial cell types together with our EM result showing

prevalence of inter-oligodendrocytic gap junctions (Li et al., 1996) provides further reason to consider oligodendrocytes as part of a composite glial syncytium.

Given evidence for numerous gap junctions between astrocytic and oligodendrocytic processes surrounding the outer turn of myelin (Massa and Mugnaini, 1982; Ochalski et al., 1996), it is perhaps not surprising to find an oligodendrocytic connexin, e.g. Cx32, distributed along myelinated fibers. Coupling between oligodendrocytic processes as well as between these and astrocytes along internodal regions of myelin may be important in view of their continuity with the outer paranodal loops of myelin. The role these loops may play in potassium ion homeostasis during axonal activity at peripheral nodes of Ranvier (Chiu, 1991; Mi et al., 1996) may be operative at central nodes. One scenario for potassium movement that includes an oligodendrocytic gap junctional contribution is accumulation of ion by paranodal loops, flow through gap junctions that have been demonstrated between these structures (as discussed below) and subsequently into oligodrendrocytic cytoplasm at the outer surface of myelin towards internodal regions, and then transfer into astrocytic or other oligodendrocytic processes via gap junctions along these regions. If Cx32 along myelinated fibers subserves such communication pathways, then this might explain its selective localization along particular fiber types having a potentially greater demand for ion redistribution.

Another role for Cx32 along CNS myelinated fibers may be considered in light of its localization in Schwann cells in peripheral nerve, and specifically at paranodal regions at nodes of Ranvier and at Schimdt-Lantermann incisures (Bergoffen et al., 1993; Spray and Dermietzel, 1995). It has been speculated that a radial pathway through gap junctionally coupled pockets of cytoplasm at incisures as well as at paranodal loops may enable critical

Schwann cell functions by allowing a direct flow of small molecules and ions radially across compact myelin through channels provided by gap junctions (Paul, 1995; Scherer et al., 1995; Spray and Dermietzel, 1995). Whether such speculation can be extrapolated to the CNS is currently unclear. However, in normal mammalian CNS unlike PNS, gap junctions between oligodendrocytic paranodal loops have been documented (Sandri et al., 1982). Moreover, Schmidt-Lanterman incisures of the kind seen in peripheral nerve have been described in CNS myelin (Blakemore, 1969; Conradi, 1969; Hildebrand, 1971; McDonald and Ohlrich, 1971; Hildebrand et al., 1993). Consequently, it remains uncertain whether Cx32 along myelinated fibers is responsible solely for formation of gap junctions at the outer surface of myelin, or is also contained within CNS myelin where it serves to mediate radial junctional coupling. Harsh tissue treatments and possible immunoproduct diffusion compromised our attempts to determine ultrastructural localization of Cx32 within compact myelin and further studies of issue will require use of immunogold methods.

VI.2. Oligodendrocyte heterogeneity

The restriction of Cx32 to subpopulations of fibers is best exemplified by labeling in cerebellum where it was associated with myelin sheaths only on axons arising from Purkinje cells. This selectivity is unlikely due to false negative staining given the consistency of results, the robust fiber staining seen among unlabeled fibers and the labeling of only oligodendrocytes in some areas of sections processed for optimal labeling of fibers. Further, some white matter regions exhibited little myelin-associated Cx32 and the most intense labeling for Cx32 occurred along individual fibers or small bundles percolating through grey matter. The

significance of this observation remains to be determined, but bears on suggestions that ion homeostatic mechanisms may differ in white matter and grey matter (Barres et al., 1990). Heterogeneity of fiber labeling raises three points. First, while oligodendrocytic paranodal loops form gap junctions in the CNS (Sandri et al., 1982), the rarity with which we and others (Spray and Dermeitzel, 1995) observed Cx32 labeling at paranodal loops together with selectivity of Cx32 for subclasses of fibers suggests either that not all nodal loops possess gap junctions or, if they do, that not all such junctions or only a fraction are composed of Cx32 supporting the possibility that oligodendrocytes may express additional connexins (Dermietzel and Spray, 1993). It is clear from the present results that a significant proportion of homologous inter-oligodendrocyte gap junctions are composed of Cx32. Heterologous astro-oligodendrocyte gap junctions may be composed of Cx43/Cx32 if these connexins form functional junctions (Werner et al., 1989; Swenson et al., 1989). If they do not, as more recently reported (White et al., 1995b), then such junctions may be composed of Cx32 in oligodendrocyes and an alternative astrocytic connexin as we have suggested elsewhere (Nagy et al., 1996).

Second, determining whether only subpopulations of oligodendrocytes express Cx32, or all do but not all transport the protein to their distal processes will require Cx32 double immunofluorescence with oligodendrocyte markers that do not produce massive myelin labeling, which tends to obscure somatic staining. In any event, the selective occurrence of Cx32 along certain fibers, arising from either differential Cx32 expression or targeting to discrete cellular sites, may be governed by axons and represent divergent responses of a uniform oligodendrocyte population to different properties of the axons they myelinate during

development. Alternatively, oligodendrocyte subtypes may be preprogrammed to express particular phenotypic characteristics, including connexin expression, and in this way are predetermined to myelinate particular classes of axons. There is evidence that oligodendrocytes are a heterogeneous population comprising several subtypes and that different subtypes may myelinate different caliber axons (Bjartmar et al., 1994; Stensaas and Stensaas, 1968; Remahl and Hildebrand, 1990). Two biochemically and morphologically distinct oligodendrocyte subtypes have been demonstrated in rat anterior medullary velum (Butt et al., 1995). Small (< 10 mm) carbonic anhydrase II (CAII)-positive cells with multiple, frequently branched processes formed myelin sheaths around numerous, small diameter (4 μm or less) fibers and large (15-20 μm) CAII-negative cells with fewer, rarely branched processes ensheathed larger diameter (4-12 μm) fibers. The Cx32-positive oligodendrocytes observed in our material appear to be of similar size as the CAII-positive subtype. In rat cerebellum, two ultrastructurally distinguishable oligodendrocytic subtypes have been identified (Monteiro, 1983). The distribution of Cx32-ir cerebellar oligodendrocytes in the present study corresponds that of cells designated type I (Monteiro, 1983) which were thought to myelinate most cerebellar axons, while type II oligodendrocytes were primarily Purkinje cell body satellites. Based on this, type I cells may comprise two subclasses, those that express Cx32 and myelinate Purkinje cell axons and those that lack Cx32 and myelinate most other axons. The poor morphological preservation in our material, coupled with the difficulty of establishing definitive ultrastructurally-based oligodendrocyte subclasses (Szuchet, 1995; Peters et al., 1991; Kruger and Maxwell, 1966), precludes further attempts to designate Cx32-ir cells as members of a particular cerebellar oligodendrocytic subclass.

