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Gap Junction Gene Expression In The Developing Nervous System

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GAP JUNCTION GENE EXPRESSION IN THE DEVELOPING

NERVOUS SYSTEM

by

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ABSTRACT

Gap junctions provide for the transfer of low molecular weight molecules and ions between cytoplasms of adjacent cells. Connexins exist as a multigene family with one or more members found in almost all adult tissues. In the central nervous system, gap junctions have been detected in a variety of cell types including neurons, astrocytes and oligodendrocytes. Gap junctions have been detected in the developing brain in a spacial and temporal pattern. This study examines gap junction expression and cellular specificity during development of the rodent central nervous system. To investigate the role of gap junctions during development, a model of neural development is examined with respect to its connexin expression.

In the developing rodent brain, connexin32 and connexin43 were detected by Northern blot analysis. Connexin43 mRNA was detected pre- and postnatally, whereas connexin32 mRNA was differentially expressed, being first detectable at postnatal day 10 in hindbrain and day 15 in forebrain. Western blot analysis demonstrated the presence of connexin protein during postnatal development of the rodent brain. To examine connexin expression in greater detail, *in situ* hybridization studies were performed. Connexin43 mRNA was found in the leptomeninges and astrocytes. Oligodendrocytes and select populations of neurons were shown to express connexin32 mRNA. Cultured astrocytes express cx43 confirming the in vivo findings. Initially, in culture, neurons express connexin26

protein which becomes less abundant with time. Neurons cultured for extended periods of time express cx32.

The embryonal carcinoma cell line, P19, differentiates into neurons and astrocytes following treatment with retinoic acid. Undifferentiated P19 cells express connexin26 and 43. The mRNA level of these two connexins do not change when P19 cells were exposed to retinoic acid. Connexin43 protein, however, is significantly reduced after exposure to retinoic acid. During differentiation, the neurons expressed connexin26 and astroctyes expressed connexin43.

These investigations have determined that gap junctions are differentially expressed during development of the central nervous system. Astrocytes express connexin43 whereas neurons and oligodendrocytes express connexin32. Connexin26 is present in immature neurons in culture. The studies on P19 cells further supports the presence of connexin26 in neurons and represents a model of neural differentiation that modulates connexin expression.

"Don't walk in front of me, I might not follow,

Don't walk behind me, I might not lead.

Walk beside me and be my friend."

To Margaret, whose love, compassion and understanding

has made this all possible.

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LIST OF ABBREVIATIONS

a.a.	amino acid
BSA	bovine serum albumin
CAM	cell adhesion molecule
cAMP	adenoside 3'-5' cyclic monophosphate
cDNA	complementary deoxyribonucleic acid
cpm	counts per minute
cRNA	complementary ribonucleic acid
CTP	cytosine tri-phosphate
cx	connexin
dBcAMP	dibutyryl cyclic adenosine monophosphate
DIV	days in vitro
DMEM	Dulbecco's modified Eagles medium
DNA	deoxyribonucleic acid
dPBS	Dulbecco's phosphate buffered saline
E	embryonic day
EC	embryonal carcinoma
FCS	fetal calf serum
GABA	gamma amino butyric acid
Galc	galactocerebroside
GFAP	glial fibrillary acidic protein

g	gravity
Hanks BSS	Hanks balanced salt solution
HS	horse serum
IP_3	inositol 4, 5-triphosphate
kb	kilobase
kD	kilodalton
MBP	myelin basic protein
MOPS	3-N-morpholino-propanesulphonic acid
Mr	relative molecular weight
NFDM	non-fat dry milk
NP-40	nonidet P-40
O-2A	oligodendrocyte - type 2 astrocyte
P	postnatal day
PBS	phosphate buffered saline
PIPES	1, 4-piperazinediethylene sulphonic acid
PKC	protein kinase C
RA	retinoic acid
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulphate

SSC	standard sodium citrate
UTP	uridine tri-phosphate

Units of Measure

Å	Angstrom
cm	centimeter
hr	hour
IU	international units
kg	kilogram
μg	microgram
μl	microliter
μm	micrometer
μM	micromolar
mm	millimetre
mM	millimolar
min	minute
M	molar
nm	nanometer

CHAPTER 1

Historical Review

1.1 Introduction

The evolution of multicellular organisms necessitated the development of cellular interactions in order to coordinate the function of groups of cells such as those making up tissue layers and organ systems. Different modes of cell interactions exist to provide a range of functions. Cell-cell interactions involve long range signalling by means of ligand-receptor mechanisms such as hormones and growth factors whereas short-range local phenomena are more likely to involve direct cell-cell contact via intercellular junctions (Chailakhyan, 1990). Intercellular junctions have been characterized at the ultrastructural (Staehelin, 1974; Mugnaini, 1986; Brightman and Reese, 1969) and molecular level (Stevenson and Paul, 1990; Legan et al. 1992; Anderson et al. 1993) and perform specific functions between cells such as adhesion (desmosomes or macula adherens; Legan et al. 1992), regulation of transcellular permeability (tight junctions or zonulae occludentes; Anderson et al. 1993; Stevenson and Paul, 1990), or intercellular communication (chemical synapses and gap junctions; Jessell and Kandel, 1993; Bennett et al. 1991; Dermietzel et al. 1990). The following dissertation will describe the characterization of one specific intercellular junction,

1

the gap junction, its distribution and potential function in the developing central nervous system (CNS).

1.2 Historical Perspecitve

One of the first studies suggesting direct intercellular communication was performed by Furshpan and Potter (1959) using the giant motor synapse of the crayfish. In their report, Furshpan and Potter (1959) describe a novel form of synaptic transmission which involved the rapid transfer of electrical activity from the pre- to post-synaptic fibre. They suggested that the synaptic membrane may contain structural differences which would account for the presence of electrical and chemical synapses in the giant motor synapse (Furshpan and Potter, 1959). Similar observations of electrotonic junctions were seen in the vertebrate CNS (Bennett et al. 1963; Furshpan, 1964). Again, the possibility of unique membrane specializations for electrical versus chemical transmission was suggested (Bennett et al. 1963; Furshpan, 1964). Dewey and Barr (1962; 1964) characterized sites of intimate membrane contact between smooth muscle cells of the intestine. The nexus was proposed to be a specialization of the membrane providing direct electrical connection between cells without intervening extracellular space (Dewey and Barr, 1962; Dewey and Barr, 1964). Kanno and Loewenstein (1964) were the first to describe intercellular communication between non-excitable cells. Fluoroscein-sodium diffused readily between the cytoplasm of salivary gland cells

of *Drosophila* suggesting the presence of a direct low resistance pathway between cells (Kanno and Loewenstein, 1964; Loewenstein and Kanno, 1964).

The structure responsible for the passage of ions and other small molecules was unequivocally demonstrated by Revel and Karnovsky (1967) in liver and heart tissue, using novel preparations for electron microscopy. The presence of a small, 2 nm (20 Å) gap between the external leaflets of plasma membrane distinquished this junction from other forms of intercellular junctions (Revel and Karnovsky, 1967). Tangential sections of lanthanum stained material revealed an array of hexagonally packed structures, each subunit containing an electron opaque core (Revel and Karnovsky, 1967). These observations elaborated on results described by Roberston (1963) in Mauthner cell synapses of goldfish. Revel and Karnovsky (1967) coined the term "gap" junction to distinquish it from tight junctions and desmosomes.

1.3 Structural Features of Gap Junctions

Revel and Karnovsky (1967) described what is now considered the typical feature of gap junctions in transmission electron microscopy; a septalaminar appearance containing 4 electron opaque layers and 3 electron transparent layers, one of these comprising the 2 nm gap characteristic of gap junctions. This observation provided a clear distinction from tight junctions which do not contain any extracellular space between outer leaflets of contacting membrane (Staehelin,

1974). Prior to finding the 2 nm gap, investigators confused tight and gap junctions considering both as pentalaminar structures in electron microscopy (Dewey and Barr, 1962; Dewey and Barr, 1964; Robertson, 1963; McNutt and Weinstein, 1970).

The entire thickness of the septalaminar gap junction containing membrane region is 15-19 nm (reviewed by Larsen, 1977; Peracchia, 1980). Revel and Karnovsky (1967) and Robertson (1963) described a polygonal pattern of gap junction packing within the membrane. Negative staining of gap junctions also displays a similar pattern (Larsen, 1977; Goodenough, 1975). The gap junction structure appears hexagonal with an electron opaque pit in the center. The center-to-center spacing is approximately 8.5-9 nm and each particle is approximately 6-8 nm in diameter (Larsen, 1977; Goodenough, 1975).

Gap junctions have a very characteristic appearance in freeze fracture replicas of plasma membranes (McNutt and Weinstein, 1970; Peracchia, 1980). They are seen as beads or particles on the protoplasmic face and corresponding pits on the exoplasmic face (Peracchia, 1980). The sizes of particles agrees with electron microscopic studies, averaging 8 nm with a center-to-center distance of 10 nm. Utilizing x-ray diffraction studies, Makowski et al. (1977) proposed a model for the transmembrane composition of gap junction channels consisting of six identical units encircling a central pore, confirming previous electron microscopic studies.

1.3.1 Nomenclature

Negative staining reveals a hexagonal arrangement of subunits comprising each particle of the gap junction (Peracchia, 1980). Goodenough (1974a) proposed the term "connexin" to describe each subunit. The structural unit of the gap junction, consisting of the 6 connexin subunits, has been termed the "connexon" (Goodenough, 1975). Connexons from each unit membrane abut one another to form a gap junction channel. Numerous gap junction channels are present in the gap junction plaque which, in itself, can vary in size (Goodenough, 1975). Beyer et al. (1987) have elaborated on this nomenclature adding the molecular weight of the protein to the term connexin. This aspect will be discussed in more detail in subsequent sections.

1.3.2 Isolation of Gap Junction Proteins

In order to study the biology of gap junctions in greater detail, it was necessary to devise methods of isolation and characterization of gap junction related proteins. Specific methods have been developed for the study of many kinds of intercellular juctions (Hertzberg et al. 1992).

a) Liver gap junction proteins

Taking advantage of the gap junction's unique insolubility in detergents,

Goodenough (1974b; 1975) isolated purified fractions of gap junction protein from mouse liver. The resultant protein migrated as three bands at 34 000, 18 000, and 10 000 daltons on sodium dodecyl sulfate - polyacrylamide gels. In experiments by Henderson et al. (1979), purified junctional proteins were isolated having a molecular weight (Mr) of 26 000 and 21 000 daltons. The 26-27 kilodalton (kD) protein was confirmed to be a gap junction protein by other investigators using different isolation methods (Hertzberg, 1984). The use of alkali extraction of non-junctional membranes instead of detergent greatly improved the protein yield, allowing for the production of antibodies to the isolated gap junction proteins and localization of the antibody to punctate sites on the plasma membrane of hepatocytes and cells of other tissues (Hertzberg and Skibbens, 1984).

The smaller protein regularly found in liver gap junction preparations (Henderson et al. 1979) was confirmed to be another gap junction protein by Nicholson et al. (1987). The 21 kD protein was shown to have partial homology with the 26 kD protein and both proteins were co-localized to the same junctional plaques (Nicholson et al. 1987).

b) Heart gap junction proteins

The presence of numerous gap junctions at the intercalated discs of heart tissue prompted the investigation and isolation of cardiac gap junction proteins employing similar methods to those used in liver (Kensler and Goodenough, 1980). Seven major proteins ranging from 28 - 57 kD were isolated. Manjunath

et al. (1984) further modified the procedure using inhibitors of proteases in their isolation procedures. Without the addition of protease inhibitors, the Mr of the isolated cardiac gap junctions were 29 500 and minor bands at 44 000 - 47 000 daltons (Manjunath et al. 1984). In the presence of protease inhibitors, Manjunath et al. (1984) demonstrated that the 29 500 dalton band was markedly reduced in abundance and the 44 - 47 kD bands were increased in intensity suggesting the lower Mr species was a degradation product. Similar sized proteins were observed in human heart tissue (Manjunath et al. 1987).

Comparisons of the partial amino acid (a.a.) sequences of the liver and heart gap junction proteins revealed that the two proteins were unique but maintained some degree of sequence homology (Guo et al. 1992; Nicholson et al. 1985). In addition, both proteins contained a hydrophobic domain suggestive of a transmembrane segment (Nicholson et al. 1985).

1.4 The gap junction gene family

Gap junctions are present in many organs and have been shown to be unique in their amino acid sequence in at least two tissues (Nicholson et al. 1985). Further analysis of gap junctions resulted in the isolation and sequencing of the complimentary deoxyribonucleic acid sequence (cDNA) for the liver gap junction (Paul, 1986; Kumar and Gilula, 1986). The isolated cDNA encoded a 283 a.a. protein with a predicted Mr of 32 007 (Table 1; Paul, 1986).

TABLE 1   The connexin gene family

Characterization of avian and mammalian connexin genes from a variety of species. The features described include amino acid (a.a.) length, predicted molecular weight (Mr) based on analysis of the sequence, size of the messenger RNA, and location of the connexin genes on chromosomes. In addition, alternative methods of classifying connexin genes are included. The type A classification scheme is that proposed by Bennett et al. (1991), type B has been proposed by Risek et al. (1990) and Gimlich et al. (1988). The superscript numbers are references in which the information was obtained and are listed below.

1(Zhang and Nicholson, 1989), 2(Hsieh et al. 1991), 3(Hennemann et al. 1992b), 4(Nishi et al. 1991), 5(Hennemann et al. 1992a), 6(Hennemann et al. 1992c), 7(Schwarz et al. 1992), 8(Haefliger et al. 1992), 9(Paul, 1986), 10(Kumar and Gilula, 1986), 11(Willecke et al. 1991), 12(Reed et al. 1993), 13(Beyer et al. 1992), 14(Hennemann et al. 1992d), 15(Kanter et al. 1992), 16(Beyer, 1990), 17(Beyer et al. 1987), 18(Fishman et al. 1990), 19(Musil et al. 1990), 20(Paul et al. 1991), 21(Beyer et al. 1988), 22(White et al. 1992), 23(Rup et al. 1993), 24(Hoh et al. 1991), 25(Lee et al. 1992)

	Species	Protein Length (a.a.)	Mr (daltons)	mRNA size (kb)	Chromosome Location	Connexin Grouping A	B
cx26	rat[1] mouse[3,4] human[25]	226	26 453	2.5 2.4, 2.8	14[2]	I	β_2
cx30.3	mouse[5]	266	30 391	1.9, 3.2	4[5]		
cx31	mouse[6] rat[24]	270 270	30 905 30 390	1,9, 2.3 1.7	4[5,7]	I	
cx31.1	rat[1] mouse[5]	271 271	31 049 31 198	1.5 1.6	4[5,8]	I	
cx32	rat[9] mouse[4] human[10]	283	32 007	1.6	X[2,8]	I	β_1
cx33	rat[8]	286	32 863	2.3	X[8]	II	
cx37	rat[8] mouse[11] human[12]	333 333 333	37 559 37 600 37 283	1.5 1.7 1.7	4[8]	II	
cx40	rat[8,13] mouse[14] dog[15]	356 358 357	40 237 40 418 39 937	3.4 3.5 2.6	3[8,7]	II	
cx42	chicken[16]	369	41 748	3.0			
cx43	rat[17] mouse[4] human[18] chicken[19]	382 382 381	43 036 43 009 43 177	3.0 3.1 3.0	10[2,8]	II	α_1
cx45	mouse[6] dog[15] chicken[16]	396 396 394	45 671 45 538 45 376	2.2 2.1 2.0	11[7]		
cx46	rat[20,21]	416	46 000	2.8	14[2]	II	α_3
cx50	mouse[22]	440	49 602	8.5			
cx56	chicken[23]	510	55 857	8.0			

Subsequent to the isolation of the liver gap junction cDNA, Beyer et al. (1987) screened a rat heart cDNA library and isolated a cDNA encoding a 382 a.a. protein with a predicted Mr of 43 026 (Table 1). Beyer et al. (1987) also proposed a system of nomenclature for the family of gap junction genes. The term connexin (Goodenough, 1974b) was reintroduced followed by the predicted Mr of the protein. The liver gap junction was called connexin32 (cx32) and the heart gap junction, connexin43 (cx43).

Comparisons of the two connexin cDNAs revealed regions of high homology and other regions of little or no homology (Beyer et al. 1987). Hydrophathicity plots demonstrated that regions of the cDNA encode for hydrophobic and hydrophilic domains (Beyer et al. 1987). Each connexin contains four prominent hydrophobic regions separated by hydrophilic regions. It was proposed that the hydrophobic regions span the membrane four times (Beyer et al. 1987). Alignment comparisons of cx32 and cx43 show that the transmembrane regions are highly homologous whereas the amino terminus, cytoplasmic loop and particularly the carboxy terminus are divergent (Figure 1.1; Beyer et al. 1987).

Using low stringency hybridization on cDNA or genomic libraries, numerous investigators have isolated and cloned various gap junction genes from a diverse source of tissues and species. Table 1 summarizes the known vertebrate connexins isolated from chicken, mouse, rat, dog and human. The mammalian gap junction gene family presently consists of 12 unique connexins, each conforming to the basic feature described in the cloning of cx32 and cx43 (Paul,

FIGURE 1.1 The gap junction protein

Schematic view of gap junction proteins from adjacent cells abutting one another in the extracellular space. Each connexin consists of 4 transmembrane domains and 2 extracellular loops which are highly conserved among different gap junction proteins. The amino and carboxy terminus and the intracellular domain are not conserved among connexins, in particular, the carboxy terminus. The extracellular space narrows at the site of gap junction contact to approximately 2 nm. The transmembrane regions cross both layers of the plasma membrane, a distance of approximately 10 nm.

Amino terminus   Carboxy terminus   Cytoplasm

Plasma membrane

Extracellular loop   2 nm   Extracellular space

Intracellular loop

■ Conserved among different connexins
▨ Divergent among different connexins
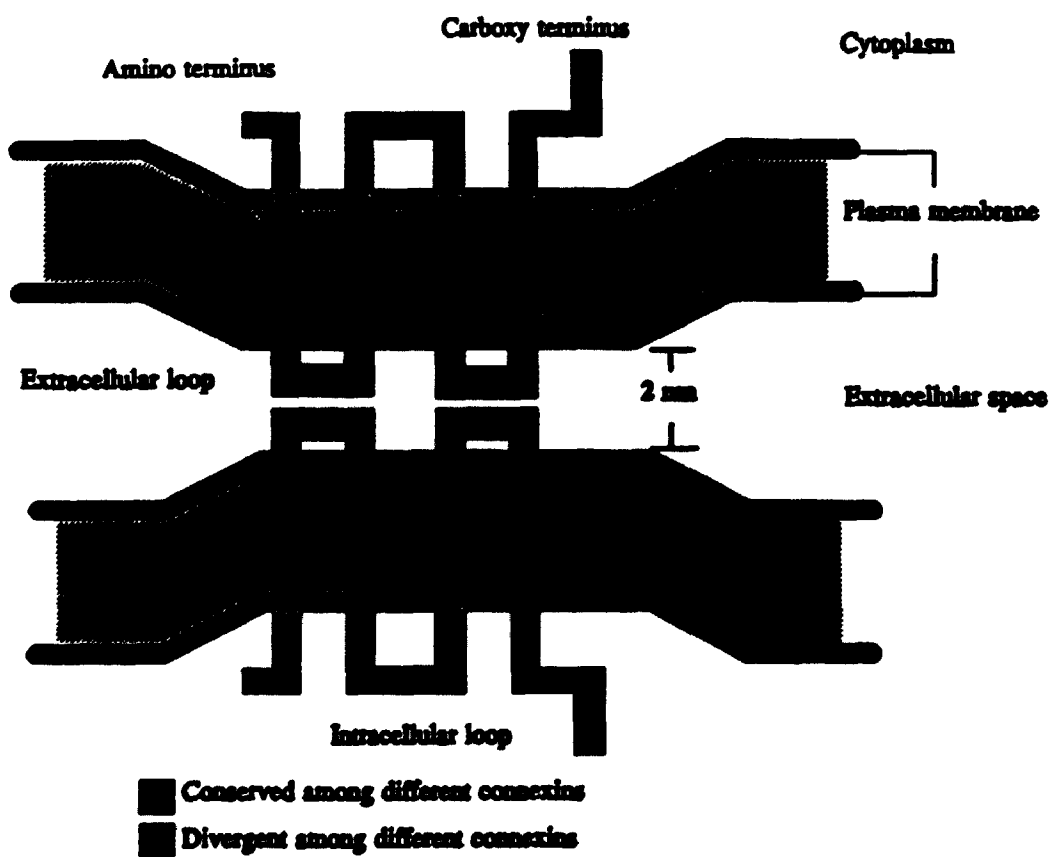
1986; Beyer et al. 1987). They possess four transmembrane domains and have their amino and carboxy termini on the cytoplasmic surface. Functional analyses involving the expression of connexin mRNAs in *Xenopus* oocytes or transfection into communication incompetent cells demonstrate that these cDNAs produce gap junction proteins which, in turn, form channels that are dye and electrically coupled (Paul et al. 1991; Hennemann et al. 1992d; Reed et al. 1993; Willecke et al. 1991; Fishman et al. 1990).