VI.3. Neuronal Cx32 or Cx32-related proteins in CNS

Although generally consistent results were obtained with the present set of anti-Cx32 antibodies, labeling of fine puncta in olfactory bulb and hippocampus with affinity-purified polyclonal antibody 7B illustrates that the CNS distribution of Cx32 or Cx32 related proteins is not straightforward. Results of other recently developed anti-Cx32 antibodies tested, but not shown here, included labeling of varicose axons similar to that we previously reported with earlier antibody reagents (Yamamoto et al., 1989). Moreover, we have obtained labeling of Golgi apparatus in most neurons with two different antibodies (not listed in Table 1), one directed against a peptide corresponding to the carboxy terminus and another against a conserved extracellular domain of Cx32 (unpublished results). The Western blot data with antibody 2C2, in addition to demonstrating recognition of Cx32, indicated detection of a faster migrating protein (Li et al., 1996). This warrants mention since two other anti-Cx32 antibodies, not listed in Table 1 and distinct from those that labeled neuronal Golgi apparatus, recognized a similar co-migrating protein (not shown). These anatomical and biochemical results could simply reflect cross reaction of antibodies with irrelevant proteins. On the other hand, such results have occurred with sufficient consistency with various anti-Cx32 antibodies directed against various epitopes to suggest the existence in brain of hitherto unidentified Cx32-related connexins that form gap junctions, or alternatively, connexin-related proteins that may subserve structural or communicative functions at neuronal structures that have some resemblance to gap junctions, such as the axon-glial junctions (Rosenbluth, 1995) or subsurface cisterns (Rosenbluth, 1962a,b; Yamamoto et al., 1990b, 1991).

VII. TABLES

Table 1. Summary of anti-Cx32 antibodies tested[1]

Anti-Cx32 Ab	Origin and sequence specificity	Dilution
72F	Monoclonal against whole Cx32	1:100
92B	Monoclonal against aa's 224-234 (C-terminus)	1:5000
48E	Monoclonal against aa's 179-186 (extracellular loop II)	1:500
6B10	Monoclonal against aa's 106-117 (cytoplasmic loop)	1:500
7C-7	Monoclonal against aa's 235-246 (C-terminus)	1:100
7B	Polyclonal (affinity purified) against aa's 235-246 (C-terminus)	1:250
2C-2	Monoclonal against proprietary sequence (cytoplasmic loop)	1:100

[1]All antibodies were provided by Dr. E.L. Hertzberg except 2C-2 which was obtained from Zymed Laboratories Inc.

Table 2. Summary of fixation conditions tested[2]

Number	Prefixtive solution	Fixative solution	Postfixation duration
1.	50 mM PB; 0.9% NaCl; 0.1% sodium nitrite; 0.01% heparin (4°)	0.1 M PB containing 4% PF, pH 7.4 (4°)	2 hr 12 hr
2.	50 mM PB; 0.9% NaCl; 0.1% sodium nitrite; 0.01% heparin (4°)	0.1 M PB containing 4% PF, pH 7.5 (4°); 50 mM borate buffer containing 4% PF, pH 9.0 (4°)	2 hr 12 hr
3.	50 mM PB; 0.9% NaCl; 0.1% sodium nitrite; 0.01% heparin (4°)	0.1 M PB containing 2% PF, pH 7.5 (4°); 50 mM borate buffer containing 2% PF, pH 9.0 (4°)	2 hr
4.	50 mM PB; 0.9% NaCl; 0.1% sodium nitrite; 0.01% heparin (37°)	0.1 M PB containing 4% PF, pH 7.5 (first 50 ml fixative solution at 37°, the rest at 4°); 50 mM borate buffer containing 4% PF, pH 9.0 (4°)	2 hr
5.	50 mM PB; 0.9% NaCl; 0.1% sodium nitrite; 0.01% heparin (4°)	0.1 M PB containing 4% PF, 0.2% picric acid, pH 7.4 (4°)	2 hr 12 hr
6.	10 mM PB; 0.8% NaCl; 0.1% sodium nitrite; 0.01% heparin (RT.)	0.1 M PB containing 4% PF, pH 7.4 (RT.)	without postfixation

(continued)

Table 2. Summary of fixation conditions tested (continued)

Number	Prefixtive solution	Fixative solution	Postfixation duration
7.	10 mM PB; 0.8% NaCl; 0.1% sodium nitrite; 0.01% heparin (RT.)	0.1 M PB containing 4%, 3% or 2% PF, pH 7.5 (RT.); 50 mM borate buffer containing 4%, 3% or 2% PF, pH 9.0 (RT.)	without postfixation
8.	10 mM PB; 0.8% NaCl; 0.1% sodium nitrite; 0.01% heparin (37°)	0.1 M PB containing 4% PF, pH 7.5 (first 50 ml fixative solution at 37°, the rest at 4°); 50 mM borate buffer containing 4% PF, pH 9.0 (4°)	without postfixation
9.	10 mM PB; 0.8% NaCl; 0.1% sodium nitrite; 0.01% heparin (RT.)	0.1 M PB containing 3% PF, 0.1% glutaraldehyde and 15% picric acid (RT.)	without postfixation

[2]Note: animals tested for without postfixation were given 0.1 M PB containing 10% sucrose following fixative solution.

Abbreviations: PB-phosphate buffer; PF-paraformaldehyde; RT.-room temperature.

Table 3. Sumary of antigen retrieval methods tested

Treatment	Concentration	Temperature	Duration
Target unmasking fluid (serotech.)	1:3 (diluted in distilled water)	90°C	10 min.
		50°C	10 min.
		Room temperature	8, 24 hr.
Ethanol	15%, 50% (diluted in distilled water)	Room temperature	0.5 hr.
Methanol	1:10 (diluted in PB containing 0.9% saline)	Room temperature	0.5 hr.
Saponin	0.3% (diluted in PBST)	4°C	16 hr.
Saponin plus target unmasking fluid	Saponin: 0.3% (in PBST)	4°C	3 hr.
	Target unmasking fluid: 1:3 (diluted in distilled water)	Room temperature	24 hr.
Protein kinase	1,5,10 μ/ml (diluted in 0.1 M tris buffer, pH 8.0 containing 50 mM EDTA)	37°C	0.5 hr.
Photo -flo 200 solution (Kodak)	0.3% (diluted in PB containing 0.9% saline)	4°C	16 hr.
Triton X-100	0.5% (diluted in PB containing 0.9% saline)	4°C	16 hr.