1.4.1 Chromosomal Localization

The majority of the mammalian connexins have been mapped to rodent chromosomes. In some cases, more than one gene is found on the same chromosome (Table 1); cx's 30.3, 31, 31.1 and 37 are on chromosome 4 (Hennemann et al. 1992a; Schwarz et al. 1992; Haefliger et al. 1992), cx26 and 42 are on chromosome 14 (Hsieh et al. 1991) and cx's 32 and 33 are on the X chromosome (Hsieh et al. 1991; Haefliger et al. 1992). Connexins 37 and 40 have been mapped to human chromosome 1 and cx26 and 46 to human chromosome 13 (Willecke et al. 1990).

1.4.2 Alternative nomenclature and connexin grouping

Although it is widely accepted to name connexin genes based on the

predicted Mr of the protein, some investigators have proposed a different system. Risek et al. (1990) and Gimlich et al. (1988) utilize the greek letters α and β to differentiate between two main groups of gap junction genes. The α/β system is based on genetic origin and sequence similarities at the nucleotide and amino acid level (Risek et al. 1990). Cx32 (Paul, 1986; Kumar and Gilula, 1986) and cx26 (Zhang and Nicholson, 1989) have greater homology to each other than they do with cx43 (Beyer et al. 1987). The proposed nomenclature termed cx43 an α gap junction and supplied the subscript 1 representing cx43 as the first α gap junction isolated (Risek et al. 1990; Gimlich et al. 1988). Cx32 is termed β_1 and cx26, β_2 (Table 1; Risek et al. 1990; Gimlich et al. 1988).

Based on the sequence similarities and differences, Bennett et al. (1991) proposed the grouping of connexin genes into two groups, I and II (Table 1). The division of gap junction genes into each group parallels the distinction made by Risek et al. (1990) and Gimlich et al. (1988) with respect to their α/β classification. An evolutionary tree demonstrating the relatedness of each connexin has been presented using the group I - group II classification scheme (Haefliger et al. 1992).

1.5 Distribution of gap junction proteins

Gap junctions can be found in almost all tissues except circulating blood cells and adult skeletal muscle. With the recent isolation of several novel

connexin genes, investigators have examined the tissue specific expression of many

gap junction proteins.

1.5.1 Plant kingdom

Gap junctions are not only present in mammals but also in organisms in

other phyla and classes. Intercellular communication between plant cells occurs

through structures called plasmodesmata (Meiners et al. 1988), providing a low-

resistance pathway between the cells. Investigations of connexin-like proteins has

revealed the presence of a 29 kD protein in soybean root with homology to cx32

(Meiners and Schindler, 1989). In addition, a cDNA encoding a 32 kD protein

having some degree of homology with cx32 has been isolated from soybean

(Meiners et al. 1991).

1.5.2 Animal kingdom

a) Invertebrates

In the animal kingdom, the first demonstration of intercellular

communication via gap junctions was in invertebrates (Furshpan and Potter, 1959;

Kanno and Loewenstein, 1964). Although slightly different in their structural

features when compared to mammalian gap junctions, arthropod gap junctions are

analagous structures (McVicar and Shivers, 1985; Berdan, 1987) and may contain

unique gap junction proteins (Buultjens et al. 1988).

b) Vertebrates

The presence of gap junctions span all vertebrate classes including the bony fishes, teleosts, which were shown to have electrotonic junctions at sites of synaptic contact (Robertson, 1963; Bennett et al. 1963). Cx32 has been demonstrated immunocytochemically throughout the electrosensory system of electic fish (Yamamoto et al. 1989).

Ebihara et al. (1989) isolated a novel connexin protein from *Xenopus* embryos. The 38 kD protein, named cx38, was present in oocytes (Ebihara et al. 1989). Gimlich et al. (1988) analyzed *Xenopus* embryonic development in detail and described three connexin proteins, α_1 (homologous to mammalian cx43), α_2 (cx38 described by (Ebihara et al. 1989) and β_1, a 30 kD protein homologous to cx32 of rat.

In birds, Beyer and colleagues have described the presence of three connexins in chicken heart (Musil et al. 1990; Beyer, 1990). They cloned the homologue to cx43 and two novel connexins, cx42 and cx45 (Table 1; Beyer, 1990). These connexins are differentially expressed in the developing heart and are found in various adult tissues including liver, stomach and pectoral muscle (Beyer, 1990). A connexin specific to chick lens has also recently been isolated and is called cx56 (Rup et al. 1993).

In mammals connexin genes have been isolated and sequenced from

rodents, dog, and human. During the molecular investigations of gap junction genes investigators examined the tissue distribution of these newly characterized members of the gap junction gene family.

1.5.3   Gap junction expression in various tissues

Table 2 demonstrates the variety of organs in which connexin genes have been identified. Much of the work has examined expression at the message level and in many cases, tissues contain more than one connexin. The following is a description of the connexin expression in select organs where sufficient data has been obtained.

a) Liver

In the organ where gap junctions were first isolated and sequenced, cx26 and cx32 are present in the same junctional plaques (Nicholson et al. 1987; Kuraoka et al. 1993) where these gap junction proteins contribute to intercellular communication, as assayed by function blocking antibodies (Hertzberg et al. 1985; Traub et al. 1989). Evidence suggests there is a regional expression of cx26 and 32 in periportal versus pericentral hepatocytes (Traub et al. 1989; Rosenberg et al. 1992). Cx26 protein and mRNA is more abundant in the periportal regions of the liver whereas cx32 is equally distributed throughout the tissue (Traub et al. 1989; Rosenberg et al. 1992). The only other connexin detected so far in liver is cx37

TABLE 2   **Tissue distribution of connexin genes**

The distribution of gap junction genes in various tissues. In many organs more than one connexin is expressed. Each connexin is compared individually with respect to abundance in many organs and cannot be directly compared with expression of other connexins in the same system. Single asterisks (*) indicate low levels, ** indicate moderate levels, and *** indicate high levels of expression. The dash (—) indicates that the particular connexin is not expressed in that tissue. Blank boxes mean that the connexin was not examined.

	liver	heart	kidney	spleen	vessel	uterus	ovary	testis	lung	stomach	brain	intestine	skin	leg
cx26	***	I	**	*	I			*	*	**	*	**	**	
cx30.3	I	I	I	I					I		I	I	**	
cx31	I	I	I	I				*	I		I	I	**	
cx31.1	I	I	I	I			I	*	I	I	I	I	***	I
cx32	***	I	**	*	I	*	I	*	*	**	**	*	I	I
cx33	I	I	I	I			I	***	I	I	I		I	I
cx37	*	**	*	*		*	*	*	***	*	*	*	*	I
cx40	I	**	*	I	**	*	**	I	***	I	I	*	I	I
cx43	I	***	*		**	*	**	*	*	*	***	*	*	*
cx45	I	**	*	I							*	*	I	
cx46	I	**	*	I		I	I				I			***
cx50	I	I	I	I			I	I	I	I	I		I	**

and it has only been examined at the message level (Haefliger et al. 1992; Willecke et al. 1991).

b) Heart

Cardiac gap junctions are found at intercalated discs where they function in the propagation of electrical activity associated v.ith contraction of the muscle (Page, 1992). The primary gap junction protein in heart is cx43, first described by Beyer et al. (1987; 1989). As seen in liver for cx32, inhibition of coupling occurred with the addition of antibodies to cx43 (Yancey et al. 1989). Table 2 shows that heart tissue also contains cx's 37, 40, 45 and 46 (Willecke et al. 1991; Haefliger et al. 1992; Kanter et al. 1992; Hennemann et al. 1992c; Paul et al. 1991). Cx40 and 45 are homologous to cx42 and 45, respectively, in chicken and the corresponding gap junction proteins are found at intercalated discs of canine ventricular myocytes when detected by immunocytochemistry (Kanter et al. 1992).

In developing heart, the cx43 protein is present from embryonic day 13 onward at which time it is detectable in myocarium of atria and ventricles (van Kempen et al. 1991; Fishman et al. 1991). The message for cx43 is similarly present in early heart development and increases in abundance until shortly after birth when it begins to gradually decreas. (Fromaget et al. 1990; Fishman et al. 1991). Gourdie et al. (1992) described the presence of MP70 (a lens gap junction protein) within the valves of adult heart tissue.

c) Lens

The lens is an avascular tissue composed of lens epithelial cells and lens fiber cells, which are interconnected by numerous intercellular junctions. Because of the avascular nature of lens, gap junctions are likely essential in the transport of nutrients throughout the tissue. Two membrane proteins have been isolated and characterized from lens fibers, a 26 kD membrane protein (MP26; Paul and Goodenough, 1983; Gorin et al. 1984) and a 70 kD membrane protein (MP70; Kistler et al. 1988), the latter sharing homology with gap junction proteins (Beyer et al. 1987; Donaldson and Kistler, 1992). In addition, MP70 shares similar structural features to that of gap junctions as well (Kistler et al. 1990) and recently the gene thought to encode MP70 has been isolated (White et al. 1992). Immunocytochemistry for mouse cx50 shows immunoreactivity to punctate regions of membrane in lens fiber cells but not epithelial cells (White et al. 1992). Other connexins present in lens are cx46 (Paul et al. 1991; Beyer et al. 1988) in lens fibers and cx43 found in lens epithelium (Beyer et al. 1987; Beyer et al. 1989).

d) Nervous system

Gap junctions are widely distributed in the central nervous system as detected by morphological and electrophysiological criteria (Dudek et al. 1983; Jaslove and Brink, 1987). Investigators have shown junctional complexes between oligodendrocytes (Brightman and Reese, 1969; Dermietzel et al. 1978), astroctyes (Brightman and Reese, 1969; Morales and Duncan, 1975; Massa and Mugnaini,

1982; Mugnaini, 1986) and neurons (Pinto da Silva and Martinez Palomo, 1975) by ultrastructural methods. These same cells are functionally coupled by gap junctions as demonstrated by electrical or dye coupling experiments. Neurons in the neocortex (Gutnick and Prince, 1981), striatum (Cepeda et al. 1989), inferior olive (Llinas, 1985), hippocampus (Andrew et al. 1982; MacVicar et al. 1982), and hypothalamus (Andrew et al. 1981) have been shown to be coupled. Intercellular communication is present in non-neuronal cells including oligodendrocytes (Von Blankenfeld et al. 1993; Ransom and Kettenmann, 1990; Kettenmann and Ransom, 1988), astrocytes (Gutnick et al. 1981; Kettenmann and Ransom, 1988; Giaume et al. 1991; Naus et al. 1991a; Dermietzel et al. 1991), ependyma (Jarvis and Andrew, 1988; Bouille et al. 1991), pinealocytes (Saez et al. 1991) and leptomeninges (Spray et al. 1991). The abundance of intercellular communication in the CNS adds to the complexity of cellular interactions that exist in the form of synaptic, trophic and molecular mechanisms. The molecular characterization of the gap junction gene family has increased the complexity further.

Dermietzel and Spray (1993) have recently reviewed the expression of various gap junction genes in the CNS. Protein or mRNA for numerous connexins have been documented in brain including cx26 (Zhang and Nicholson, 1989), cx32 (Paul, 1986; Nagy et al. 1988b; Shiosaka et al. 1989; Yamamoto et al. 1989a), cx37 (Haefliger et al. 1992; Willecke et al. 1991), cx43 (el Aoumari et al. 1990; Yamamoto et al. 1990a; Naus et al. 1991a), and cx45 (Hennemann et al. 1992c). The distribution of three of these connexins (cxs 26, 32 and 43) has been

examined in some detail, whereas the cell types of the CNS which express cx37 or cx45 are presently unknown.

Nagy et al. (1988) described the expression of cx32 in many regions of the CNS. The mRNA for the protein is present throughout the CNS as well (Micevych and Abelson, 1991). Immunocytochemical evidence and *in situ* hybridization studies suggest that cx32 is expressed in neurons (Yamamoto et al. 1989a; Shiosaka et al. 1989; Micevych and Abelson, 1991) and oligodendrocytes (Micevych and Abelson, 1991).

In primary cell culture, astrocytes express cx43 exclusively (el Aoumari et al. 1990; Giaume et al. 1991; Naus et al. 1991a; Batter et al. 1992; Dermietzel et al. 1991). In vivo, cx43 has been demonstrated immunocytochemically (Yamamoto et al. 1990b; Yamamoto et al. 1990a), the immunopositive cells being identified as astrocytes. The distribution of cx43 mRNA was also suggestive of astrocytic expression (Micevych and Abelson, 1991). The expression of cx43 is heterogenous in various areas of the adult CNS (Willecke et al. 1990; Carr et al. 1989). A similar heterogeneity was demonstrated in cultured astrocytes from different brain regions (Batter et al. 1992). This may represent region-specific syncytium formation leading to differential levels of spatial buffering as postulated by Mugnaini (1989).

Other non-neuronal cells have also been studied with respect to their connexin expression. Ependymal and leptomeningeal cells co-express cx26 and cx43 (Dermietzel et al. 1989; Spray et al. 1991) and pinealocytes express cx26

(Saez et al. 1991).

The function of gap junctions in the adult brain is not completely understood. In neurons, gap junctions are the morphological correlate of the electrical synapse, providing for the rapid transmission of electrical activity (Xie et al. 1992; Sotelo and Korn, 1978; Leitch, 1992; Jessell and Kandel, 1993). Astrocytes are thought to provide a syncytium whereby extraneuronal ions could be dissipated (Orkand, 1977). Recent evidence supporting this hypothesis shows that Ca^{2+} waves are propagated through astroctyes via gap junctions (Charles et al. 1991; Cornell-Bell et al. 1990; Finkbeiner, 1992; Charles et al. 1992). It is possible that gap junctions transfer nutrients or signalling molecules between neural cells but this has not been investigated in detail.

e) Other tissues

The previous sections described the distribution of gap junctions in select tissues. Many other tissues contain connexin genes as summarized in table 2 and deserve brief mention.

The endothelial cells and vascular smooth muscle of blood vessels express two connexins, cx40 (Beyer et al. 1992; Bruzzone et al. 1993) and cx43 (Pepper et al. 1992). Cultured endothelial cells establish intercellular communication with their neighbors, the level of which is markedly reduced when the cultures are 'wounded' (Pepper et al. 1989; Pepper et al. 1992). The level of coupling is seen to gradually return to and exceed basal level until the wound is healed (Pepper et

al. 1989). The increased coupling is accompanied by parallel increases in cx43 protein and mRNA (Pepper et al. 1992).

In reproductive organs numerous gap junctions are expressed. The uterus expresses four connexins (see table 2) but during pregnancy there is a modulation of cx32 and cx43 during implantation and onset of labour (Winterhager et al. 1988; Hendrix et al. 1992). The ovary and its surrounding cells have extensive gap junctions (Wert and Larsen, 1989; Eppig, 1991) but the recent localization of cx37 (Haefliger et al. 1992) and cx40 (Haefliger et al. 1992; Beyer et al. 1992) to ovary has not been further refined to cell type. Cx43, however, is present between ovarian granulosa cells (Beyer et al. 1989). In testis, cx43 has been localized to Sertoli and Leydig cells whereas cx26 and cx32 are present in seminiferous tubules (Risley et al. 1992). The other gap junction mRNAs present in testis (Table 2) have not been analyzed further.

In the epidermis, keratinocytes have been shown to express functional gap junction channels (Salomon et al. 1993). Numerous gap junction genes are expressed in this tissue (Table 2; Hennemann et al. 1992a,c; Haefliger et al. 1992), and expression has been localized to this cell type (Hennemann et al. 1992a,c).

1.6 Regulation of gap junction assembly and function

Metabolic cooperation has been considered one of the main functions of gap junctions since the time of their discovery (Hooper and Subak-Sharpe, 1981).

Gap junctions are permeable to many sorts of molecules including ions, nucleotides, amino acids and second messenger molecules such as Ca^{2+} and inositol 1,4,5-triphosphate (IP_3) (Saez et al. 1989; Spray and Burt, 1990; Saez et al. 1993). IP_3 mobilizes internal stores of Ca^{2+} (Berridge, 1993) and can pass through gap junctions to alter Ca^{2+} levels in neighboring cells (Saez et al. 1989). Alternatively, Ca^{2+} itself can be transferred via gap junctions (Saez et al. 1989). Numerous investigators have shown that waves of Ca^{2+} moving from cell to cell correlate with gap junctional coupling (Cornell-Bell et al. 1990; Charles et al. 1992). Prior to metabolic coupling, cells need to produce gap junction proteins and transport them to the membrane where channels can form, open and initiate communication.

1.6.1 Assembly and Disassembly of gap junctions

As cells come into contact with each other, they develop various intercellular junctions between their apposed membranes, including gap junctions. One of the initial steps prior to cell contact involves cell attraction by cell adhesion molecules (CAMs). Investigators have shown a correlation between the expression of CAMs and gap junction proteins (Keane et al. 1988; Jongen et al. 1991; Meyer et al. 1992). Transfection of E-cadherin into communication deficient cell lines that did not express CAMs resulted in an induction of gap junctional intercellular communication in E-cadherin expressing clones (Jongen et al. 1991).

Meyer et al. (1992) demonstrated a relationship between intercellular communication and N-cadherin by interrupting adhesion with function blocking antibodies directed against extracellular epitopes. This abolished gap junctional communication between the cells (Meyer et al. 1992). Presumably, intercellular contact induces events related to the formation of gap junction channels. If gap junctions in the membrane are influenced to bind to neighboring connexons or if they are induced to be transported to the membrane is presently unknown.

With respect to connexin trafficking, there is evidence that connexin proteins pass through cellular organelles on route to the membrane. Cx43 has been shown to enter the Golgi apparatus in studies using the protein trafficking inhibitor, monensin (De Sousa et al. 1993; Puranam et al. 1993). Once in the membrane, connexons must associate with their counterparts in the adjacent cell. This may involve disulphide bond formation either at the intramolecular or intermolecular level (Rahman and Evans, 1991; el Aoumari et al. 1991; Goldbeter et al. 1990).

During disassembly of gap junctions, usually one cell separates taking the whole gap junction complex, which is internalized in the forms of vesicles (Larsen and Tung, 1978; Mazet et al. 1985). The vesicles are associated with acid phosphatase activity (Larsen and Tung, 1978; Naus et al. 1993) suggesting that gap junction proteins are broken down rather than reutilized. This course of events is reasonable considering that gap junction proteins have relatively short half lives, ranging from one to three hours (Traub et al. 1987; Traub et al. 1989; Laird et al.

1991).

1.6.2 Functional Regulation

There are many levels at which gap junctions can be regulated including transcriptional and translational control, as well as post-translational events. The function of the gap junction channel itself, that is, its open or closed state (a process referred to as gating) can be influenced by a number of factors such as ion concentration, acidification and anesthetics (reviewed by Saez et al. 1993; Spray and Burt, 1990). Retinoids have been shown to upregulate and downregulate gap junctions depending on concentration and cell type (Mehta et al. 1989; Brummer et al. 1991; Pitts et al. 1986; Dewey and Barr, 1962). Rogers et al. (1990) have demonstrated that retinoids elevate cx43 mRNA and protein levels. Adenoside 3',5'-cyclic monophosphate (cAMP) is another substance that modulates intercellular communication, either decreasing (Sakai et al. 1992), or increasing gap junctional conductance (Burt and Spray, 1988). In examining the effects of cAMP on gap junctional communication, Loewenstein (1985) proposed that cAMP enhances communication through some phosphorylation event, possibly phosphorylating the gap junction protein directly.

a) Phosphorylation of connexin proteins

Phosphorylation of gap junctions was first shown by Saez et al. (1986) who

demonstrated that cAMP increased the incorporation of radiolabeled phosphate into cx32 protein and enhanced junctional conductance. This work suggested the involvement of a cAMP-dependent protein kinase. Further studies examined other kinases and detected similar phosphorylation events by protein kinase C (PKC) and Ca^{2+}/calmodulin-dependent protein kinase II (Saez et al. 1990). The phosphorylation of cx32 occured on serine233 (Saez et al. 1990).

Musil et al. (1990) described the phosphorylation of cx43 where the native protein of approximately 42 kD increased in Mr to ~45 kD. No shift of Mr was reported for cx32 (Saez et al. 1986; Traub et al. 1987). The apparent shift in Mr was shown to be due to the phosphorylation event (Musil et al. 1990). Musil and colleagues (Musil and Goodenough, 1990; Musil and Goodenough, 1991) have further characterized the phosphorylation of cx43 showing that various states of phosphorylation exist. Cx43 can exist as a non-phosphorylated protein (cx43-NP) and a ~44 kD cx43-P_1 state and a ~46 kD cx43-P_2 state, these proteins being phosphorylated on serine (Musil et al. 1990). The cx43-P_2 form is concentrated in gap junctional plaques whereas cx43-NP is predominantly intracellular (Musil and Goodenough, 1991). The role of phosphorylation in connexin assembly (Musil and Goodenough, 1991; Laird et al. 1991) or as a regulator of channel gating (Saez et al. 1993) is still unresolved. Possibly, phosphorylation may play a part in both functions.