Table 4. Documentation of Cx32-immunostaining density in mouse and rat CNS[3]

	Labeled oligodendrocyte	Labeled myelinated fiber
Neocortex	+2	+3
Internal capsule	+1	+4
Corpus callosum	+1	+4
Olfactory bulb	+1	+3
Hippocampus		
Alveus	+2	+3
Paramidal layer	+3	+2
Stratum oriens	+1	+1
Stratum radiatum	+1	+1
Striatum lacunosum molecular	+3	+1
Dentate gyrus		
Granule layer	+2	+1
Polymorphic layer	+2	+1
Dorsal hippocampal commissure	ND	+4
Fimbria	ND	+4
Subiculum	+3	+3
Septum		
Fornix	ND	+3
Bed nucleus of the stria terminalis	+1	+1
Lateral septal nucleus	+1	+1
Medial septal nucleus	+1	+1
Stria medularis	ND	+4
Islands of Calleja	ND	+4
Basal ganglia		
Nucleus caudate/putamen	+1	+1
Globus pallidus	+3	+3
Substantia innominata	+3	+3
Substantia nigra		
Compact part	+3	+3
Reticular part	+3	+3
Ventral pallidum	+1	+3

(continued)

Table 4. Documentation of Cx32-immunostaining density in mouse and rat CNS (continued)

	Labeled oligodendrocyte	Labeled myelinated fiber
Thalamus		
Habenula	+2	+3
Lateral nuclei		
Dorsomedial nucleus	ND	+4
Ventrolateral nucleus	+2	+3
Midline nuclei	+2	+3
Reticular nuclei	+2	+4
Hypothalamus		
Anterior hypothalamic nuclei		
Lateral zone	+3	+1
Medial zone	+3	+1
Mammillary nuclei	+3	+1
Optic tract	+3	+4
Midbrain		
Central gray	+2	0
Cerebral peduncles	+1	+4
Colliculi		
Inferior	+4	+2
Superior	+4	+2
Interpeduncular nucleus		
Pontine nuclei	+3	+4
Ventral tegmental area	+3	+4
Cerebellum		
Granule cell layer	+2	+3
Molecular cell layer	0	0
Purkinje cell layer	+1	+2
White matter	ND	+4
Cerebellar peduncle	0	+2
Deep cerebellar nuclei	ND	+4

(continued)

Table 4. Documentation of Cx32-immunostaining density in mouse and rat CNS (continued)

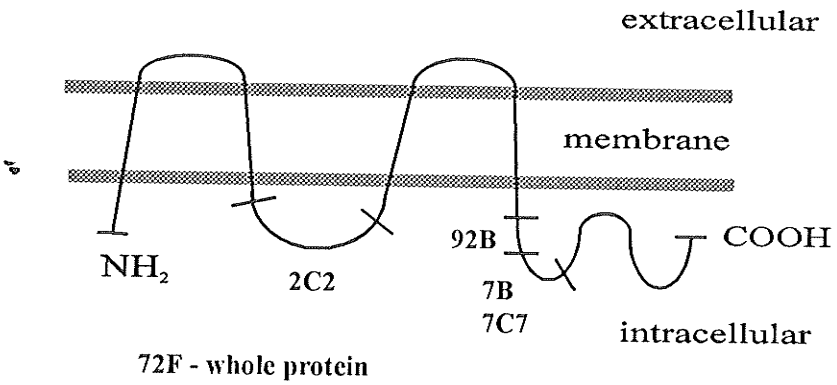
	Labeled oligodendrocyte	Labeled myelinated fiber
Hindbrain		
Ventral cochlear nucleus	+3	+4
Facial nucleus	+3	+2
Hypoglossal nucleus	ND	+2
Inferior olivary complex	+2	+2
Lateral superior olive	+3	+4
Raphe obscurus nucleus	+2	+2
Raphe pallidus nucleus	+2	+2
Trigeminal complex		
Spinal tract V	ND	+2
Neucleus	+3	+4
Medial vestibular nucleus	+3	+3
Posterodorsal tegmental nucleus	+3	+2
Probst's bundle	+3	+2
Superior paraolivary nucleus	+3	+4
Spinal cord		
White matter	+3	+3
Grey matter	+3	+3

[3]Scale: 0 = no Cx32-immunostaining observed; +1 = scattered labelled cells or fibers; +2 = moderate number of labelled cells or fibers; +3 = high number of labelled cells or fibers; +4 = very high number of labelled cells or fibers; ND = not determined.

VIII. FIGURES

VIII. Figure 1

Fig. 1. Illustration of sequences against which anti-Cx32 antibodies were directed as depicted with respect to membrane orientation of Cx32. The antibodies indicated are listed in Table 1.



72F - whole protein

Fig.1

VIII. Figure 2

Fig. 2. Immunodetection of Cx32 in liver and specificity controls for some of the antibodies listed in Table 1. **A-F:** Sections of rat liver processed with antibodies 7B (A), 7C7 (C) and 2C2 (E) or with these antibodies preadsorbed with peptide (B,D,F, respectively). Typical punctate immunoreactivity around hepatocytes is eliminated after antibody preadsorption. Magnifications: A,C, x300; B, x160; D,F, x210; E, x400.

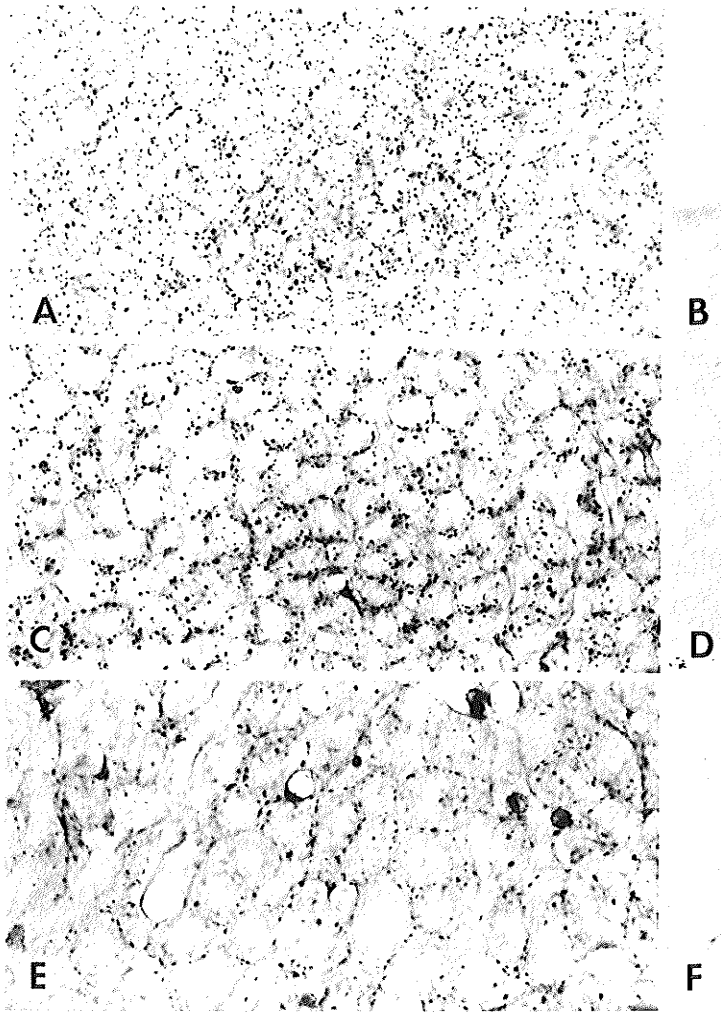


Fig.2

VIII. Figure 3

Fig. 3. Immunodetection of Cx32 in mouse and rat cerebellum and specificity controls with some of the antibodies listed in Table 1. **A-F:** Immunolabeling in cerebellar folia with anti-Cx32 antibody 7B (A, mouse), 2C2 (B, mouse), 7C7 in mouse (C) and rat (D), 72F (E, mouse) and 92B (F, rat). Depending on fixation conditions, immunoreactivity is localized to oligodendrocyte cell bodies (arrowheads) and fibers (arrows) in both white matter and the granule cell layer, or is associated with only fibers in these areas (D,F, arrows). **G-I:** Examples showing elimination of labeling in cerebellum after peptide preadsorption of antibody 7B (G, mouse), 7C7 (H, rat) or 92B (I, rat). ML, molecular layer; gcl, granule cell layer; Pcl, Purkinje cell layer. Magnifications: A, x150; B,C, x170; D,I, x70; E,F, x100; G,H, x90.