Although cx43 is phosphorylated on serine there was no information with regard to the specific kinases involved. Saez et al. (1993) have recently provided

evidence suggesting the involvement of PKC. Synthetic peptides corresponding to the cytoplasmic region of cx43 were phosphorylated only by PKC and not cAMP-dependent protein kinase nor Ca^{2+}/calmodulin dependent protein kinase II (Saez et al. 1993).

Cx43 is also phosphorylated on tyrosine as shown in experiments using pp60[v-src] transformed cells (Swenson et al. 1990; Crow et al. 1992). The tyrosine kinase, pp60[v-src], phosphorylated cx43 on tyrosine resulting in the inhibition of gap junctional intercellular communication. Replacing tyrosine 265 with phenylalanine abolished the phosphorylation of cx43 and restored gap junctional communication (Swenson et al. 1990).

b) Gap junctional regulation in pathological conditions

The regulation of gap junctions occurs at many levels and involves a complex interaction of events. What is the result of improperly controlled gap junction regulation? It has long been suggested that the lack of intercellular communication is correlated with cancerous growth (Loewenstein and Kanno, 1967). This observation has been made in many cases of tumorigensis and is the subject of numerous reviews (Yamasaki, 1990; Klaunig and Ruch, 1990). The involvement of gap junctions in tumor formation has been studied by transfecting connexin genes into communication deficient tumor cell lines. Both cx32 (Eghbali et al. 1991; Eghbali et al. 1993) and cx43 (Mehta et al. 1991; Zhu et al. 1991) have been examined in this paradigm. Transfection with cx43 results in a significant

reduction of growth rate in culture (Mehta et al. 1991; Zhu et al. 1991) and in vivo (Naus et al. 1992). Cx32 transfectants, however, were unaffected in vitro but grew much more slowly in vivo when compared to their untransfected counterparts (Eghbali et al. 1991). It cannot be determined if decreased intercellular communication is the cause or effect of carcinogenesis, but it appears to be part of the process of tumor development.

Epilepsy is a disease state where a large population of neurons are sychronized to discharge abnormally, resulting in loss of control of voluntary motor movement. It has been postulated that altered electrical coupling in combination with shifts in extracellular ion concentration might contribute to epileptiform discharges (Dudek et al. 1986; Dudek et al. 1983). Recently, Naus et al. (1991b) have observed increased levels of cx32 and cx43 mRNA in biopsy samples from patients experiencing uncontrollable seizures, supporting the involvement of gap junctions in seizure disorders.

1.7 Gap junctions in development

Gap junctions are present in many adult tissues as well as developing systems (Bennett, 1973) where they can be found at the earliest stages of embryogenesis (Kidder, 1987; 1992). The unique characteristic of gap junction channels as a route for intercellular passage of low molecular weight molecules has been thought to play a role in pattern formation and compartmentation of the

developing organism (Warner, 1987; Fraser, 1990; Rivera et al. 1988; Caveney, 1985).

The developing mouse embryo has been the focus of much investigation of the role gap junctions play in this phenomenon (Kidder, 1987). Cx43 is detected very early in embryogenesis, its mRNA present in the four cell embryo (Valdimarsson et al. 1991; Nishi et al. 1991) and protein, arranged in gap junction-like plaques, at the 8 cell stage (Valdimarsson et al. 1991; De Sousa et al. 1993). Cx26 or cx32 message is not detectable in the early embryo (Nishi et al. 1991). Both protein and mRNA for cx43 is present during organogenesis in the mouse (Nishi et al. 1991; Ruangvoravat and Lo, 1992; Yancey et al. 1992) and is detected in many developing organs. The expression of cx26 and cx32 were more restricted during organ development (Nishi et al. 1991). Some of the most recently described connexin genes are differentially expressed in the developing versus adult organs. Connexins 31, 31.1, 37, 40 and 45 have been detected in embryonic skin and kidney, where the level of expression differs from that of the mature tissue (Hennemann et al. 1992a,c; Haefliger et al. 1992).

The role of gap junctions in development has been tested experimentally by the injection of antibodies or antisense RNA into early embryos (Warner, 1987; Fraser, 1990). Lee et al. (1987) and Bevilacqua et al. (1989) injected antibodies or antisense RNA, respectively, into blastomeres of the 2-4 cell stage embryo. This resulted in decompaction of the embryo or exclusion of the affected cell if only one blastomere was injected. In *Xenopus* embryos, Warner et al. (1984;

1987) injected antibodies to gap junction proteins into a single blastomere. Unlike mouse embryos which decompacted, the *Xenopus* embryo continued to develop but the resultant tadpole displayed abnormalities in regions normally derived from the injected blastomere (Warner et al. 1984; 1987). These results suggest that gap junctional intercellular communication is important for the proper maintenance of developmental events and interference results in termination of development (decompaction of mouse embryos) or generation of abnormalities in tissue organization (*Xenopus* embryos).

1.7.1 Muscle development

Adult skeletal muscle is unique in that it does not possess gap junctions. However, there is well documented evidence for the existence of gap junctions during the development of muscle. Kelly and Fallon (1983) demonstrated gap junctions in the limb bud of chick embryos. The limb bud also displays a gradient of gap junctional communication, assayed by dye transfer (Coelho and Kosher, 1991). Recently, the expression of cx32 and cx43 was detected in the developing mouse limb, and the two proteins were localized to separate regions of the limb bud (Laird et al. 1992). Balogh et al. (1993) have examined cultures of a muscle cell line and demonstrated that myoblasts express cx43 mRNA and protein and are dye coupled. The level of cx43 decreases as the cells differentiate and fuse forming myotubes and myofibres (Balogh et al. 1993). Muscle development

provides an interesting example of eliminating intercellular communication during the differentiation of mature tissue.

1.7.2 Development of the nervous system

The CNS contains morphologically defined gap junctions from the earliest stages of development (Mollgard and Moller, 1975; Revel and Brown, 1975). Gap junctions are expressed in numerous cell types as discussed in an earlier section (chapter 1.5.3). During development functional coupling, mediated through gap junctions, is modulated. Astrocytes are poorly coupled in the early stages of culturing, the extent of coupling increasing as the astrocytes mature (Fischer and Kettenmann, 1985). The coupled cells form an extensive syncytium coincident with maturation (Fischer and Kettenmann, 1985). In vivo, coupling among astrocytes was observed to increase substantially during postnatal development of the rat visual cortex (Binmoller and Muller, 1992). Binmoller and Muller (1992) could not detect dye spread prior to postnatal day 10 and suggested that functional coupling is a characteristic of mature astrocytes. Similarly, in cultures of the oligodendrocyte lineage, dye or electrical coupling was not detectable in immature stages of development (Von Blankenfeld et al. 1993). In the final stage of oligodendrocyte differentiation, dye coupling could be detected in 40% of the cells (Von Blankenfeld et al. 1993). The development of coupling in oligodendrocytes does not occur until it is committed to becoming an

oligodendrocyte. The developing precursor to oligodendrocytes has the potential to develop along a novel astrocyte lineage (O-2A; (Raff et al. 1983; Raff, 1989), where gap junctions have been detected morphologically (Waxman and Black, 1984; Black and Waxman, 1988) but intercellular communication could not be detected (Sontheimer et al. 1991b).

Neurons have been shown to be dye coupled in development but the pattern of coupling is reversed to that of glia. As the brain matures, less coupling is seen between neurons in the neocortex. Connors et al. (1983) described the reduction of coupling, assayed with Lucifer Yellow, from approximately 70% of neurons at postnatal day 1-4, to 40% at day 10, and 20% in the adult. The extent of coupling ranged from 3-7 neurons clustered around the injected cell. Similar observations were made by Walsh et al. (1989) in the developing neostriatum and LoTurco et al. (1991) in the embryonic cortex. In a recent study by Peinado et al. (1993), the tracer Neurobiotin was used to increase the sensitivity in detecting intercellular communication. A similar proportion of injections resulted in coupling (66% at postnatal day 5) but clusters involved many more neurons (up to 80). Coupling was observed until day 16, at which time it could not be detected (Peinado et al. 1993). These clusters of neurons may represent developmental columns (Peinado et al. 1993). Yuste et al. (1992) described the spontaneous increase in intracellular calcium in discrete clusters of neurons in the developing cortex. Discrete communication compartments likely serve a developmental role as such coupling is absent from mature cortex (Peinado et al. 1993; Yuste et al.

1992).

The molecular charaterization of gap junction expression in the developing CNS has described the presence of connexins 26, 32, 37, 40, 43 and 45 in embryonic or postnatal brain (Dermietzel et al. 1989; Yamamoto et al. 1992; Hennemann et al. 1992a,c; Willecke et al. 1991). The recently described connexin genes, cx37, cx40 and cx45 have only been detected at the tissue level, therefore no cellular distribution is presently known. In each case, the abundance of mRNA in the embryo is substantially higher than that found in the adult brain (Hennemann et al. 1992a,c; Willecke et al. 1991). Cx40 was not detectable in mature brain whereas it was present in the embryonic CNS (Hennemann et al. 1992d).

Detailed studies have examined the expression of cx26, cx32 and cx43 during development. Immunocytochemistry for each connexin during pre- and postnatal development showed differential expression of the proteins (Dermietzel et al. 1989). Cx43 and cx26 were present prenatally, cx43 continuuing to be expressed until adulthood whereas cx26 decreased rapidly following birth and was barely detectable beyond six days postnatal. Cx32 was not present in embryonic brain and was first detected at day 6 increasing thereafter (Dermietzel et al. 1989). Cx43 was attributed to astrocytes and leptomeninges, cx32 to oligodendrocytes and neurons, and cx26 to pinealocytes, leptomeninges and ependyma (Dermietzel et al. 1989). The presence of cx43 in developing astrocytes was examined in greater detail in an immunocytochemical study of the developing rodent brain (Yamamoto

et al. 1992) and the olfactory system (Miragall et al. 1992). Additionally, cx32 was

demonstrated in oligodendrocyte like cells of the developing olfactory system

(Miragall et al. 1992) and cx32 mRNA was found by *in situ* hybridization using

cDNA probes in neurons, glia and ependyma of the two day old rat (Matsumoto

et al. 1991).

1.8 Objectives

From this discussion, it is clear that gap junctions are a diverse family of

genes expressed in many tissues of the adult and during development. Gap

junctions are controlled at many levels including post-translationally and may

modulate developmental signals during the maturation of organ systems. The

CNS expresses numerous connexins during its development, some of which are

localized to specific cell types. The objective of the following investigation is to

examine the differential expression of gap junction genes during the development

of the CNS and to further examine the cellular specificity of connexin expression

in the different cell types of the brain. The expression of gap junction mRNA and

protein is examined in a model of neural development, P19 embryonal carcinoma

cells, for its potential in investigating the role of gap junction gene expression

during cell differentiation.

CHAPTER 2

Developmental expression of gap junction genes in rodent brain

2.1 Introduction

Gap junctions are intercellular membrane channels composed of connexons, hexameric assemblies of integral membrane proteins (Loewenstein, 1981; Loewenstein, 1987). These channels provide an avenue for intercellular communication, allowing for the passage of small molecules and ions. In the adult, the protein subunits of connexons (i.e. connexins) appear to be expressed in a partially tissue-specific fashion (Table 2). The characterization of gap junction protein in the brain provides evidence for cx32 (Paul, 1986; Dudek et al. 1988; Nagy et al. 1988; Dermietzel et al. 1989), cx43 (Sasaki et al. 1988; Dermietzel et al. 1989) and cx26 (Dermietzel et al. 1989).

During development, there is extensive regulation of the spatial and temporal expression of gap junctions, this regulation being implicated in the control of differentiation and growth (Revel and Brown, 1975; Bennett et al. 1981; Caveney, 1985; Kidder et al. 1987). In general, there is high intercellular coupling during early development, with a loss or attenuation of coupling during terminal differentiation (Fujisawa et al. 1976; Bennett et al. 1981). Dramatic evidence for

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the necessity of gap junctions during embryonic development has been provided by utilizing antibodies to gap junction proteins. Injection of such antibodies into amphibian (Warner et al. 1984; 1987) or mouse (Lee et al. 1987) embryos results in severe developmental anomalies.

In the mature mammalian nervous system, gap junctions appear to be restricted to specific areas, being identified in neurons, ependymal cells, astrocytes and oligodendrocytes (Mollgard and Moller, 1975; Dermietzel et al. 1978; MacVicar et al. 1982; MacVicar and Dudek, 1982; Kosaka, 1983a; Kosaka, 1983b; Dudek et al. 1983; Mugnaini, 1986; Nagy et al. 1988; Massa et al. 1984; Massa and Mugnaini, 1982; Yamamoto et al. 1990b; Dermietzel et al. 1989). In the neocortex and hippocampus, evidence suggests that there is some intercellular coupling of neurons in vivo (Sloper and Powell, 1978; Sotelo and Korn, 1978; MacVicar et al. 1982; Kosaka, 1983a,b). Examination of intercellular coupling using dye transfer or electrical recording in vitro suggests this phenomenon is more extensive. In slice preparations of adult rodent neocortex, 20% of neurons were coupled (Gutnick et al. 1981; Connors et al. 1983). More recently, immunocytochemical evidence from both immunocytochemical and *in situ* hybridization analysis suggests that gap junctions are distributed throughout various regions of the adult CNS (Nagy et al. 1988; Shiosaka et al. 1989; Yamamoto et al. 1989a; Micevych and Abelson, 1991).

During neocortical development, there is a high degree of intercellular coupling between neurons. Thus, in slices of postnatal day 4 (P4) rat neocortex,

as many as 80% of the neurons examined were dye-coupled, declining to 20% in the adult (Connors et al. 1983). In the ventricular zone of embryonic day 18 rats, clusters of neurons are coupled and as these cells migrate from the ventricular zone, the degree of coupling decreases (Lo Turco and Kriegstein, 1991). Peinado et al. (1993) observed that clusters of coupled neurons in the neonatal rat cortex follow a developmental decrease in the number of coupled cells found in a cluster. This early electrotonic coupling via gap junctions may function in providing necessary electrical activity for subsequent differentiation. Alternatively, the possibility of intercellular transfer of some morphogen cannot be excluded (Bennett et al. 1981; Caveney, 1985).

The objective of the present study was to examine the developmental appearance of gap junction mRNA and protein in the mouse and rat brain by Northern and Western blotting techniques. The regional expression of gap junction mRNA was also examined in the adult by Northern blot analysis.

2.2 Materials and Methods

2.2.1 RNA Isolation

Total cytoplasmic RNA was extracted from the forebrain or hindbrain (i.e. rostral or caudal to the midbrain-diencephalic junction, respectively) of 5 to 8 BALB/c mice at embryonic day 18 (plug date considered day 0) and postnatal days 0, 5, 10, 16, 20, 30 and adult, and 2 to 4 Sprague-Dawley rats at postnatal days 1, 5, 10, 15, 20, 30 and 60. Total cytoplasmic RNA was isolated from freshly dissected tissue by extraction with phenol-chloroform-isoamyl alcohol (Schibler et al. 1980). The tissue was finely chopped with RNase-free scissors (baked at 250C for 5 hr), washed once with RNase-free PBS (10mM Na_2HPO_4, 150 mM NaCl, pH 7.2) followed by a wash with cell lysis buffer (20mM Tris-HCl [pH 8.8], 200 mM NaCl, 20 mM $MgCl_2$). Using a douce homogenizer, the tissue was homogenized in 10 ml of fresh lysis buffer by passing the plunger up and down 8-10 times. The resultant cell suspension was transferred to a 30 ml corex centrifuge tube and centrifuged at 5 000 rpm for 10 min at 4C to remove cell debris. The supernatant was transferred to a 50 ml falcon "Blue Max" tube (Canlab) containing an equal volume of a second buffer (2% SDS, 40 mM EDTA, 100 mM NaCl, pH 8.0) and 10 μl of proteinase K (10 mg/ml) and left at room temperature for 20 min prior to extraction. Two and one half volumes of phenol-chloroform-isoamyl alcohol was used to extract the tissue. After vigorously

shaking the samples were centrifuged at 3 500 rpm for 15 min at room temperature. The aqueous layer (upper layer) was removed to another tube and the procedure repeated until the interface was clear. A final extraction with chloroform-isoamyl alcohol was performed to remove residual phenol. The samples were precipitated with 0.3 M $NaC_2H_3O_2$ and 95% ethanol at -20 C.

Total cytoplasmic RNA was extracted from various regions of 4 adult Sprague-Dawley rat brains in order to determine the regional distribution of connexin mRNA in adult CNS. The areas dissected included the cerebellum, pons, medulla oblongata, midbrain, thalamus/striatum, amygdala/pyriform cortex, hippocampus, frontal cortex, parietal/occipital cortex, and olfactory bulb.

2.2.2 Northern Blot Analysis

For detection of cx32 and cx43 mRNA by Northern blot hybridization analysis, 10 μg aliquots of total cytoplasmic RNA, as determined by measurements of optical density at 260 nm, were dissolved in 50% formamide containing MOPS (20 mM 3-N-morpholino-propanesulphonic acid) and denatured with 3.7% formaldehyde at 55°C for 15 min. The reaction was stopped on ice and loading buffer was added (0.4% bromphenol blue, 1mM EDTA, 50% glycerol). Samples were subjected to electrophoresis on a denaturing agarose gel for 3 hr (Rave et al. 1979) and transferred overnight by capillary action to nitrocellulose using 10X SSC as a buffer (Thomas, 1980). After baking in a vacuum oven at 80°C for 2 hours,

the blots were prehybridized for 3 hr at 42°C in a solution containing 50%

formamide, 5 X Denhardt's solution (1X= 0.02% each of Ficoll, bovine serum

albumin (BSA) and polyvinylpyrrolidone; (Denhardt, 1986), 5 X PIPES (750 mM

NaCl, 25 mM 1,4-piperazinediethylene sulphonic acid, 25 mM EDTA, pH 6.8),

0.2% sodium dodecyl sulphate (SDS) and 100 μg/ml herring sperm DNA.

a) Radiolabeling of DNA probes

The same blots were hybridized with the cx32 cDNA (1.5 kb) and cx43 cDNA (1.4

kb) probes (kindly provided by Dr. David Paul, Harvard University; see Paul,

1986; Beyer et al. 1987). The cDNAs were radiolabeled by nick translation (Rigby

et al. 1987) or random priming (Feinberg and Vogelstein, 1983) using kits from

Gibco-BRL. One μg of plasmid DNA was used for nick translation and 25 ng

linearized DNA used for random priming. The method followed that provided by

the manufacturer.

b) Hybridization and post-hybridization washes

Hybridization was performed overnight at 42°C in a solution containing 50%

formamide, 5 X Denhardt's, 5 X PIPES, 0.2% SDS. Following hybridization,

membranes were washed at room temperature for 45 min in 2X standard saline

citrate (SSC), 0.2% SDS, followed by 45 min. in 0.5X SSC, 0.2% SDS at 37°C, and

0.15X SSC, 0.2% SDS at 65°C. Autoradiographs were obtained by exposing

Kodak XAR film to the hybridized membranes. To determine the equivalence of

RNA amounts in each lane, the same membranes were hybridized with a cDNA

probe for 18S rRNA (provided by Dr. D. Denhardt, Rutgers University).

2.2.3 Quantitative Analysis of Connexin mRNAs

Following hybridization with the radiolabeled cDNAs for cx32 and cx43, the

blots were left to decay completely then rehybridized with the cDNA for 18S

rRNA. This was done to determine that equal amounts of RNA were loaded into

each lane. The relative levels of cx32 and cx43 mRNA were calculated for each

time point using an LKB Densitometric scanner and normalized with reference to

the level of 18S rRNA. The Northern analysis was repeated three times on the

same samples to ensure that the same pattern was observed.

2.2.4 Western Blot Analysis

A plasma membrane-enriched protein fraction was isolated from the brain

of two to four Sprague-Dawley rats at postnatal day 5, 12, 20 and adults and from

adult liver and heart. The brain was divided into forebrain and hindbrain samples

as described for the RNA isolation. The procedure used was that of Hertzberg

(1984; 1992). Tissue was homogenized in 20mM NaOH, centrifuged twice at

20,000 rpm, and resuspended in 1mM $NaHCO_3$. Following a third centrifugation,

the protein was resuspended in a minimal volume of bicarbonate buffer and

stored in loading buffer (2% SDS, 1mM phenylmethyl sulfonyl fluoride, 5% ß-mercaptoethanol, 20% glycerol, in 80mM Tris-HCl pH 6.8) at -20°C. Due to interference from myelin in the brain samples during electrophoresis, these samples were treated to remove myelin prior to the NaOH extraction procedure. The method followed was that of Cuzner et al .(1965). The tissue was homogenized in 0.32 M sucrose, 1 mM EDTA (pH 7.4) and centrifuged briefly at 5,000 rpm to remove nuclei and cell debris. The supernatant was decanted to a new tube and centrifuged at 10,500 rpm for 15 min to give a pellet containing most of the myelin. The supernatant was diluted with one volume of NaOH and centrifuged at 20,000 rpm. The remainder of the protocol followed that of Hertzberg (1984).