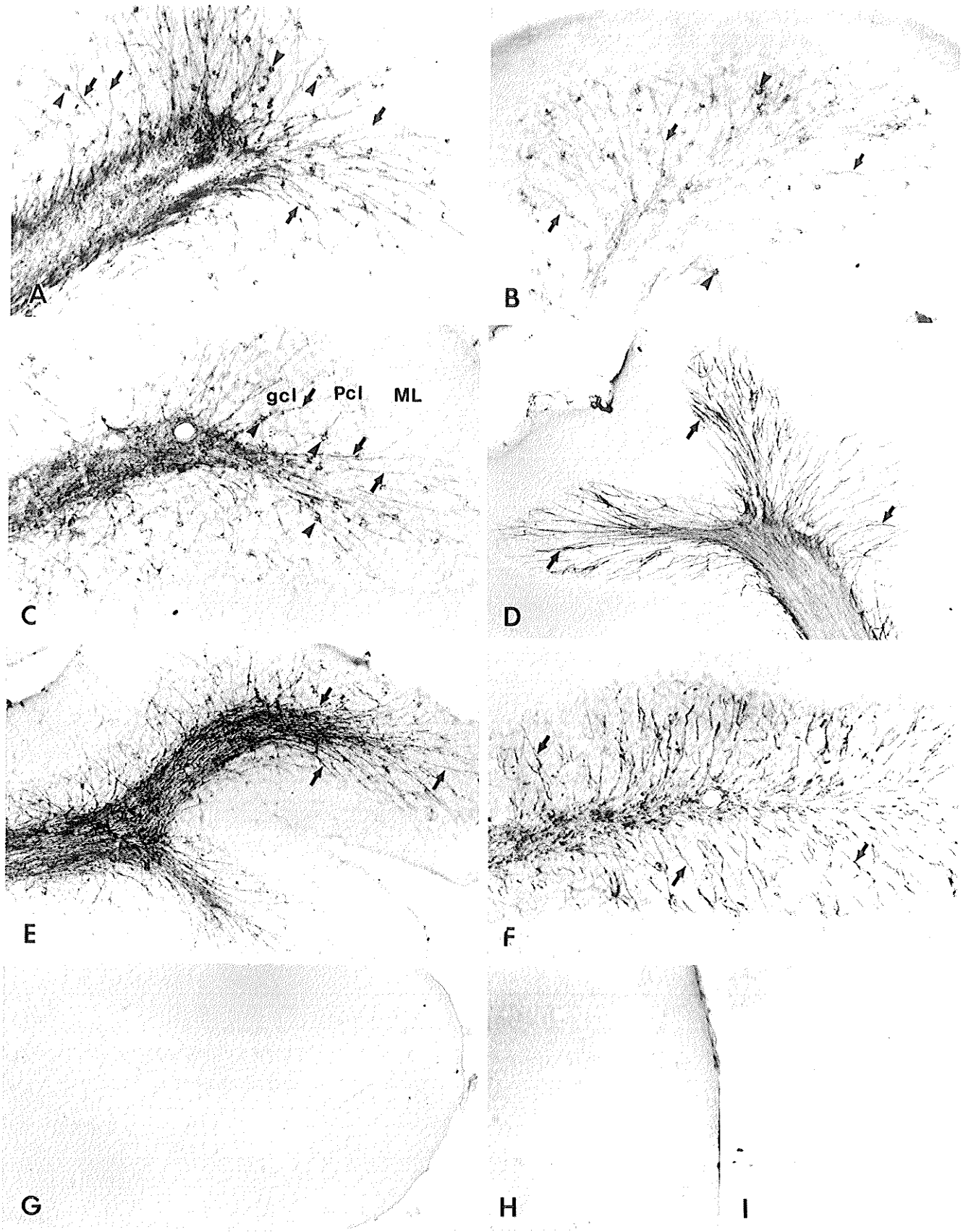


Fig.3

VIII. Figure 4

Fig. 4. Immunofluorescence localization of Cx32 in oligodendrocytes of mouse brain. **A,B:** Micrographs of a field in the granule cell layer (gcl) of a cerebellar section double-labeled for Cx32 (A) and the oligodendrocyte marker CNPase (B). Corresponding arrows indicate Cx32-positive cells that are also CNPase-positive. **C,D:** Cx32-ir cells in the gcl extend several processes (C,D, arrows) which contact labeled fibers (D, arrowheads). **E-H:** Cx32-ir cells in lateral hypothalamus (E), lateral mammillary nucleus (F), and stratum lacunosum molecular of the hippocampus (G, shown at higher magnification in H). Note punctate appearance of most labeling associated with cell bodies and processes. A,C-E,G,H, antibody 7B; F, antibody 72F. Magnifications: A,B, x560; C, x500; D, x680; E,G, x210; F, x290; H, x770.

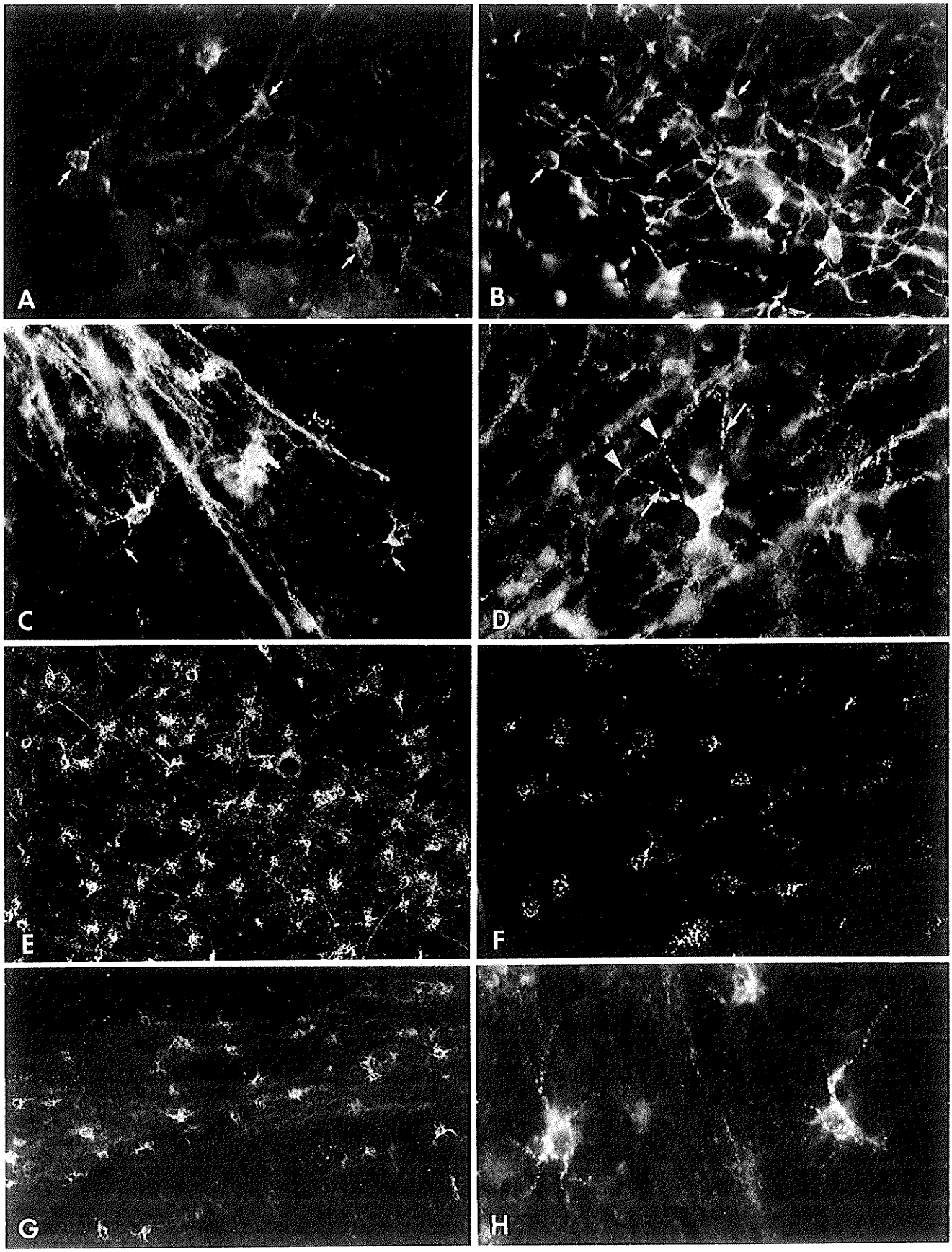


Fig.4

VIII. Figure 5

Fig. 5. Immunoperoxidase localization of Cx32 (antibody 7B). **A,B:** Distribution of Cx32 immunoreactivity in rat subcortical and brainstem structures. Labeling is sparse in striatum (St) and lateral septal nuclei (Sep) (A), but is dense and heterogeneously distributed in thalamus (THAL) (A), midbrain (MB) (A) and medulla (B). **C-G:** Cx32-ir oligodendrocytes in regions of mouse brainstem including superior colliculus (SC) (C), dorsal tegmental area (D), inferior colliculus (E), peripheral margin of the central gray area (CGA) (F) and facial nucleus (G). Note differential density of labeled cells among regions, but relatively uniform cellular distribution within any particular region. Magnifications: A, x12; B, x20; C, x40; D, x60; E,G, x160; F, x330.