Samples for immunoblotting were resolved on 12% SDS polyacrylamide gels (Laemmli, 1970). Fifty μg of protein, based on Lowry determinations, were loaded into each lane, and gels were run in triplicate. One of the gels was stained with Coomassie Blue (Fig. 2.4A). Electrophoretic transfer to nitrocellulose was carried out in a BioRad apparatus at 75 volts for one hour in Laemmli buffer (25 mM Tris, 192 mM glycine pH 8.3, 20% methanol). The nitrocellulose blot was incubated in 5% non-fat dry milk (NFDM) in phosphate buffered saline (PBS - 120mM $NaH_2PO_4 \cdot H_2O$, 95mM NaOH, 70mM NaCl, pH 7.4) for one hour at 37°C. The blot was incubated overnight at 4C with a monoclonal antibody to cx32 (antibody M1213; Goodenough et al. 1988) diluted 1:10 in PBS, then washed in PBS and incubated with a secondary antibody (biotinylated anti-mouse IgG -

Vectastain ABC kit, Vector Laboratories) in PBS-NFDM for one hour, followed

by several washes in PBS and incubated with Avidin DH and biotinylated Alkaline

Phosphatase reagent. To visualize antibody binding, the membrane was reacted

with 3.3 mg nitroblue tetrazolium, 1.65 mg 5-bromo 4-chloro 3-indolyl phosphate

in 10 ml alkaline phosphatase buffer (100 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$

pH 9.5). Duplicate blots were incubated for two hours at room temperature with

a polyclonal antibody against cx43 (directed against a.a. 252-271; Beyer et al.

1989), diluted 1:1000 in PBS-NFDM. The blot was then treated with Vectastain

ABC kit as described above. For each of the connexin antibodies, three

immonublots were processed to determined the developmental pattern of

connexin protein expression.

2.3 RESULTS

2.3.1 Developmental Pattern of Connexin43 mRNA

Northern blot analysis of RNA isolated from mouse and rat brain at various developmental times revealed a relatively high level of cx43 mRNA in both the forebrain and hindbrain (Fig. 2.1A, 2.2A upper band). The cDNA hybridized to a single mRNA band of approximately 3.0 kb. This is similar to the mRNA present in adult heart, with no message detectable in adult liver (Fig. 2.1A). The lower band in the liver lane, at 1.6 kb, represents residual radioactivity from previous hybridization of this blot with the cx32 cDNA (Fig. 2.1B). Cx43 mRNA is detected in brain as early as embryonic day 12 (data not shown) and readily detectable by E18 (Fig. 2.1A). Postnatally, there appears to be an increase in the level of this mRNA from birth (PO) to P30 or adult. This pattern is observed in both the forebrain and hindbrain and reproducible in each of the three Northern blots analyzed.

2.3.2 Developmental Pattern of Connexin32 mRNA

Hybridization of these same Northern blots with the cDNA for cx32 mRNA revealed a single RNA band, of approximately 1.6 kb, present in liver but absent in heart (Fig. 2.1B). In hindbrain samples of mouse, cx32 mRNA is barely detectable

FIGURE 2.1 Northern blot analysis of connexin32 and 43 in developing mouse brain

Northern blot analysis of the mRNA for the gap junction proteins connexin43 and connexin32 in the developing mouse brain. Approximately 10 μg of total RNA was loaded into each lane. A) Connexin43 mRNA is easily detectable in embryonic day 18 (E18) brain. The cDNA for connexin43 mRNA hybridizes to a single band of RNA in brain, similar in size (approximately 3.0 kb) to the mRNA recognized in heart (H). No connexin43 mRNA is detectable in liver (L). The lower band in liver represents connexin32 mRNA from a previous hybridization with the cDNA for connexin32. In RNA isolated from forebrain, connexin43 mRNA gradually increases with development, from birth (PO) to postnatal day 30 (P3O), decreasing slightly in the adult (A). In hindbrain, connexin43 mRNA is also detectable at these early times, its expression level increasing with age. The arrow represents position of the 28S ribosomal RNA. Exposure time was 2 days. B) The cDNA for connexin32 mRNA hybridizes intensely to a single band of approximately 1.6 kb in RNA isolated from liver (L) but not from heart (H). This mRNA is not readily detectable in forebrain until P16. In contrast, connexin32 mRNA is easily detectable in hindbrain by P10. Again, the level of this mRNA decreases in adult forebrain and hindbrain. The arrow represents the position of the 18S ribosomal RNA. Exposure time was 5 days. C) Hybridization of the same Northern blot with a cDNA probe to 18S rRNA shows similar levels of RNA have been loaded onto each lane.

FOREBRAIN   HINDBRAIN

E18 L H P0 P5 P10 P16 P30 A P0 P5 P10 P16 P30 A

A.

B

C
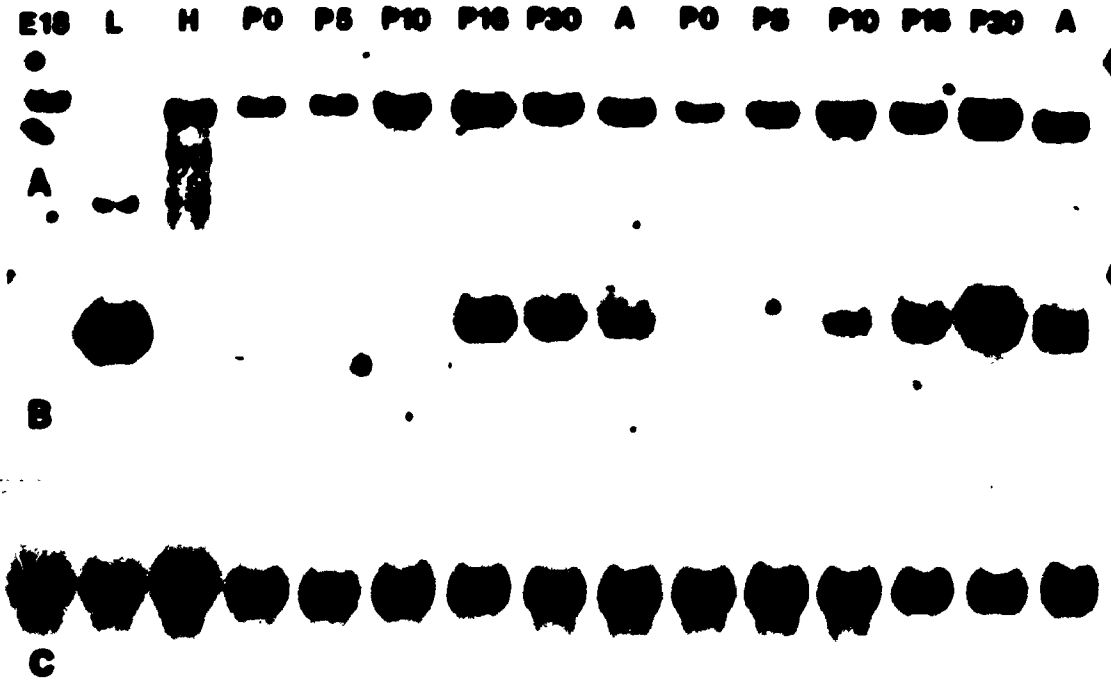
FIGURE 2.2 Northern blot analysis of connexin32 and 43 in developing rat brain

Northern blot analysis of the mRNA for the gap junction proteins connexin43 and connexin32 in the developing rat brain. Approximately 10 μg of total RNA was loaded into each lane. A) Connexin43 mRNA (upper band) is detectable by postnatal day 1 (P1) and continuously increases in abundance to day 30 (P30), slightly decreasing by postnatal day 60 (P60). The accumulation of mRNA for connexin32 (lower band) shows a similar pattern to that seen in the mouse, being readily detectable at approximately P10 in the hindbrain and P15 in the forebrain. The levels of this mRNA also increase with development. The filled arrow represents position of the 28S ribosomal RNA, and the open arrow the position of the 18S ribosomal RNA. Exposure time was 3 days. B) Hybridization of the same blot with a 18S rRNA cDNA probe shows that similar levels of total RNA were loaded onto the lanes.

FOREBRAIN   HINDBRAIN
P1 P5 P10 P15 P20 P30 P60   P1 P5 P10 P15 P20 P30 P60

A

B

at P5, increasing substantially beyond P5 (Fig. 2.1B). In the hindbrain of rat, the level of cx32 mRNA increases rapidly beginning at P10 (Fig. 2.2A lower band). The level of cx32 mRNA in the foreb ·.n at this time is not readily detectable, but increases substantially by P15-16 (Fig. 2.1B, 2.2A lower band).

The developmental expression of cx26 was also examined by Northern analysis. The signal for cx26 was weak at all ages examined and particularly low after postnatal day 10.

2.3.3   Relative levels of Connexin mRNAs

The relative amount of connexin mRNA in the rat and mouse forebrain and hindbrain at different postnatal times was determined by densitometric scanning of autoradiograms shown in Figure 2.2. To assure equivalence in the amount of total RNA per lane, hybridization of the same blots was carried out with a cDNA probe for 18S rRNA (Fig. 2.1C, 2.2B). The results of the densitometric scanning are summarized in Figure 2.3. The intensity of the hybridization reflects the relative amount of connexin mRNAs at each time point. Values for the relative levels of connexin mRNAs were normalized with reference to the level of 18S rRNA. Cx43 mRNA in the rat brain is detectable at birth (P0) and increases thereafter. The temporal pattern of increasing mRNA is obvious in the rat but less so in mouse. In contrast, cx32 mRNA is not readily detectable until P10 in the hindbrain and P15 in the forebrain of rat. In mouse, however,

FIGURE 2.3 Relative level of connexin32 and 43 in developing rodent brain

Histogram depicting the relative level of connexin43 and connexin32 mRNA in the forebrain and hindbrain of the rat and mouse at different postnatal times. The relative amount of hybridization signal was determined using densitomentric scanning of the autoradiograms from the Northern blots in Figures 1 and 2 and normalized to 18S rRNA signal. While connexin43 mRNA is readily detectable from birth onwards, the level of connexin32 mRNA becomes detectable only after P5 or P10. In addition, connexin32 mRNA appears in the hindbrain earlier than in the forebrain.

Mouse - Connexin 43

Mouse - Connexin32

Rat - Connexin43

Rat - Connexin32

Postnatal Development

Forebrain   Hindbrain
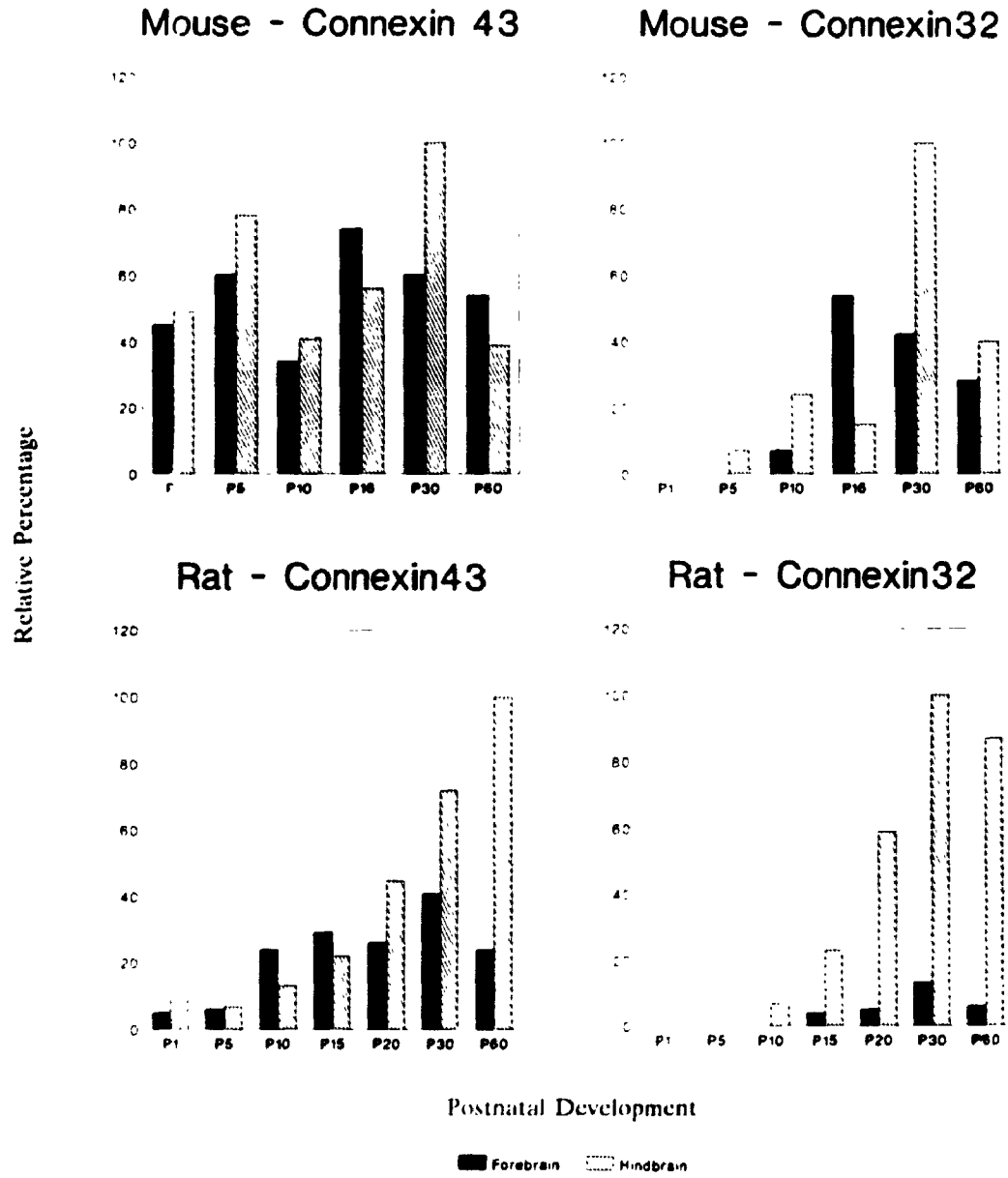
cx32 is present at P5 in the hindbrain and P10 in forebrain. Longer exposure times were required to detect cx32 mRNA in P5 forebrain. It should also be noted that the level of connexin mRNA is generally higher in hindbrain than forebrain samples.

2.3.4 Western Blot Analysis of Connexin Proteins

The appearance of connexin protein was examined by Western blotting utilizing antibodies specific for liver and heart gap junction proteins. Immunoblots of membrane-enriched protein fractions show cx32 protein in liver (Fig. 2.4B; apparent Mr 28 kD). In addition, an aggregate of cx32 is present at approximately 48 kD, as previously reported (Paul, 1986). Brain samples showed detectable levels of cx32 at P5 through to adult (Fig. 2.4B). The level of cx32 protein detectable in adult forebrain was less than that in adult hindbrain (Fig. 2.4B). The adult samples showed a doublet at approximately 27-28 kD. Each brain sample also shows a 33 kD band which is not seen in the liver lane. This may be an aggregation product of connexin proteins caused by the altered isolation method as it was not seen in Western blots of protein samples not treated to remove myelin. It is also possible that this is crossreactivity with the 33 kD breakdown product of cx43 (Manjunath et al. 1984) since this band is not seen in liver, which has no cx43 protein. A similar pattern of appearance of this protein was observed with a polyclonal antibody against cx32 (Paul, 1986)(data not

FIGURE 2.4 Western blot analysis of connexin32 and 43 in developing rat brain

Western blot analysis of membrane-enriched protein samples. Approximately 50 μg of protein was loaded into each lane. The liver and heart lanes had only 25 μg each. Amersham rainbow molecular weight standards were used. A) Coomassie blue stained replica gel. The amount of protein loaded in each lane is approximately equal except for P5 hindbrain which is slightly underloaded. B) When incubated with an antibody against connexin32, an intense 28 kD band was observed in the liver lane (L). In addition, a 48 kD band represents a larger molecular mass aggregate of this protein. The antibody reacted with a 28 kD band in each of the brain samples examined (postnatal day 5 (P5) to adult) in fore- and hindbrain. The adult lanes show a doublet at 27-28 kD. In addition to the 28 kD band, a second faint band was detected with a molecular weight of approximately 33 kD, possibly crossreactivity with a breakdown product of connexin43 (see text for more details). C) With a connexin43 antibody, a 43 kD band was detected in the heart lane (H). The antibody reacted primarily with a 43 kD polypeptide in P5 brain samples. Later postnatal brain samples showed a 41 kD protein, and a 43 kD component. The doublet is more distinquishable in hindbrain samples.

FORE   HIND

P5 PI2 P20 A   P5 PI2 P20 A   L

A

46kD◊
30kD◊

B

H

46kD◊

C
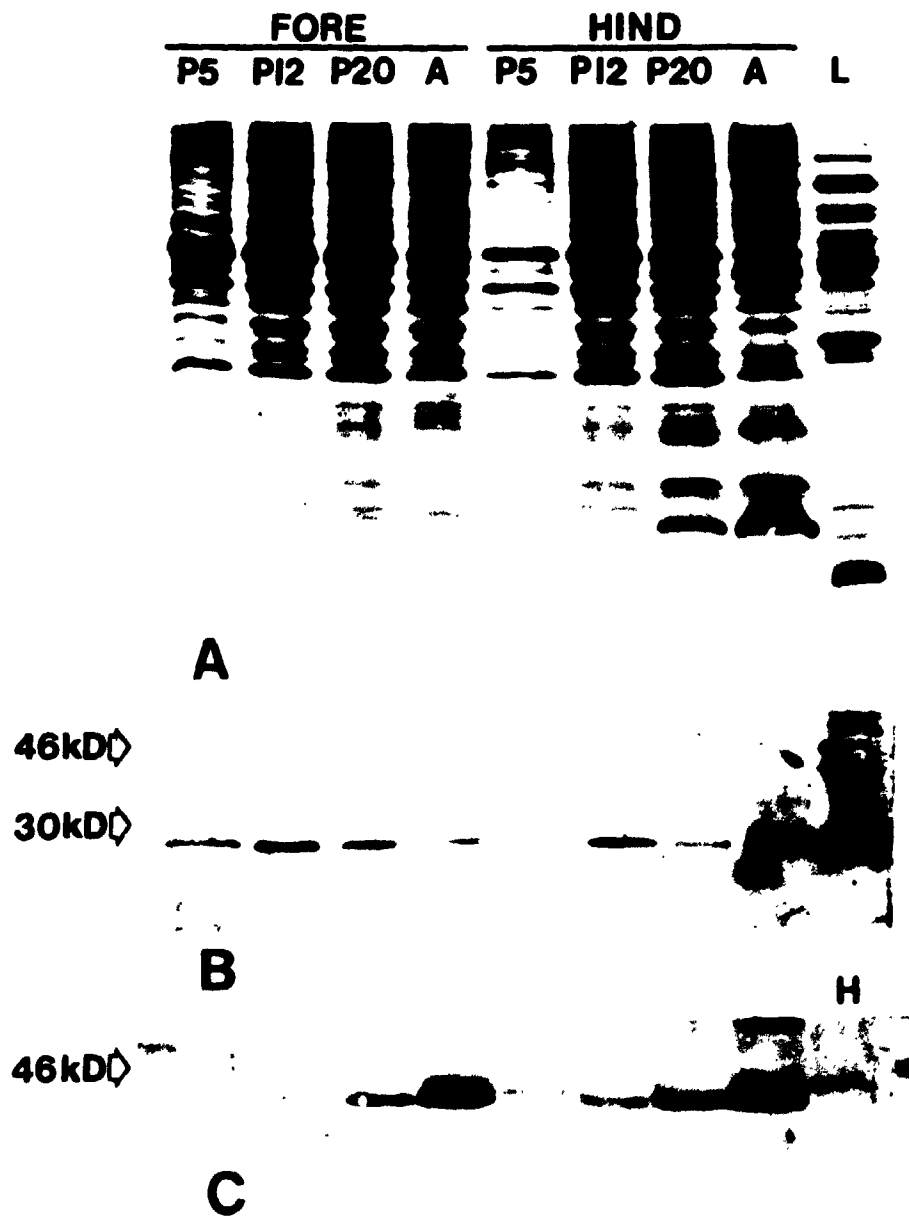
shown).