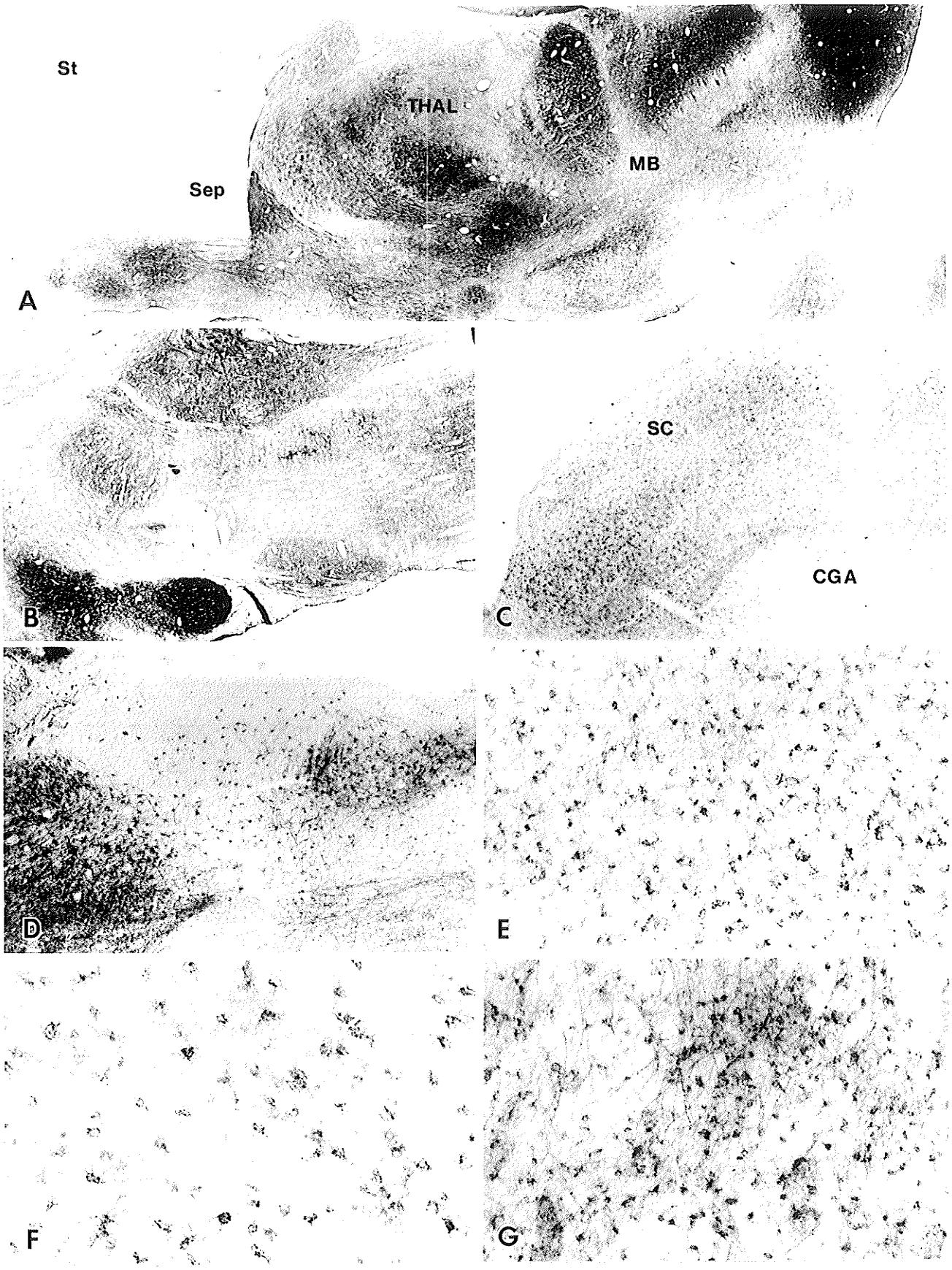


Fig.5

VIII. Figure 6

Fig. 6. Pairs of immunofluorescence micrographs of mouse brain sections showing double-labeling for Cx32 (left column, antibody 7B) and CNPase (B,D) or Rip (F,H) in the cerebellar granule cell layer (A,B), a cerebellar folium (C,D), layers 3-5 of motor cerebral cortex (E,F), and substantia nigra (G,H). **A-D:** Individual fibers are double-labeled for both Cx32 (A) and CNPase (B) (corresponding arrows), but Cx32-positive fibers are less widely distributed (C) than those labeled for CNPase (D). **E-H:** Cx32-ir fibers in cerebral cortex (E) and substantia nigra (G) are also more sparsely distributed than Rip-ir fibers in these areas (F,G, respectively). ML, molecular layer; Pcl, Purkinje cell layer, gcl, granule cell layer. Magnifications: A,B, x410; C,D, x140; E,F, x210; G,H, x230.