Western blots incubated with polyclonal antibody to cx43 revealed the presence of this gap junction protein in heart (Fig. 2.4C). Cx43 was detectable in all brain samples analyzed, with increasing amounts of protein from P5 to adult in both forebrain and hindbrain (Fig. 2.4C). While the cx43 in heart had an apparent Mr of 43 kD, the protein recognized by this antibody in brain had a Mr of approximately 41-43 kD. Postnatal day 12 and 20 brain samples had predominately a protein doublet of 43 kD and 41 kD with the doublet more pronounced in hindbrain. The adult samples may have a doublet but the intensity of the signal makes it difficult to be certain. Postnatal day 5 samples showed only a single band at approximately 43 kD.

2.3.5 Regional expression of Connexin32 and 43 mRNA

The level of connexin43 mRNA was found to be generally equivalent in the different brain regions examined in the adult (Fig. 2.5A). In contrast, connexin32 mRNA appears heterogeneously distributed in the brain (Fig. 2.5A,B). It is evident that brainstem regions, including the midbrain, pons and medulla, contain higher levels of connexin32 mRNA than cerebellum and forebrain regions.

Densitometric analysis of these Northern blots provides relative quantitative data concerning regional mRNA distribution. The densitometric values were normalized with respect to the level of 18S ribosomal RNA (Fig. 2.5C). In

FIGURE 2.5 Regional analysis of connexin expression in CNS

Northern blot analysis of the mRNA isolated from adult cerebellum (1), pons (2), medulla (3), midbrain (4), striatum/thalamus (5), amygdala/pyriform cortex (6), hippocampus (7), parietal/occipital cortex (8), frontal cortex (9) and olfactory bulb (10). Ten μg of total RNA was loaded into each la..e. The connexin43 cDNA hybridizes to a single mRNA at 3.0-kb in size (A, upper band). This mRNA appears to be evenly distributed in different regions of the brain. The connexin32 cDNA hybridizes to a single 1.6-kb mRNA (A, lower band) and is differentially distributed throughout these regions of the brain. Connexin32 mRNA is most readily detected in the midbrain/pons/medulla sample while other regions of the brain contain considerably less connexin32 mRNA. The same blot was first hybridized with connexin43 cDNA, followed by connexin32 cDNA, and then exposed to X-ray film for 5 hours. When this same blot was exposed for 18 hours, connexin32 mRNA is readily detectable in all brain regions examined (B). To ensure that equivalent amounts of RNA were loaded into each lane, the same blot was hybridized with the cDNA for 18S ribosomal RNA (C).
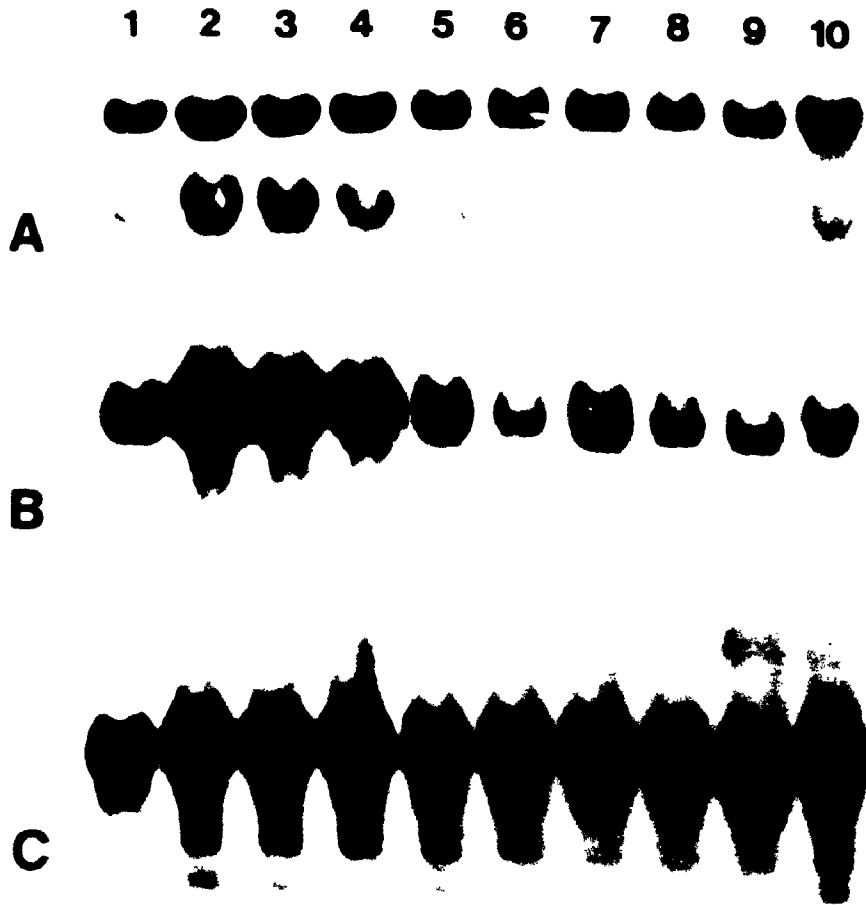
general, the level of connexin43 mRNA varies little between the regions examined (Fig. 2.6A). Only the olfactory bulb sample differs substantially, with a much higher level of this mRNA. In contrast, the level of connexin32 mRNA varies greatly between these different regions, being particulary abundant in the hindbrain regions (Fig. 2.6B).

Since the cDNAs are of equivalent size, and were labeled to similar specific activities, hybridization of the same Northern blot followed by exposure of the same length of time allow for an estimate of the relative abundance of these mRNAs. After a 5 hour exposure, it is evident that the level of connexin32 mRNA is generally lower than connexin43 mRNA (Fig. 2.5A), and longer exposure times are required to detec. connexin32 mRNA (Fig. 2.5B).

FIGURE 2.6 Relative levels of connexin32 and 43 mRNA in adult rodent brain

Densitometric analysis of the Northern blots indicates that the level of connexin43 mRNA is equivalent in most regions examined, with the exception of the olfactory bulb (A). In contrast, the level of connexin32 mRNA varies dramatically between these different regions (B). The abbreviations are as follows: CE, cerebellum; HIND, brainstem minus pons; mid, midbrain, ST, striatum/thalamus; AP, amygdala/piriform cortex; HI, hippocampus, PO, parietal/occipital cortex; FC, frontal cortex; OB, olfactory bulb.

Relative Level of Connexin43 mRNA
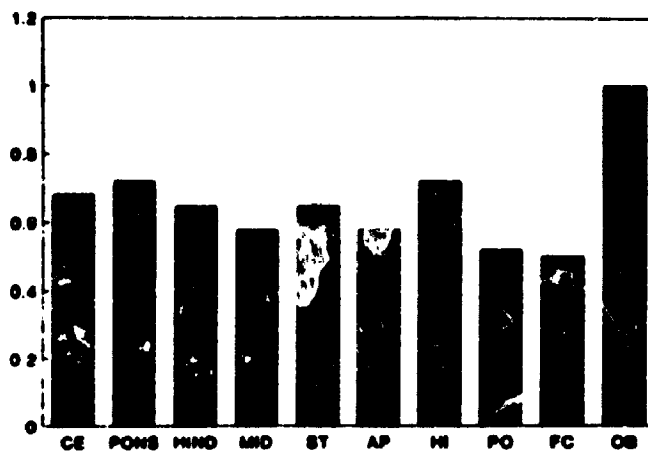
A

Relative Level of Connexin32 mRNA
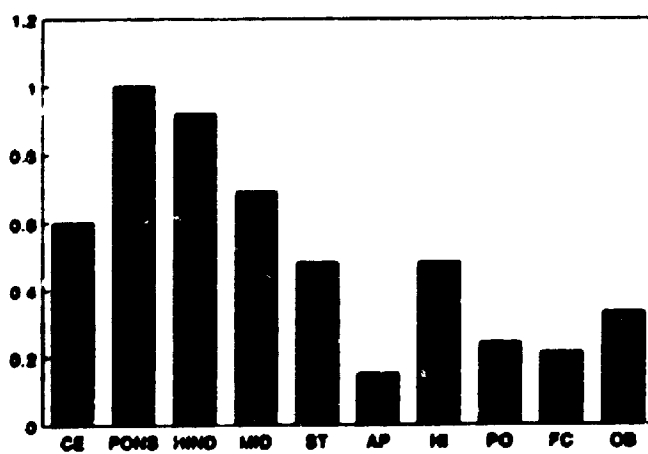
B

2.4 DISCUSSION

Gap junctions were previously considered prevalent in invertebrate and lower vertebrate nervous systems, presumably functioning as a "primitive" form of interneuronal communication (Eccles, 1964). Initial observations in the mammalian central nervous system in vivo, based primarily on electrical coupling and ultrastructural observations, indicated the presence of gap junctions in various cranial nerve nuclei and the inferior olivary nucleus (reviewed by Llinas, 1985). In addition to intercellular coupling between neurons, morphological data indicated the existence of gap junctions between glial cells, including astrocytes, oligodendrocytes and ependymal cells (Dermietzel et al. 1978; Mugnaini, 1986). Later studies showed the presence of neuronal dye-coupling or gap junction-like immunoreactivity in other areas of the CNS in vivo, such as the hippocampus (MacVicar et al. 1982; Kosaka, 1983b; Nagy et al. 1988; Shiosaka et al. 1989; Yamamoto et al. 1989a) and neocortex (Gutnick and Prince, 1981; Sloper and Powell, 1978). In general, the in vivo electrophysiological and morphological demonstration of gap junctions in the mammalian CNS suggested a relatively restricted distribution. The presence of gap junctions has been more widely demonstrated in slice preparations of the mammalian CNS in vitro (Takato and Goldring, 1979; MacVicar and Dudek, 1980; Andrew et al. 1981; Gutnick et al. 1981; Gutnick and Prince, 1981; MacVicar and Dudek, 1981; Andrew et al. 1982; Dudek et al. 1983). Such studies have suggested that as many as 20% of neurons

are dye-coupled in slices of adult neocortex, with levels as high as 80% in neonatal

cortex (Andrew et al. 1982). Electrophysiological and morphological evidence for

such extensive intercellular coupling is less conclusive. The possibility of

intercellular coupling in the absence of morphologically identified gap junctions

cannot be excluded (Williams and DeHaan, 1981). Alternaively, given the

multitude of factors interacting in the preparation of brain slices (Reid et al.

1988), it has been suggested that the in vitro slice preparation may induce some

artefactual increase in dye-coupling (Gutnick et al. 1985).

Due to the recent isolation of gap junction proteins, and the cloning of

some of their mRNAs, it is now obvious that there is a family of gap junction

proteins, encoded by more than one gene (Paul, 1986; Beyer et al. 1987; Kumar

and Gilula, 1986; Willecke et al. 1990; Willecke and Traub, 1990; Bennett et al.

1991; Dermietzel and Spray, 1993; Dermietzel et al. 1990). Beyer et al.(1987)

have introduced a nomenclature for the various gap junction proteins, based on

their molecular weights. Thus, the protein initially isolated from liver has been

termed cx32 and is encoded by a 1.6 kb mRNA, while the protein isolated from

heart is cx43, encoded by a 3-kb mRNA. Other proteins associated with gap

junctions have been isolated, for example connexin26 (2.5 kb mRNA; Nicholson et

- 1987). While there is evidence for some tissue specificity in the distribution of

these proteins, reports of the characterization of gap junction mRNA and protein

in the mammalian brain have been confusing. Both cx32 mRNA and

immunoreactivity have been demonstrated in adult rat brain (Paul, 1986; Nagy et

al. 1988; Shiosaka et al. 1989; Yamamoto et al. 1989a; Micevych and Abelson, 1991; Dermietzel et al. 1989), and cultured neurons and astrocytes (Dudek et al. 1988). Other studies have suggested that cx43 mRNA and immunoreactivity are also present (Dermietzel et al. 1989; Dupont et al. 1988; Yamamoto et al. 1990b; Yamamoto et al. 1990a; Micevych and Abelson, 1991; Naus et al. 1991a). Cross reactivity of rat cardiac gap junction antibodies with a 41 kD protein has been observed in adult rat brain (Dupont et al. 1988; el Aoumari et al. 1990; Yamamoto et al. 1989a).

In the present study, a protein of similar molecular weight was found in P12, 20 and adult rat brain. Postnatal day 5 brain samples had a protein of 43 kD, similar to the size in heart. The presence of the doublet only at certain postnatal times may suggest its association with specific developmental events. Musil and colleagues (Musil et al. 1990; Musil and Goodenough, 1990; Musil and Goodenough, 1991) have shown that the cx43 protein is phosphorylated resulting in bands ranging from 42-45 kD in chick lens. The observed 27-28 kD doublet in adult brain samples reacted with the cx32 antibody may represent a similar posttranslational modification of gap junction proteins associated with developmental events. Cx32 has also been reported to be phosphorylated in hepatocytes (Saez et al. 1986; Traub et al. 1987) although no apparent shift in Mr was associated with the phosphorylation. The developmental pattern seen in the Western analysis utilizing the cx43 antibody and to a lesser extent, the cx32 antibody, follows that of the Northern analysis data.

The present study indicates the presence of both cx32 and cx43 mRNA in adult brain, confirming these previous reports. Our detection of cx32 mRNA in adult brain but not in the neonatal brain before P10 suggests that there is developmental regulation of the expression of this gene in the brain. In contrast, cx43 mRNA is readily detectable in perinatal as well as adult brain. Both connexin proteins are present in rodent brain and appear to be developmentally regulated. Cx43 protein and mRNA is more abundant than cx32, an observation noted during development and in the adult. The apparent discrepancy between the RNA and protein analysis for cx32 can be resolved when one examines longer exposures of x-ray films of the Northern blots. Such exposures reveal the presence of cx32 mRNA in samples of P5 rat and mouse brain mRNA (data not shown). The monoclonal antibody and Western analysis of membrane enriched protein samples may allow greater sensitivity in detection than Northern analysis of total RNA.

The earlier detection of cx32 mRNA in the mouse CNS presumably reflects a slight difference in the state of neonatal maturity compared to the rat. Due to a relative delay in implantation in the rat, gestation is lengthened by 1.5 days when compared to the mouse (Butler and Juurlink, 1987). While embryonic development thus appears to proceed with a slight delay in the rat compared to the mouse, any lag in postnatal development would become less apparent with increasing time after birth. Alternatively, species differences have been described for various gap junction proteins in liver (Traub et al. 1989). Differences of this

nature may exist in the gap junction distribution and abundance in the CNS of mouse and rat during development.

The increase in the level of cx32 mRNA not only parallels neural maturation, but also follows a caudal to rostral gradient of development in the brain, with hindbrain levels increasing prior to those in the forebrain and remaining elevated in the adult. Ultrastructural studies of developing neocortex of E16 mouse suggest the presence of small "atypical" gap junctions which decrease with maturation (Shoukimas and Hinds, 1978). Little morphological data are available concerning the presence of gap junctions during postnatal brain development. Recently, Dermietzel et al. (1989) described the immunocytochemical distribution of several of the connexin proteins during neural development. They reported that cx43 and cx26 immunoreactivity are present in the embryonic neuroepithelium. During postnatal development, the cx26 immunoreactivity disappears while cx43 continues to be present in astrocytes. In contrast, cx32 immunoreacitivity is not detectable until after birth, being present in some neurons and oligodendrocytes. Our data are generally consistent with this pattern of connexin appearance with the observed profiles of cx43 and cx32 mRNAs. We also detected very low levels of cx26 mRNA during postnatal development of the rodent CNS. Taken together, these studies suggest an apparent developmental regulation of the expression of gap junction mRNA and protein in the rodent brain. Changes in intercellular coupling with development occur in many systems, apparently playing some role in tissue differentiation

(Revel and Brown, 1975; Warner et al. 1984; Caveney, 1985; Kidder et al. 1987). The temporal appearance of cx32 mRNA and protein coincides with major postnatal developmental events, including glial and neuronal maturation, synaptogenesis, myelination and vascularization (Jacobson, 1991). Given the suggestion that some of the connexins are cell-specific (Dermietzel et al. 1989), the pattern of cx43 expression may reflect astrocyte and endothelial maturation, while cx32 is more likely associated with expression in neuronal and oligodendrocytic maturation. The possible role of gap junctions in these and other developmental processes remains to be determined.

The expression of connexin mRNAs in various regions of the adult CNS agrees with findings by Mycevich and Abelson (1991) where they observed a relatively even distribution of cx43 mRNA, as detected by *in situ* hybridization, throughout regions of the rodent brain. Cx32, however, was more abundant in certain regions. Our detection of higher levels of cx32 in the cerebellum and hindbrain agrees with our in situ hybridization results discussed in chapter 3.

In summary, Northern and Western blot analysis indicate that the genes for both cx32 and cx43 are actively expressed in the adult brain, while the expression of these genes are differentially regulated during neural development. This differential expression of gap junction genes suggests a possible developmental role for gap junctions as they parallel other phenomena such as synaptogenesis, myelination and vascularization of the CNS.

CHAPTER 3

Cellular localization of gap junction mRNAs in developing rat brain

3.1 Introduction

The gap junction protein forms a transmembrane channel that provides a pathway for the exchange of small metabolites and ions between adjacent cells (Loewenstein, 1981). The presence of gap junctions has been reported in a large variety of mature tissues (reviewed by Dermietzel et al. 1990), with the exception of adult striated muscle and circulating blood cells, as well as in developing systems. Gap junctions are thought to represent one potential mechanism of cell-cell interaction during development (Bennett et al. 1981; Caveney, 1985; Fraser, 1990; Schultz, 1985). In more primitive organisms, Caveney (1985) described the role of gap junctions during compartmentalization of the insect larva and good evidence exists for the importance of gap junctions during Hydra development (Fraser, 1990; Guthrie and Gilula, 1989). The presence of gap junctions in vertebrate development has been demonstrated in many organ systems. Gap junctions have been described in the preimplantation embryo (Kidder, 1987; Barron et al. 1989; Nishi et al. 1991; Dale et al. 1991; Flores and Lane, 1991; De Sousa et al. 1993), during developmental stages of both cardiac (Fishman et al. 1991; Gourdie et al. 1992; Fromaget et al. 1990) and skeletal muscle (Kalderon et

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al. 1977; Balogh et al. 1993; Laird et al. 1992), during neuromuscular junction formation (Allen and Warner, 1991), and in the developing CNS(Shoukimas and Hinds, 1978; Connors et al. 1983; Bergmann and Surchev, 1989; Walz and Hertz, 1983; Lo Turco and Kriegstein, 1991; Yuste et al. 1992; Yamamoto et al. 1989a). Evidence demonstrating a role for gap junctions in development has been obtained by using perturbation experiments with function blocking antibodies to gap junctions or anti-sense RNA technology experiments in amphibian (Warner et al. 1984) or mouse embryos (Lee et al. 1987; Bevilacqua et al. 1989).

In the CNS, however, the only evidence for the role of gap junctions during development has come from experiments using electrical or dye coupling as a marker for functional channels. Connors et al. (1983) showed that 70% of the neurons in the newborn rat neocortex are dye coupled, and this percentage decreased to 20% in the adult. Similar results have been reported in the developing striatum (Walsh et al. 1989). LoTurco and Kriegstein (1991) examined the rat embryonic neocortex and found that neuroblasts are highly coupled prior to their migration from the ventricular zone. In addition, discrete domains of coupled neurons extend throughout the entire thickness of the rat somatosensory cortex (Yuste et al. 1992). Astrocytes in the visual cortex of postnatal day 14 (P14) rats have been shown to transfer Lucifer Yellow more than 200 μm from the injected cell, this area of dye spread increasing to 300μm at 7 weeks of age (Binmoller and Muller, 1992). Taken together, these observations clearly

demonstrate the existence of gap junctions during the development of the mammalian CNS.

With the advent of new biochemical and molecular biological techniques, the study of gap junctions has taken on a new dimension (Dermietzel et al. 1990). Recent isolation of gap junction proteins and cloning of their complementary DNAs (cDNA) have yielded invaluable tools for the study of the cell biology of gap junctions. Using these tools, investigators have examined the distribution of connexin proteins in the CNS. Primary astrocyte cultures have been shown to contain cx43 (Dermietzel et al. 1991; Giaume et al. 1991; Naus et al. 1991a). In vivo, Yamamoto et al. (1990b) have demonstrated cx43 immunoreactivity in astrocytes, ependymal cells, pia, and arachnoid cell layers but no immunoctyochemical labelling was detected in neurons or oligodendrocytes. Connexin32 immunoreactivity has been detected in neurons of the hippocampus (Nagy et al. 1988; Shiosaka et al. 1989). Mycevich and Abelson (1991) using *in situ* hybridization, localized cx32 mRNA in fibre tract areas and certain populations of neurons while the cx43 mRNA expression pattern suggested localization to astrocytes.

In the developing CNS, Dermietzel et al. (1989) showed that cx26 and cx43 protein are present in embryonic day 18 and very early postnatal ages but as the level of cx43 protein continues to increase beyond 6 days of age, cx26 decreases dramatically. Connexin32 begins to appear after birth and increases in abundance to adulthood. A more detailed analysis of cx43 expression in the developing rat

brain has recently been done using immunocytochemistry (Yamamoto et al. 1992).
In chapter 2, it was shown that the mRNA and protein for cx32 and cx43
increases with development, however, expression of cx32 begins postnatally
whereas cx43 is present as early as embryonic day 18. In a brief investigation
using *in situ* hybridization with a cDNA probe, Matsumoto et al. (1991)
demonstrated the presence of cx32 in neural cells of the postnatal day 2 rat brain.

The aim of this study was to investigate the differential localization of cx32
and cx43 mRNA during development of the rat CNS by using *in situ* hybridization.
In addition, cell isolation techniques were used to ascertain the cell specific
expression of the two connexin genes in the CNS.

3.2 Materials and Methods

3.2.1 Tissue collection for *in situ* hybridization

Brains were collected from 3 or 4 Sprague-Dawley rats at postnatal ages 3, 10, 15, 30 and adult. The animals were overdosed with sodium pentobarbital (1.5 ml/kg body weight) and perfused transcardially with cold 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4. As controls, the liver and heart of adult rats were collected. The tissues were postfixed for 4 to 8 hours and subsequently immersed in a sucrose series of 12%, 16%, and 18% in PBS for 12 hours each. Tissues were then frozen with dry ice and cryostat sections at 10 μm thickness were collected on chrome-alum coated slides. The slides were stored at -20°C until needed. Embryonic day 20 brains were immersed overnight in 4% paraformaldehyde and then cryoprotected in sucrose as described above.

3.2.2 cRNA probe production

Three cDNA probes were used to produce riboprobes, a 1.5 kilobase (kb) clone of cx32 (Paul, 1986) in pGEM-3Z, a 1.4 kb clone of cx43 (Beyer et al. 1987) subcloned into Bluescript M13 phagemid, and a 680 base pair (bp) clone of cx26 (Zhang and Nicholson, 1989) subcloned in Bluescript KS- phagemid.. The cx32 insert was cloned such that linearization with Pvu II and transcription with the SP6

RNA polymerase produced an antisense RNA probe. Linearization with Hind III and using the T7 RNA polymerase produced the sense strand. For cx43, a BamH I cut and T3 RNA polymerase produced the antisense strand and linearization with Sal I and use of T7 polymerase would produce a sense RNA probe. The cx26 clone was linearized with XbaI and transcribed with the T7 polymerase to produce antisense probes. Sense strands of cx26 were produced by linearizing with EcoRI and transcribing with T3 polymerase. Probes were made using the Promega Riboprobe Gemini system and ^{35}S-UTP (New England Nuclear) following the procedure outlined in the technical manual provided with the kit. The only exception was a reduction in the volume of non-radioactive UTP from 2.4 μl to 1.5 μl to increase the amount of radiolabelled UTP incorporated into the probe.

Following production, the riboprobes were alkali treated to reduce their length to approximately 500 bp prior to use in the *in situ* hybridization protocol. The RNA was then precipitated with 0.3 M NaC$_2$H$_3$O$_2$ in 95% ethanol and centrifuged for 30 min at 10 000 rpm at 4 C. The pellet was dissolved in 180 μl of an alkaline bicarbonate solution (40 mM NaHO$_3$, 60 mM Na$_2$CO$_3$, pH 10.2) on ice. Once dissolved, the probe was placed at 60 C for a predetermined time to reduce the probe size to 500 bp. The probes were returned to ice and hydrolysis neutralized by the addition of 21 μl of glacial acetic acid:1M NaC$_2$H$_3$O$_2$ (pH 6.0, ratio 5:95). The samples were then re-precipitated as described above. The

specific activities of all probes were consistent, ranging between $1.0\text{-}1.3 \times 10^8$ cpm/μg RNA.

3.2.3 *In situ* hybridization

a) ^{35}S labelled cRNA probes

The *in situ* hybridization procedure followed that of Whitfield et al. (1990). All solutions used were treated with 0.1% diethyl pyrocarbonate for 8 hr to destroy nucleases. The sections were removed from the freezer, left to dry at room temperature for 15 min, then fixed in 3.7% formaldehyde in PBS for 5 min and washed in PBS for 5 min. Following fixation, the slides were acetylated in 0.25% acetic anhydride in 0.1M triethanolamine (pH 8.0) for 10 min, washed in 2X SSC, dehydrated through an ethanol series followed by a 5 min delipidation step in chloroform and then a 2 min wash in 100% and 95% ethanol. The sections were placed in a humidified chamber and coated with 50 μl of hybridization buffer containing 1.0×10^6cpm of cRNA probe. The hybridization buffer consisted of 50% formamide, 0.6M NaCl, 10 mM Tris-HCl (pH7.5), 2X Denhardt's reagent, 1 mM EDTA (pH 8.0), 0.01% denatured herring sperm DNA, 0.05% yeast transfer RNA, and 10% dextran sulfate. A coverslip was placed onto the slide and the sections incubated at 52°C overnight. Following hybridization, the coverslips were removed in 2X SSC. The slides were placed into a solution of 20 μg/ml RNase A in 0.5M NaCl, 10 mM Tris-HCl (pH 8.0), 1

mM EDTA (pH 8.0) for 30 min at room temperature and subsequently washed in

the same buffer without RNase A. The next step involved three 1 hour rinses of

decreasing salt concentration and increasing temperature (2X SSC at 50C; 0.2X

SSC at 55C; 0.2X SSC at 60C). The slides were dehydrated in an ethanol

series containing 0.3M ammonium acetate and used to expose Kodak XAR film

before being dipped in NTB-2 emulsion diluted 1:1 in distilled water. The

autoradiograms were stored desiccated for 8 to 14 days at 4C, then developed

for 2 min in D-19 developer, stopped for 30 sec in water and fixed for 5 min in

Kodak fixer. The sections were counterstained with the nuclear stain Brazilin

(ICN Biomedical), dehydrated and mounted with Entellan (Merck).

Positive signal was determined to be hybridization signal that exceeded the

background levels found on the sections hybridized with the sense strand

riboprobe. As well, negative control tissues ensured specificity of the riboprobes

to their respective connexin mRNAs.

b) *In situ* hybridization with Digoxigenin probes

In situ hybridization was performed on brain sections using the Boehringer

Mannheim Genius system for nonradioactive labelling of cRNA probes with

Digoxigenin-UTP. Labelling of connexin probes followed the method detailed in

chapter 3.2.3 (a) except ^{35}S-UTP was replaced with Digoxigenin-UTP.

The *in situ* hybridization protocol involved the following steps. The slides

were fixed for 15 min in 3.7% formaldehyde in PBS and rinsed in PBS for 10 min.

They were then treated for 5 min in 95% ethanol-5% glacial acetic acid at -20°C and rehydrated through an ethanol series. The sections were exposed to 10 μg/ml proteinase K in a buffer consisting of 50 mM EDTA (pH 8.0), 100 mM Tris-HCl (pH 8.0), and 2 mM $CaCl_2$, then washed for 10 min in 2X SSC and prehybridized for 1 hr. The prehybridization buffer contained 5X SSC, 5X Denhardt's reagent, 50% formamide, 250 μg/ml yeast transfer RNA, and 5% dextran sulfate. The prehybridization solution was washed off the slide with 2X SSC and the slides dehydrated in ethanol. Fifty microliters of hybridization buffer (same as prehybridization buffer) containing 5 ng/μl probe was added to the slide and the buffer spread over the slide by placing a coverslip on top. The slides were placed in a humidified chamber and incubated overnight at 46°C. The posthybridization washes were all done at 42°C and consisted of 2 washes in 2X SSC, 1 at 0.2X SSC and 2 in 0.1X SSC each for 15 min. The hybridization signal was detected according to the instructions of the nucleic acid detection kit (Boehringer Mannheim Biochemical) except that the antibody was used at 1:2000 dilution. The substrate (5-Bromo-4-Chloro-3-Indolyl Phosphate/Nitro Blue Tetrazolium) was left on the slides for 8 to 12 hours prior to being washed off with 2 rinses of phosphate buffer. The slides were counterstained and mounted as described above.

3.2.4   Double labelling experiments and Immunocytochemistry

Adult rat brain sections were cut at 40 μm thickness for use in free floating immunocytochemistry prior to *in situ* hybridization. Sections were blocked for 30 min in 10% normal goat serum, 1% BSA in PBS and subsequently washed 3 times at 10 min each in PBS. The primary antibody (mouse anti-glial fibrillary acidic protein [GFAP; Boehringer Mannheim Biochemical]) was diluted 1:10 in PBS containing 0.1% BSA and 0.3% Triton X-100. The sections were incubated for 24 hr at 4°C on a rotating table. The sections were washed 3 times for 15 min each in PBS and incubated with a biotinylated secondary antibody (Dimension Labs) for 1 hr at room temperature. After 3 PBS washes, the sections were treated for 1 hr with an ABC peroxidase kit (Dimension Labs). The reaction product was detected by using 1 mg/ml 3,3' diaminobenzidine tetrahydrochloride (DAB) in PBS containing 0.03% H_2O_2. Following immunocytochemistry, the sections were mounted onto slides and stored frozen until used for *in situ* hybridization as described above.

Sections of P5, P10 and P15 rat brain, 40 μm in thickness, were incubated with a mouse anti-myelin basic protein (MBP; Boehringer Mannheim Biochemical) to localize myelinating oligodendrocytes. The method of detection was the same as that for GFAP described above. These sections were compared to similar 10 μm sections on which *in situ* hybridization was performed.

3.2.5   Cell Isolation from mature rat brain

The isolation of neurons and neuroglia was performed using procedures published by Snyder et al. (1980) and Farooq and Norton (1978). In both methods, mature rat forebrain from 6 animals were used and the isolation repeated on three separate occassions. Purity of cell isolation was determined by visual observation based on criteria established by Snyder et al. (1980) and Faroog and Norton (1978). Figure 3.1 summarizes the protocols describe below.

a) Astrocyte and neuron isolation

Astrocytes and neurons were isolated according to the method of Snyder et al. (1980). Rat forebrains were sliced into 8-18 pieces (depending on age) and incubated at 37 C in 0.1% trypsin in cell isolation medium (8% glucose, 5% fructose, 2% Ficoll, 10 mM KH_2PO_4, pH 6.0) in a shaking water bath for 90 min and trypsinization stopped with trypsin inhibitor (0.1%). The medium was discarded and the tissue aspirated through a nozzle of 2.2-2.4 mm diameter into a flask containing isolation medium and poured through a 420 μm screen. This represented filtrate 1. The residue remaining on the screen was aspiritated once again and passed through a screen representing filtrate 2. This was repeated two more times and all filtrates combined. The filtrate was centrifuged at 720 g for 15 min and the cell rich pellet resuspended in 7% Ficoll in cell isolation medium. The suspension was centrifuged at 280 g for 10 min to obtain a neuron-enriched pellet, P1. The supernatant was centrifuged again, at 720 g for 10 min obtaining

P2 and a final centrifugation of the supernatant at 1120 g for 15 min obtained P3, an astrocyte enriched pellet.

Each of the pellets were layered onto discontinuous Ficoll gradients of 10%-22%-28%-32% Ficoll in isolation medium and centrifuged at 8500 g for 5 min. Specific layers were removed to be examined for cell type and RNA analysis.

b) Oligodendrocyte isolation

The isolation of oligodendrocytes followed the method of Farooq and Norton (1978) as described. Forebrains of rats were minced with scissors in ice-cold isolation medium (Hanks balanced salt solution [Hanks BSS; 135 mM NaCl, 5 mM glucose, 5 mM KCl, 0.5 mM KH_2PO_4, 0.5 mM $MgCl_2 \cdot 6H_2O$, 0.5 mM $MgSO_4 \cdot 7H_2O$, 0.5 mM$Na_2HPO_4 \cdot H2O$, 1 mM $CaCl_2$] 25 mM HEPES, 1% BSA, pH 7.2). The tissue was trypsinized for 30 min at 37 C in a shaking water bath. Following trypsinization, the suspension was chilled for 5 min on ice and 0.1% trypsin inhibitor (Sigma) was added. The tissue was transferred to round bottom tubes and centrifuged for 5 min at 190 g. The overlying fluid was removed by aspiration and the pellet resuspended in isolation medium and the centrifugation/aspiration step repeated.

The resultant suspension was passed through a 145 μm nitex screen followed by 3 passages through a 75 μm screen and finally diluted 1:1 with 70%

**FIGURE 3.1 Flowchart of protocols used to isolate neural cells from mature rat
brain**

The flo⁻ charts for the isolation of astrocytes/neurons or oligodendrocytes are
based on methods described in chapter 3.2.5. In the isolation of astrocytes and
neurons, brain slices are trypsinized and passaged through nitex screen to obtain
filtrates which are centrifuged in a Ficoll discontinuous density gradient.   The
resulting pellets are used for analysis. Oligodendrocyte isolation also involves the
trypsinization of mature forebrain. The suspension is centrifuged and passed
through a series of screens with progressively smaller pores and finally centrifuged
through a sucrose density gradient, the pellet being used for analysis.

OLIGODENDROCYTE ISOLATION
Synder et al., 1980

Six Rats → Mince forebrain → Trypsinize, 37 C, 30 min. → Centrifuge 190g 5 min. → Aspirate rbc resuspend Centrifuge {repeat once} → 145 um screen → 74 um screen, 3 times → Density gradient 53%, 45% sucrose at 3065g, 15 min. → Collect pellet and layer below rbc → Dilute and pass Thru 25um screen → ANALYSIS

ASTROCYTE / NEURON ISOLATION
Faroog and Norton, 1978

Six rats → Slice brains (8–10 peices) → Trypsinize (37 C, 90 min) → Aspirate tissue → 420 um screen → Filtrate 1 (F1) → Centrifuge 720g 15 min.

Aspirate residue → Filtrate, F2 → F3, F4

Centrifuge 260g 10 min. → Pellet 1

Centrifuge 720g 10min → Pellet 2

Centrifuge 1120g 15min → Pellet 3

Density gradient 32%, 28%, 22%, 10% Ficoll

Astrocytes   Astrocytes/neurons   Astrocytes/neurons

sucrose in isolation medium (sucrose was RNase free from Merck). The next step involved centrifugation of the cell suspension through a discontinuous sucrose gradient of 53% and 45% sucrose in isolation medium. The gradient was centrifuged at 3065 g for 15 min in a swinging bucket rotor. The layer below the 45-53% layer was collected and diluted 5 fold with RNase free DNase (Boehringer Mannheim Biochemicals). Lastly, the suspension was filtered through a 25 μm screen and used for RNA analysis.

3.2.6 RNA extraction and Northern blot analysis

The cells collected in the isolation protocol described above were rinsed once in RNase-free PBS (see chapter 2.2.1), centrifuged at 2 000 rpm for 3 min and the PBS decanted. The resultant pellet was suspended in lysis buffer containing 1.0 % NP-40 and left on ice for 5 min. Following the incubation the cells were centrifuged once again to remove cell debris. The remainder of the protocol is described in chapter 2.2.1.

3.3 Results

3.3.1 *In situ* hybridization

a) Probe Specificity

To ensure that the connexin cRNA probes hybridized to their respective mRNAs, peripheral tissues known to express these gap junction mRNAs were used as positive controls. The *in situ* hybridization results obtained with probes for cx32 and cx43 mRNA showed differential patterns of hybridization within peripheral tissues and developing central nervous system. The specificity of the probes are shown in Figure 3.2 where they are hybridized to peripheral tissues known to express these connexins. The cx32 riboprobe hybridized strongly to hepatocytes of liver sections (Fig. 3.2A, C) whereas no signal beyond background was detected with the cx43 probe in liver (Fig. 3.2B, D). In areas of liver containing blood vessels, cx43 mRNA was present in the endothelial cells, but not within the liver tissue itself (data not shown). The connexin43 riboprobe hybridized to cardiac muscle (Fig. 3.2B, E) but cx32 did not (Fig. 3.2A, F). It is interesting to note that in liver, cx32 mRNA is present in virtually all hepatocytes as indicated in figure 3.2C (inset), but cx43 mRNA is found in a select number of cells within the heart muscle (Fig. 3.2E, inset; arrows). Myocardiocytes appeared to contain less connexin mRNA relative to the hepatocytes. These observations are qualitative in nature, and based on the fact that the specific activities of each

FIGURE 3.2 Specificity of connexin riboprobes

Sections of peripheral tissue hybridized with the connexin probes.
Autoradiograms showing cx32 hybridization in liver (A; I) and cx43 mRNA in
heart (B; h). Hybridization of cx32 cRNA in heart (A; h) and cx43 in liver (B; I)
is extremely low and considered background. Darkfield photomicrograph of liver
shows intense hybridization signal in all hepatocytes for cx32 mRNA (C) and no
signal for cx43 (D). The inset in (C) is a higher magnification brightfield
photomicrograph of a liver section showing individual hepatocytes (nuclei stained
with brazalin) expressing cx32 mRNA. The grains in (D) are background as
shown in the inset. Heart tissue hybridized with cx43 antisense probe (E) shows
selective myocardiocytes labelled. This is seen in the higher magnification inset
(arrows). The cx32 probe resulted in only background on heart tissue (F) with no
clear hybridization over individual myocardiocytes (inset). Magnification C-F
(x144); insets (x360).
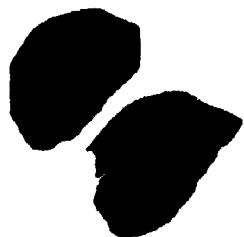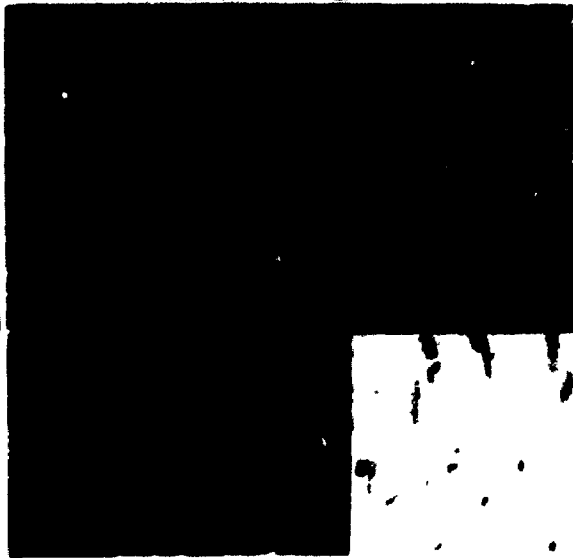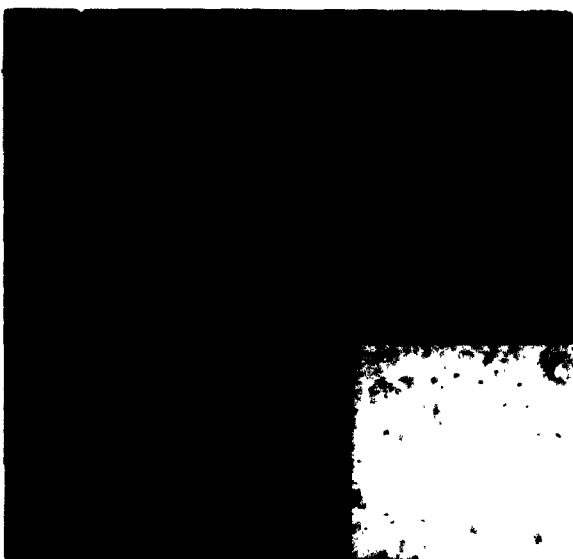
FIGURE 3.3 *In situ* hybridization - embryonic day 20 cortex

Darkfield photomicrograph of horizontal sections through the cortex of E20 showing only background with the cx32 antisense probe (A) and a strong signal in the pial layer when probed for cx43 (B). The cx43 probe also hybridizes within the layers of the cortex. Arrows show the pial surface. The cx43 signal at the pial surface is seen more clearly in brightfield at higher magnifications (C). Magnification A, B (x144); C (x360).
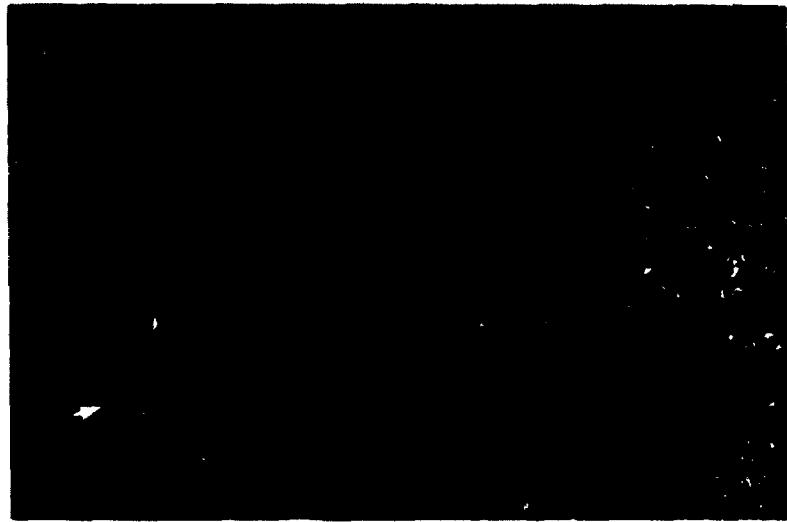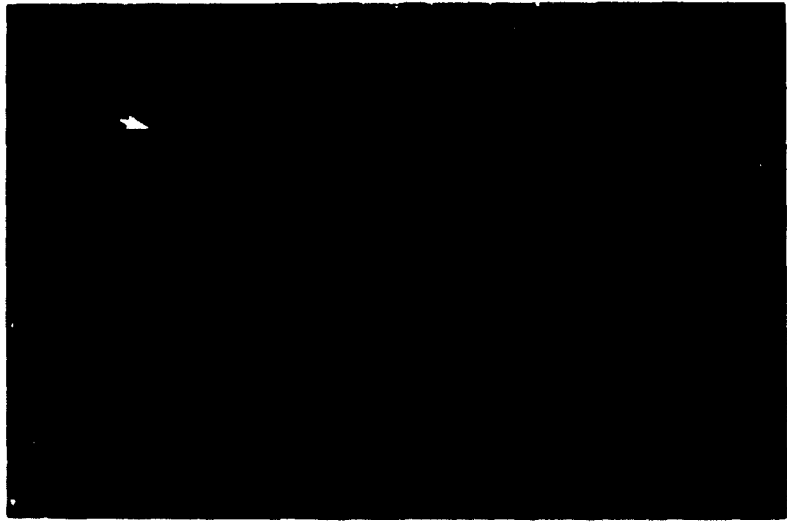
probe are equal, it is possible to compare the relative hybridization intensity of

cx43 to that of cx32. Such comparisons are made throughout the results and are

not meant to represent quantitative observations.