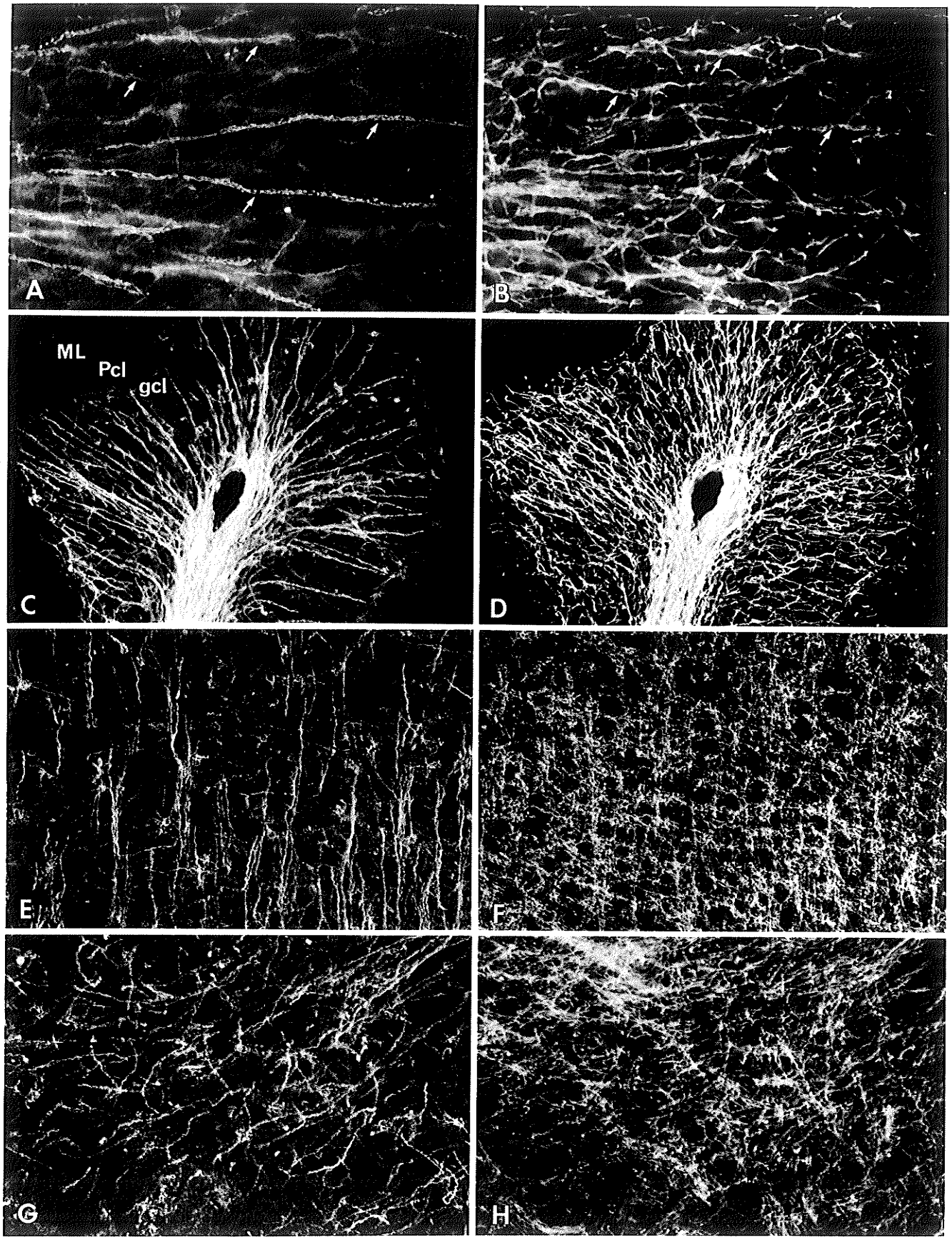


Fig.6

VIII. Figure 7

Fig. 7. **A,B:** Immunoperoxidase labeling shows Cx32-ir (antibody 7B) oligodendrocytes and fibers in various layers of the frontal cerebral cortex of mouse (A) and a relatively greater abundance of labeled fibers among pyramidal cells in the CA2 and CA3 subfields of rat hippocampus (B). Magnifications: A, x150; B, x100.

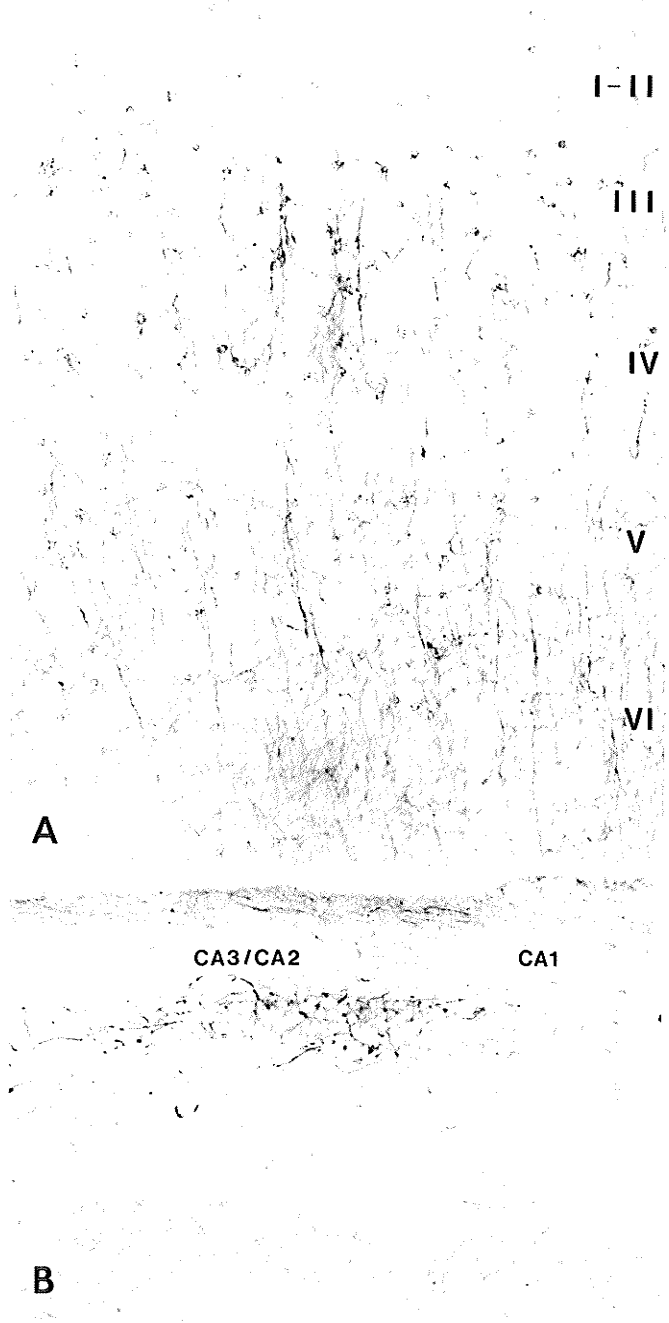


Fig.7

VIII. Figure 8

Fig. 8. Immunofluorescence of Cx32-ir fibers and associated oligodendrocytes labeled with antibody 7B in forebrain structures and thalamus of mouse. **A-D:** Islands of Calleja (ICj) (A), anterior portion of ventral pallidum (VP) (B), claustrum (C), border region between striatum (St) and globus pallidus (GP) (D). Cx32-ir oligodendrocytes are mingled with labeled fibers in each area. Note the heterogeneity of labeling characterized by areas of dense surrounded by sparse staining. **E:** Reticular thalamic nucleus and ventral lateral thalamic nucleus. Dense bundles of Cx32-ir fibers (arrows) traversed these two regions. **F,G:** Magnifications of areas in A and B showing segmental (F, arrows) or punctate (G, arrows) labeling along individual fibers. Magnifications: A-D, x160; F,G, x560; E, x190.