Sense strand riboprobes were used on sections from all ages examined and

only very low background levels were detected. A comparable low background

result was obtained when liver and heart sections were hybridized with cRNA

probes to cx43 and cx32 respectively (Fig.3.2D and F). The absence of signal in

the negative controls indicates that the riboprobes are hybridizing specifically to

the connexin mRNA of interest.

The cx26 riboprobe hybridized specifically to liver sections. During the

examination of sections of developing brain, cx26 mRNA was detected in

leptomeningeal cells of E20, P3 and P10, the only ages examined for cx26

hybridization. No signal was detected in the CNS proper, when compared to

background as determined by sense strand hybridization, therefore, the expression

of this connexin was not examined further.

b) Prenatal and Neonatal Development

At E20 no cx32 hybridization was detected above background in all areas

of the brain. Figure 3.3A shows a horizontal section through the cerebral cortex.

No hybridization can be detected from the pial layer to deeper layers of the

cortex. This was true for other areas of the brain such as the hippocampus,

midbrain and hindbrain (data not shown). The cx43 riboprobe hybridized

FIGURE 3.4 *In situ* hybridization - postnatal day 3 cortex and brainstem

Darkfield photomicrograph showing the distribution of signal in coronal sections of P3 brain. The temporal cortex shows no signal with cx32 antisense (A) but an obvious signal with cx43 (B). Connexin32 antisense signal is present in the medial vestibular nucleus (MVe) of the medulla oblongata (C) ventrolateral to the forth ventricle (4V). The signal for cx43 is more diffuse in the same region of the brainstem (D). Magnification x144.
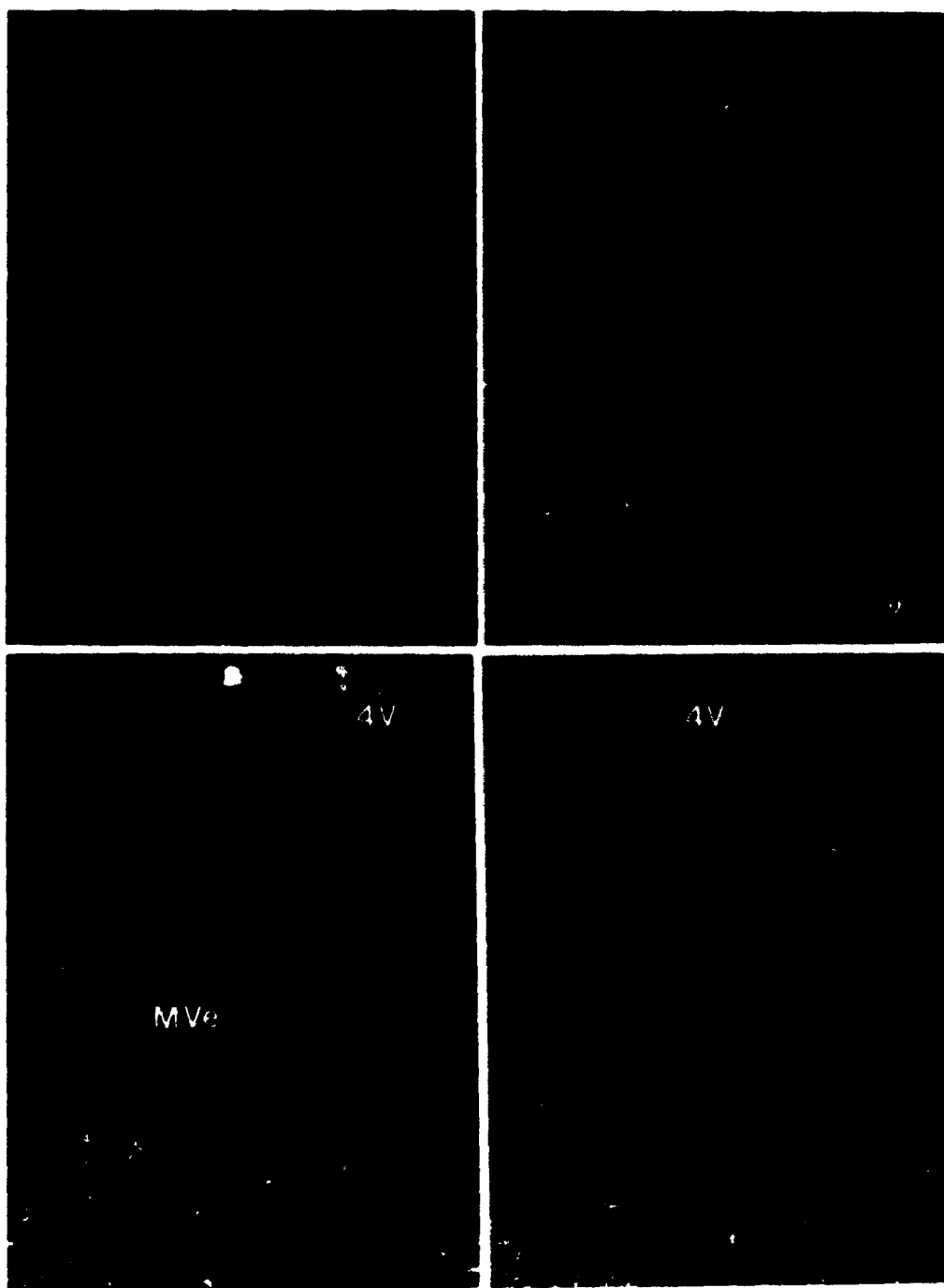
intensely to the pial cell layer (Fig. 3.3B, C). As well, the level of hybridization in deeper cortical layers was higher than the sense strand, suggesting a weak signal in cells of that area (Fig.3.3B).

At the first postnatal age examined, P3, no signal for cx32 could be detected in the temporal cortex (Fig. 3.4A). The corpus callosum, internal and external capsule showed no signal at this time. The same area probed for cx43 mRNA exhibited a distinct hybridization (Fig. 3.4B), the signal being restricted to deeper cortical layers. The hindbrain region was the first area to show signs of cx32 hybridization (Fig. 3.4C). The area of the medial vestibular nucleus shown in figure 3.4C was positive for cx32 mRNA, as was the dorsal paragigantocellul r nuclei, and parvicellular reticular nuclei. The spinal trigeminal tract had cx32 mRNA hybridization also. This same area, ventrolateral to the fourth ventricle, had cx43 positive cells (Fig. 3.4D) located more diffusely throughout the region.

c) Further Postnatal Development

In the P10 brainstem and cerebellum, there was hybridization of the cx32 riboprobe to the spinal trigeminal tract, facial nerve and white matter of the cerebellum (Fig. 3.5A), areas positive for MBP as determined by immunocytochemistry (Fig. 3.5B). There is considerable overlap between the staining pattern for MBP and cx32 hybridization suggesting the presence of cx32 in myelinating oligodendrocytes. The 'co-expression' of cx32 mRNA and MBP immunoreactivity was repeated at P15 (data not shown). Hybridization with the

FIGURE 3.5 Postnatal day 10 cerebellum; overlapping signal for connexin32 and MBP

Darkfield photomicrograph of a section of P10 cerebellum (A) showing cx32 hybridization in the cerebellar white matter (wm), spinal trigeminal tract (sp5) and facial nerve (7n). Brightfield photomicrograph of a similar region immunostained for myelin basic protein (B) shows the white matter (wm) of the cerebellum and spinal trigeminal tract (sp5) clearly labelled. Magnification x22.
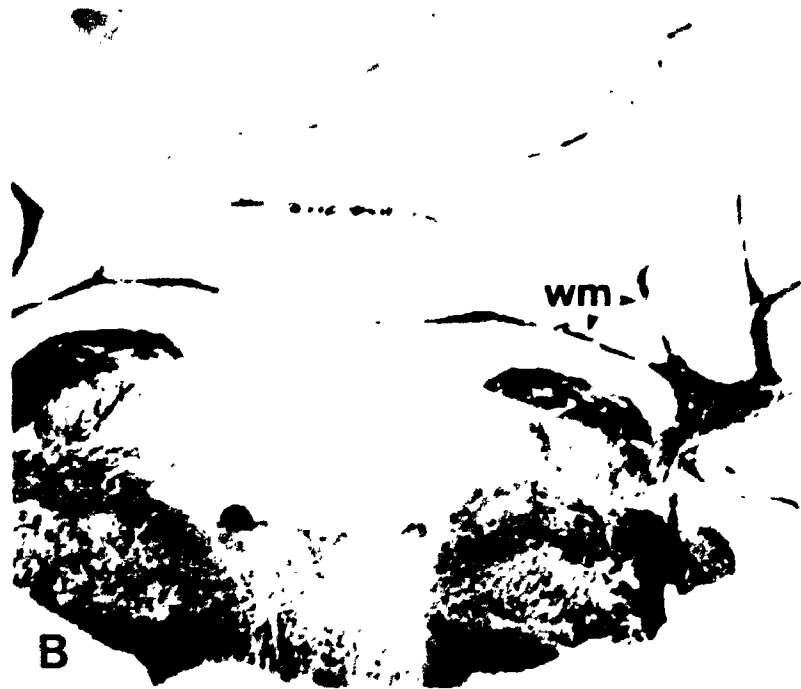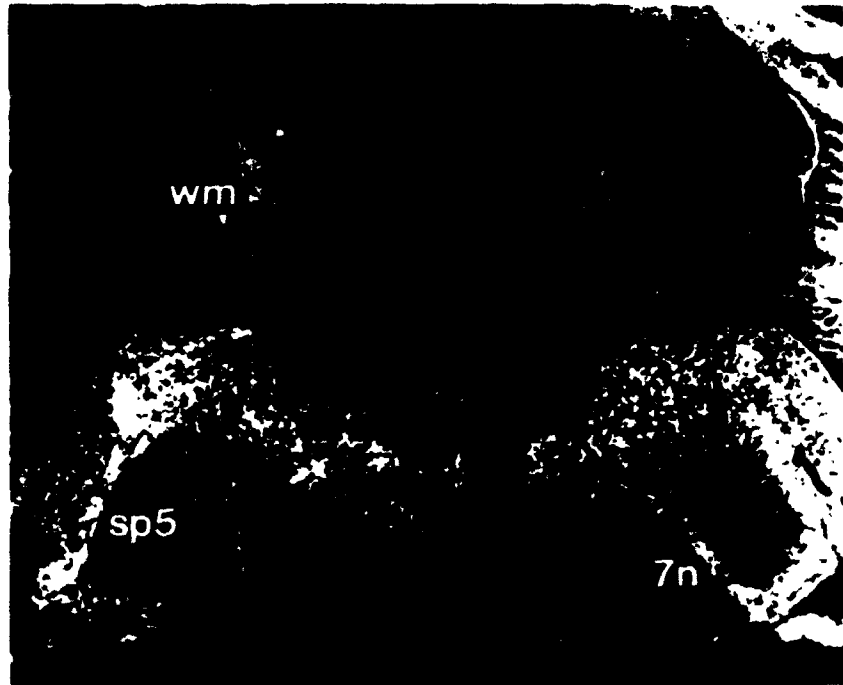
FIGURE 3.6 *In situ* hybridization - postnatal day 10 brainstem

In darkfield photomicrographs of brainstem at P10, hybridization for cx32 mRNA becomes prominent (A) showing strong labelling in the region of the dorsal paragigantocellular nucleus (DPGi) and medial longitudinal fasciculus (mlf). A similar area hybridized with cx43 antisense (B) again shows a more diffuse labelling. Magnification x144.
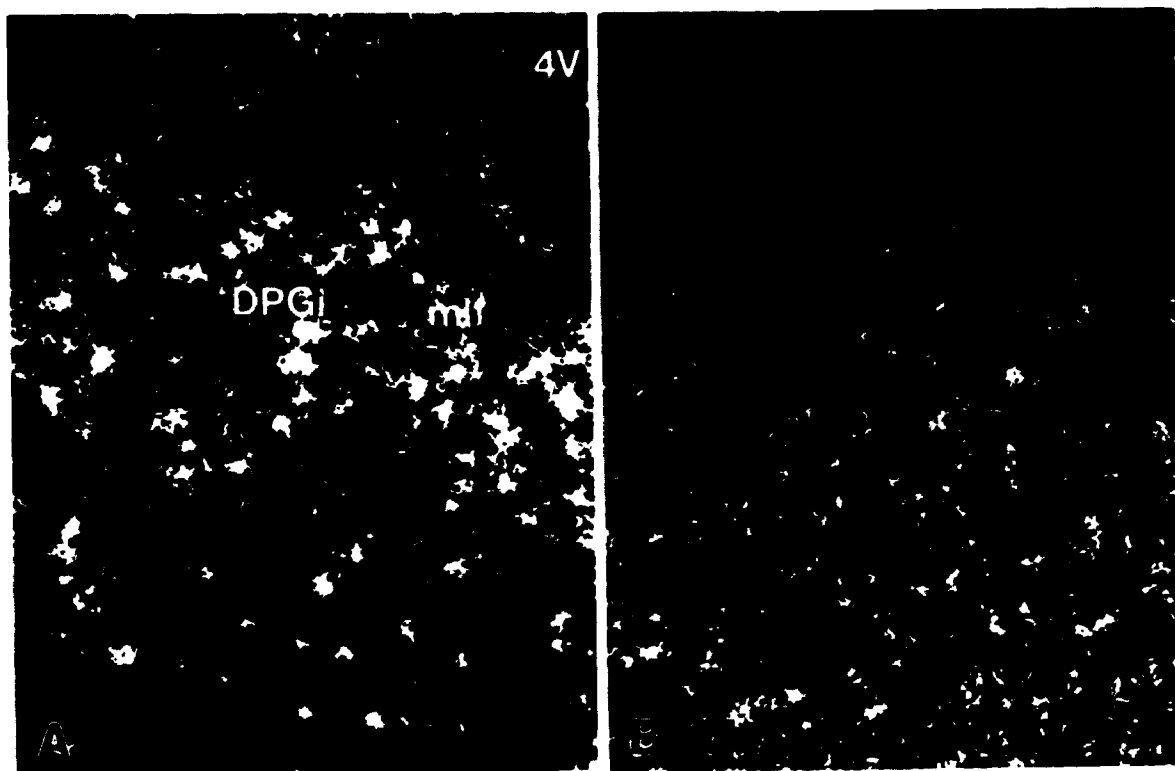
cx43 riboprobe showed the cerebellar white matter containing very few positive cells whereas the brainstem had a strong but homogeneous pattern of hybridization. Various nuclei in the medulla oblongata at P10 contained very prominent cx32 signal, such as the dorsal paragigantocellular nucleus and medial longitudinal fasciculus (Fig. 3.6A). Other nuclei containing cx32 mRNA included the medial parabrachial nucleus and mesencephalic trigeminal nucleus. Areas of the forebrain such as the cerebral peduncle and corpus callosum began to show weak hybridization for cx32 mRNA. Cx43 hybridization was found in a diffuse group of cells (Fig. 3.6B) with no indication of a particular nucleus or tract being labelled. There were very few cells of the corpus callosum that hybridized for cx43.

At P15 the hybridization signal of both probes was found in numerous locations in the CNS. In the midbrain, the distribution of cx32 and cx43 mRNA differed greatly. Cx32 mRNA was found in the decussation of the superior cerebellar peduncle (Fig. 3.7A). There was no signal in the central grey matter nor around the aqueduct. The distribution for cx43 in the midbrain region was even (Fig.3.7B), including the central grey matter and intense labelling of ependymal cells surrounding the aqueduct. The localization of signal also differed in the cerebellum with cx32 mRNA being abundant in the cerebellar white matter (Fig. 3.8A) whereas figure 3.8B shows no signal in this area for cx43. The spinal trigeminal tract was also labelled with cx32 (Fig. 3.9A). The brainstem hybridized evenly with cx43 (Fig. 3.8B), similar to earlier time points. More caudal, the

FIGURE 3.7 *In situ* hybridization - postnatal day 15 midbrain

A distinct difference in hybridization is seen between the two probes in darkfield photomicrographs of P15 sections of the midbrain. Connexin32 antisense probe (A) hybridizes strongly to cells in the decussation of the superior cerebellar peduncles (xscp) but there exists no signal near the aqueduct (Aq) in the area of the central grey matter (CG). The same area hybridized with the cx43 probe (B) shows strong labelling of ependymal cells lining the aqueduct and an even distribution of signal in all areas of the midbrain section including the central grey area. Magnification x22.
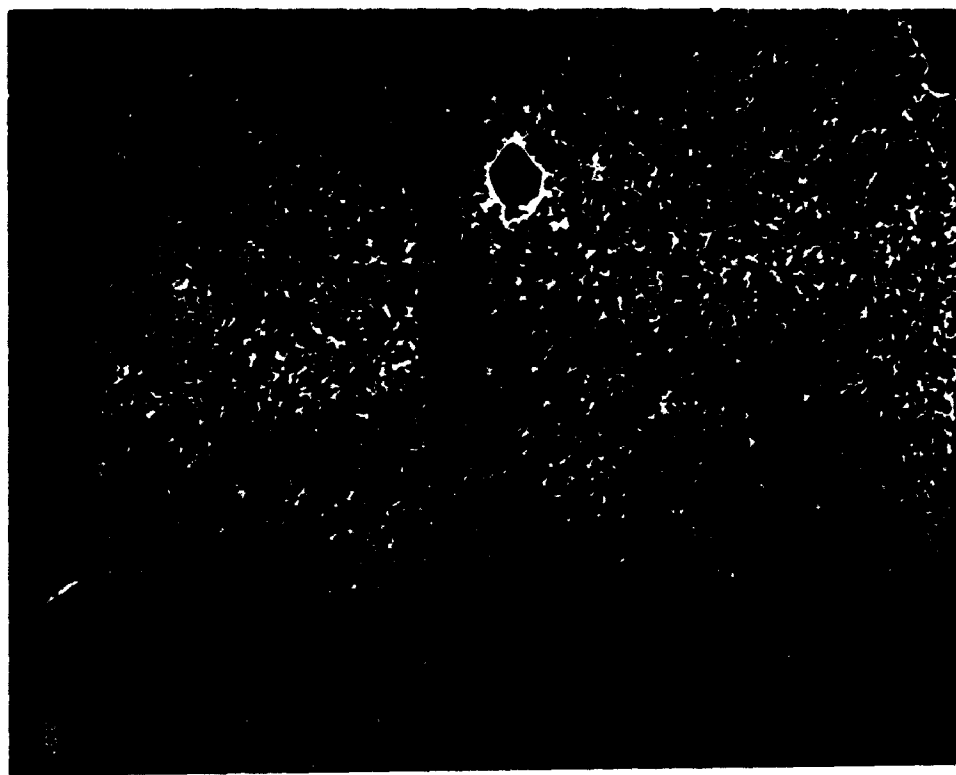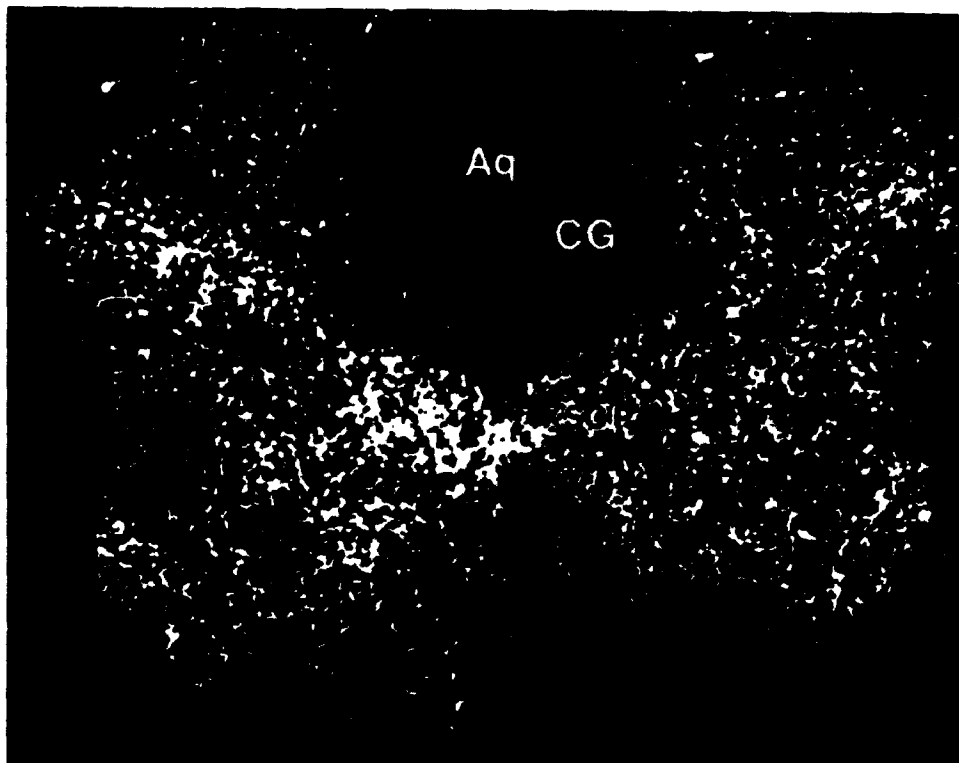
FIGURE 3.8 *In situ* hybridization - postnatal day 15 cerebellum and brainstem