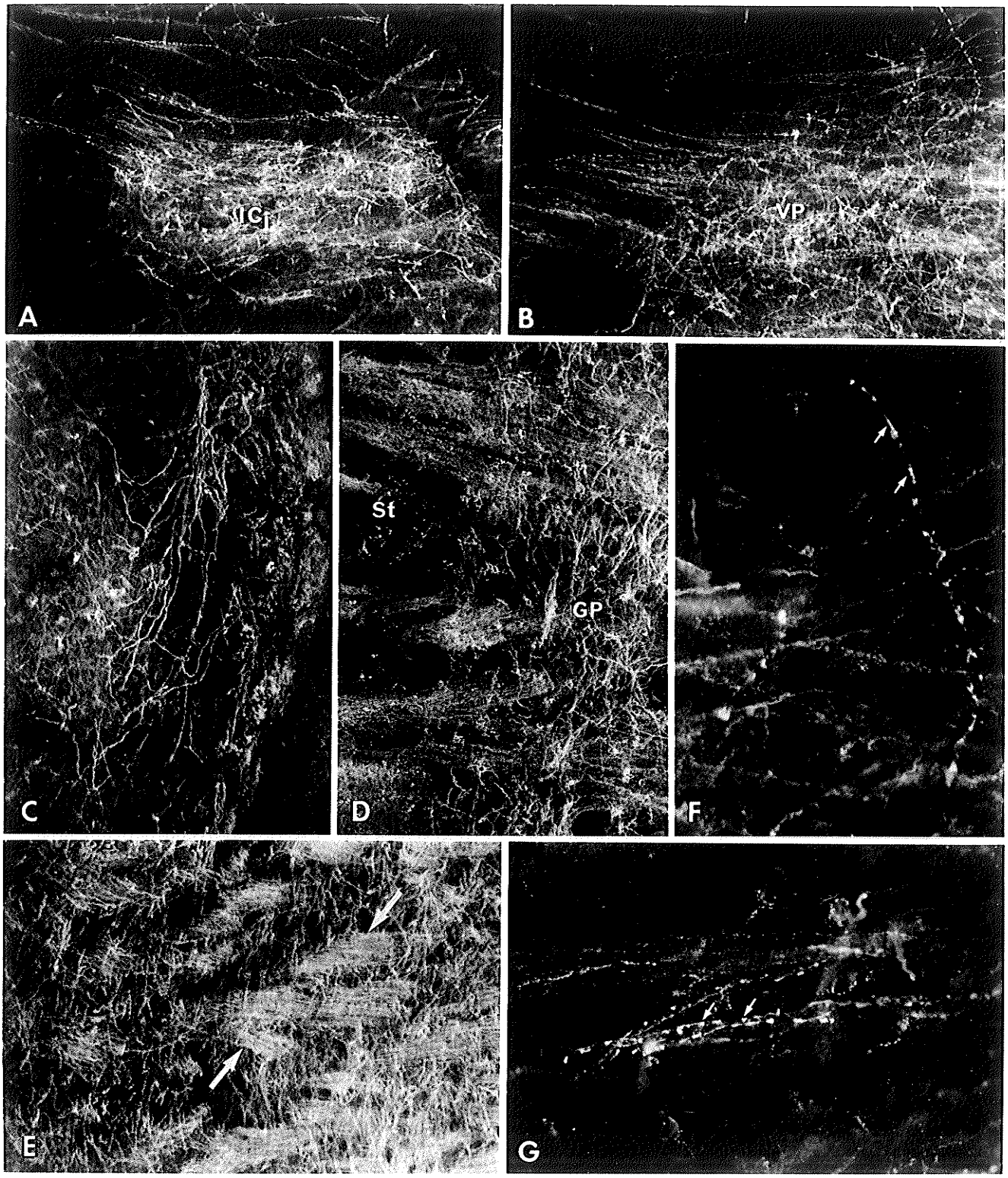


Fig.8

VIII. Figure 9

Fig. 9. Immunoperoxidase localization of Cx32 in oligodendrocytes and fibers in spinal cord. **A,B:** Sagittal sections at lumbar levels of rat cord incubated with a cocktail of antibodies 7C7, 2C2 and 72F. Many labeled cell bodies are seen in deep dorsal horn laminae III-V and very few in the substantia gelatinosa (A). Bundles of labeled fibers emerge from the dorsal column and penetrate into spinal cord gray matter (B, arrows). **C,D:** Transverse sections at a rostral cervical level in mouse cord showing distribution of Cx32-ir cells in the ventral horn (C, antibody 2C2) and higher magnification of labeled cells in the dorsal horn (D, antibody 7C7). Magnifications: A,B, x80; C, x160; D, x330.

Fig.9

VIII. Figure 10

Fig. 10. Immunofluorescence micrographs showing relatively rare patterns of Cx32 immunoreactivity in rat brain with antibody 7B. **A,B:** Puntate labeling is seen around glomeruli in olfactory bulb (A, arrows) and collections of puncta are localized in roughly circular patches in the stratum radiatum of the hippocampus (B, arrows). These puncta did not appear to be associated with either oligodendrocytes or myelinated fibers. Magnifications: A, x260; B, x210.

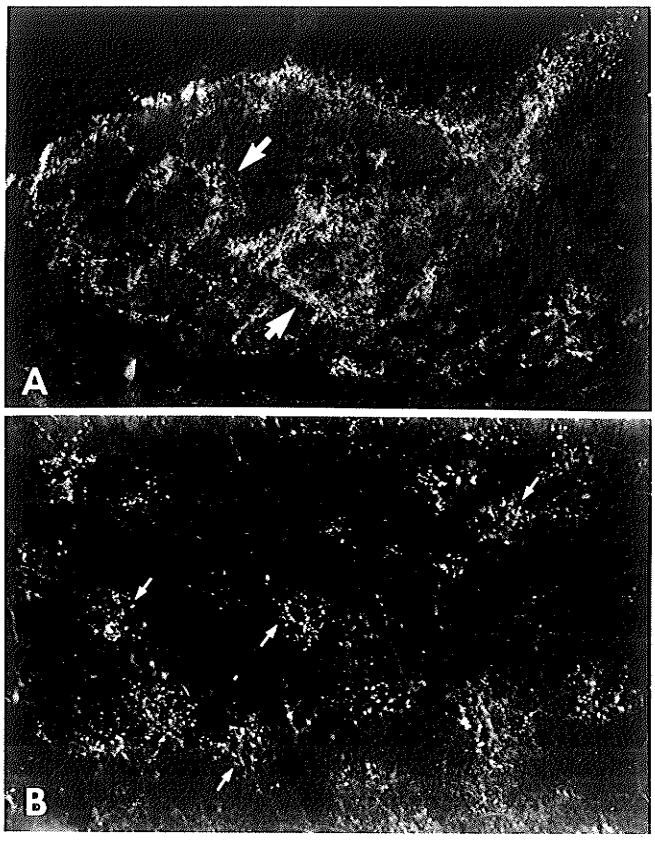


Fig.10

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