In this darkfield photomicrograph, the P15 cerebellum hybridized with cx32 (A) shows an intensely labelled cerebellar white matter when compared with cx43 (B). The spinal trigeminal tract (sp5) is positively labelled for cx32 (A). Magnification x45.
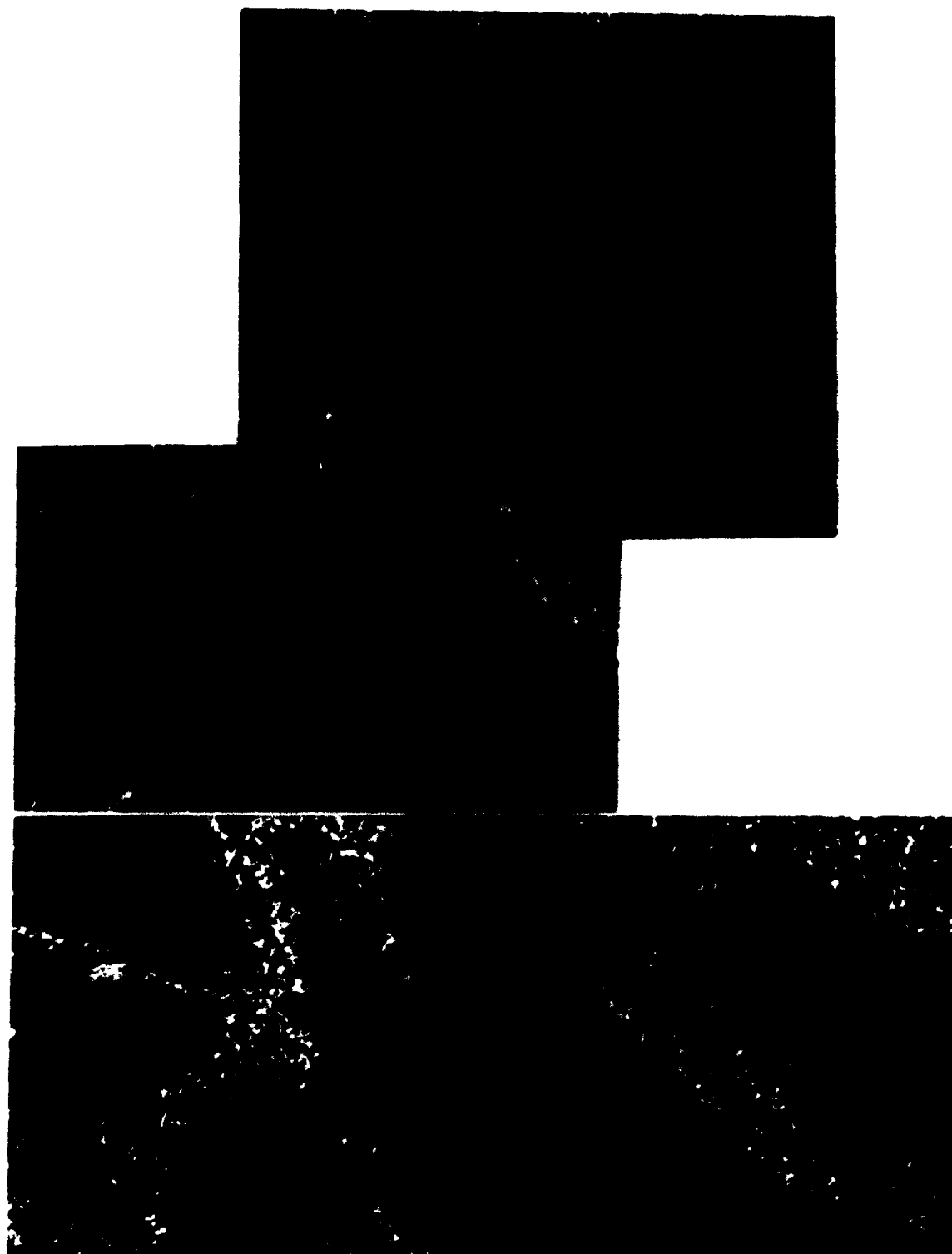
FIGURE 3.9 *In situ* hybridization - postnatal day 30 cortex and cerebellum

In these darkfield photomicrographs, the temporal cortex at P30 shows a strong signal with both connexin probes, in particular, layer III (A, cx32 and B, cx43). Hybridization with the cx32 probe resulted in signal in the Purkinje cell layer (P) of the cerebellum (C), as well as a strong signal in the cerebellar white matter (wm). Cx43 (D) hybridizes to the Purkinje cell layer (P) also as well as to cells of the granule cell layer (GC). The arrows in D demonstrate Purkinje cells hybridizing to the cx43 riboprobe. Magnification x144.
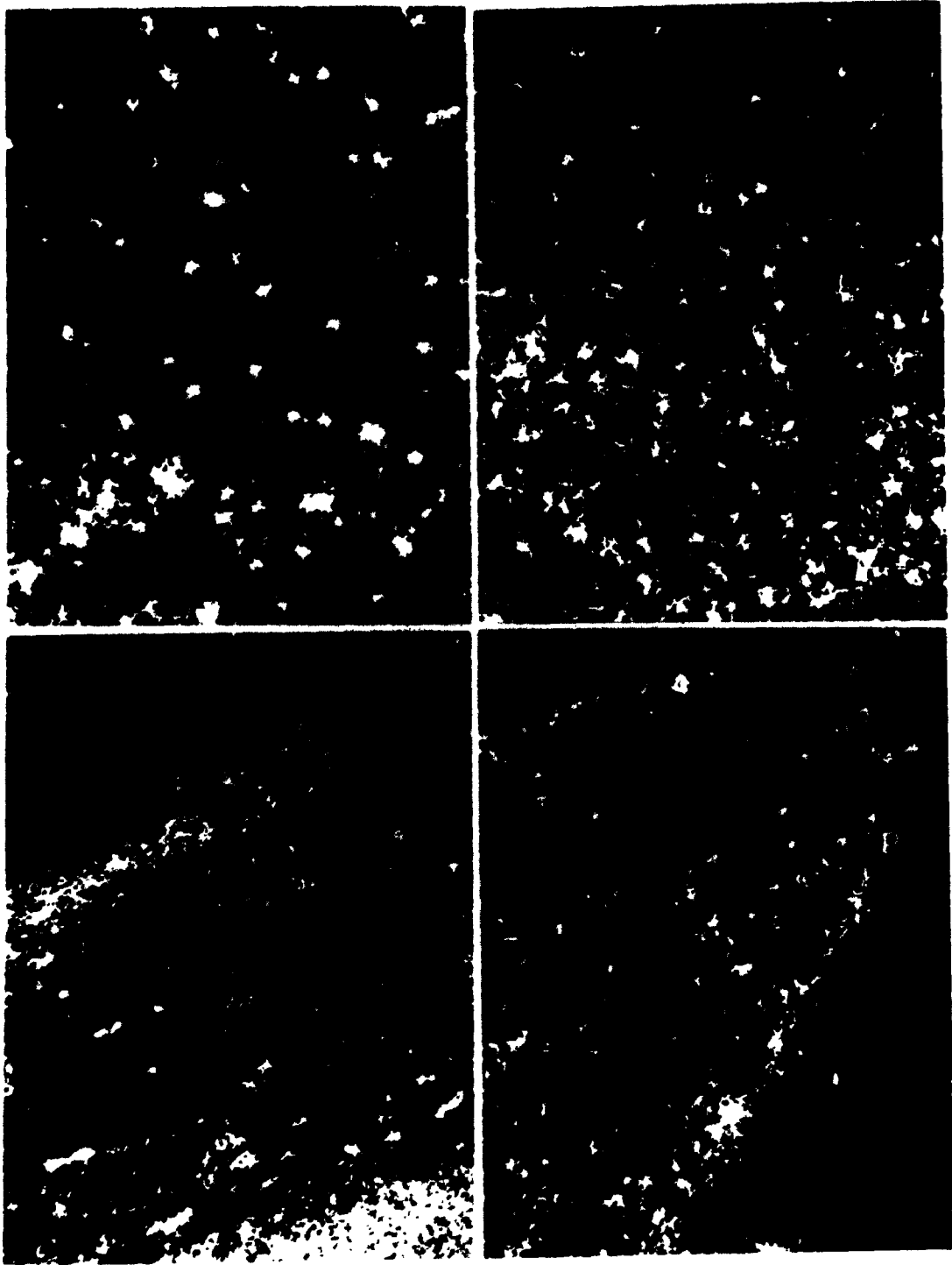
inferior cerebellar peduncle contained strong cx32 signal as did nuclei in the brainstem such as the parvicellular reticular nucleus and dorsal paragigantocellular nucleus. Forebrain regions such as the corpus callosum, internal and external capsule and optic tract showed strong hybridization to the cx32 riboprobe. At this time cx32 mRNA was detected in numerous thalamic nuclei as well. The cx43 mRNA signal was present in cortex and subcortical areas and more cells were labelled than at P10.

In the temporal cortex of P30 animals a strong hybridization was discerned with both probes but positive cells were more abundant in layer III (Fig. 3.9A, B). The CA2, 3 region of the hippocampus and dentate gyrus showed dense cx32 mRNA hybridization. The signal was present in the adult at about the same intensity. The hippocampal region also contained cx43 mRNA but no distinct layer could be detected. The fibre tracts of the forebrain including the internal capsule, external capsule and corpus callosum had fewer cx32 positive cells when compared to P15. There were few cx43 positive cells in the corpus callosum but it was abundant in the cortical layers above (Fig. 3.9B) and subcortical region below. Examination of the cerebellum revealed that the Purkinje cell layer contained signal for both cx32 (Fig. 3.9C) and cx43 mRNA (Fig.3.9D). The white matter of the cerebellum had a strong signal for cx32 at P30 (Fig. 3.9C) although slightly weaker than seen at P15.

FIGURE 3.10   Non-isotopic *in situ* hybridization - postnatal day 30 piriform
                     cortex

Non radioactive *in situ* hybridization with Digoxigenin labelled antisense connexin
riboprobes in the P30 piriform cortex. The cx43 probe (A) labelled cells of the
piriform cortex and deeper layers, whereas, layer 1b is most strongly labelled with
the cx32 probe (B). The cx43 probe in cells of the cortex shows strong reaction
product indicating hybridization. Magnification of the cortical layer shows a
cytoplasmic reaction product with the cx43 probe (C). Magnification A, B (x90);
C (x360).
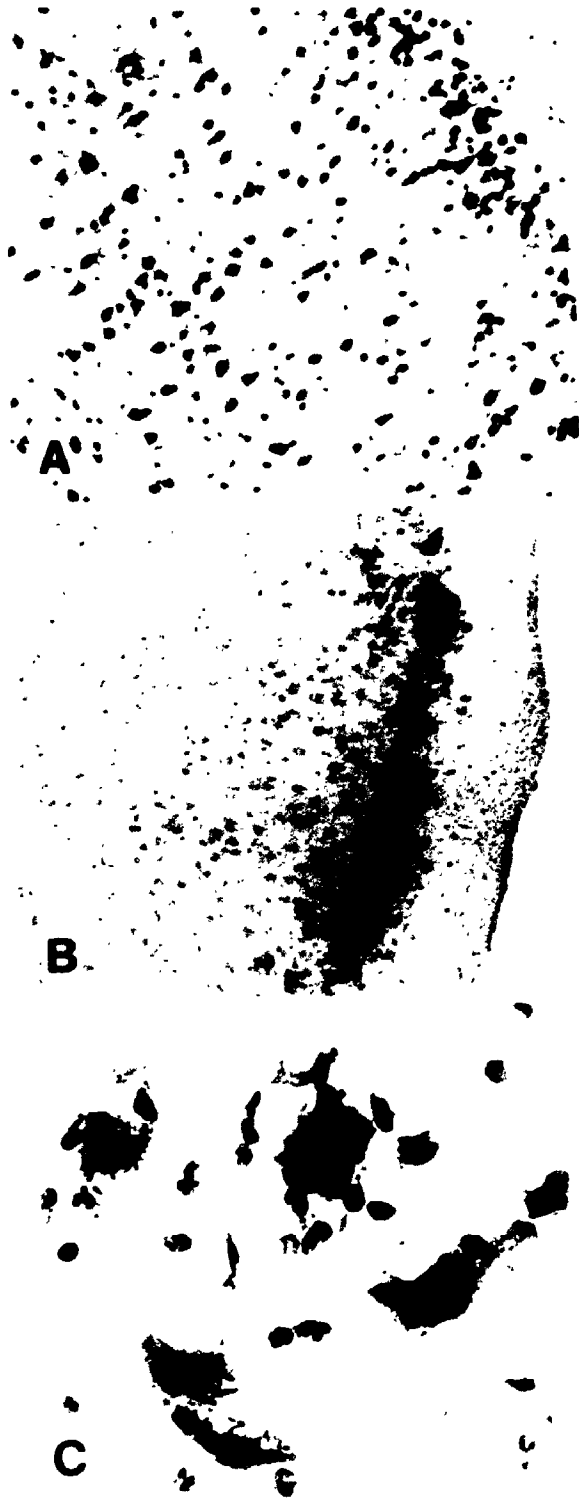
3.3.2 Digoxigenin *in situ* hybridization

The non-radioactive detection method of digoxigenin labelled nucleic acid probes confirmed the *in situ* hybridization results obtained using the radiolabelled probes. Figure 3.10A and B shows the piriform cortex of P30 rat brain hybridized with cx43 and cx32 Dig-cRNA probes, respectively. The intensity of hybridization was stronger for cx43 in deeper layers of the cortex (Fig. 3.10A) than for cx32 (Fig. 3.10B). Figure 3.10C shows a high magnification of cx43 mRNA hybridization which is localized to the cytoplasm of the cells.

3.3.3 Double labelling immunocytochemistry and *in situ* hybridization

Sections of adult brain were incubated with an antibody to GFAP followed by *in situ* hybridization for cx43. Although the hybridization signal for cx43 is much reduced per cell (Fig. 3.11A), some cells were present containing GFAP (Fig. 3.11B) and cx43 mRNA (Fig. 3.11A). The immunocytochemical procedure likely caused some loss of RNA and hence a reduced *in situ* hybridization signal. Therefore, similar sections were processed for immunocytochemistry and *in situ* hybridization separately. This method resulted in coincident patterns of cx32 mRNA and MBP staining as described above (Fig. 3.6A, B) and showed wide

FIGURE 3.11   **Double labeling immunocytochemistry and *in situ* hybridization for cx43 and GFAP**

Brightfield photomicrograph of a section reacted with both GFAP antibody and ^{35}S-labelled cx43 antisense probe showing that cx43 positive cells (A, arrows) are positive for GFAP immunoreactivity (B, arrows). Magnification x567.
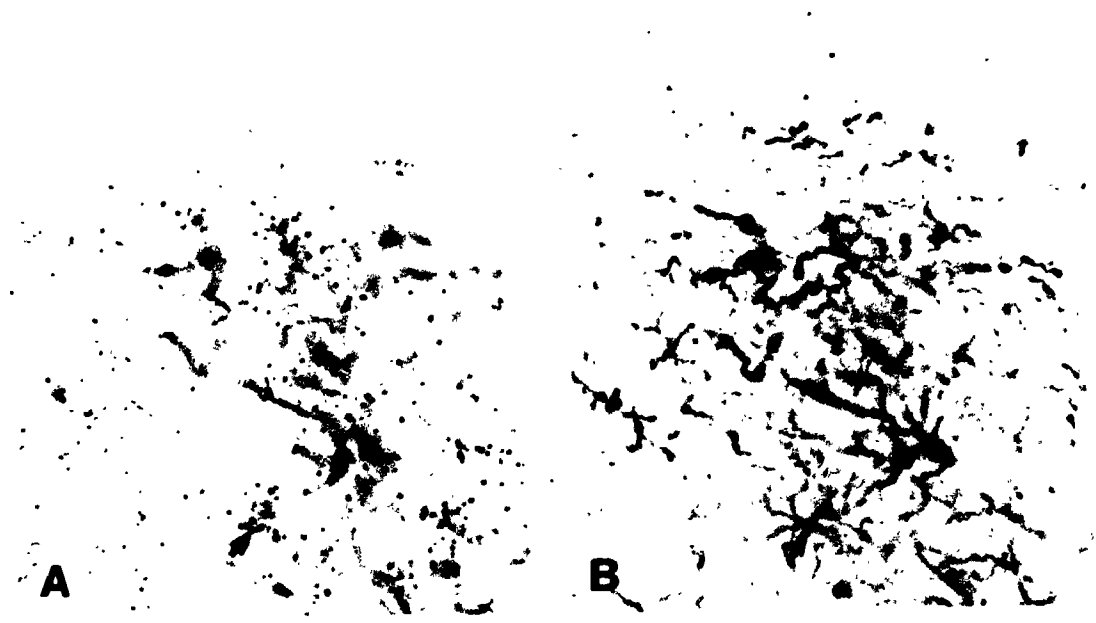
FIGURE 3.12   **Isolation of oligodendrocytes, astrocytes and neurons from adult rat brain**

Brighfield phase contrast photomicrographs of cell isolates from mature rat forebrain astrocytes possessing most of their processes (A) whereas oligodendrocytes are stripped of their processes and appear as small, round, phase-bright cells (B). Only short processes remain on neurons isolated with this method (C). Northern analysis revealed cx43 in astrocytes (D; lane 1, large arrowhead) and cx32 and 43 in the oligodendrocyte isolation (lanes 2 and 3, [large arrowhead, cx43; small arrowhead, cx32]). In lanes 2 and 3, the cx43 signal likely originates from contaminating cells found in the preparation (see text for more details) Neuron-enriched isolation resulted in degraded RNA but hybridization with a probe for cx32 resulted in a signal beginning at 1.6 kb (lane 4, small arrowhead). Lane 5 represents RNA from adult rat brain demonstrating that CNS expresses cx32 and 43. Magnification A, B, C (x144).

A

B

C

D

1 2 3 4 5
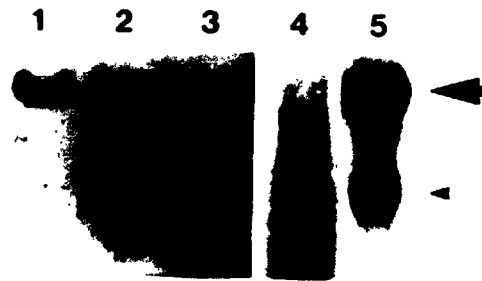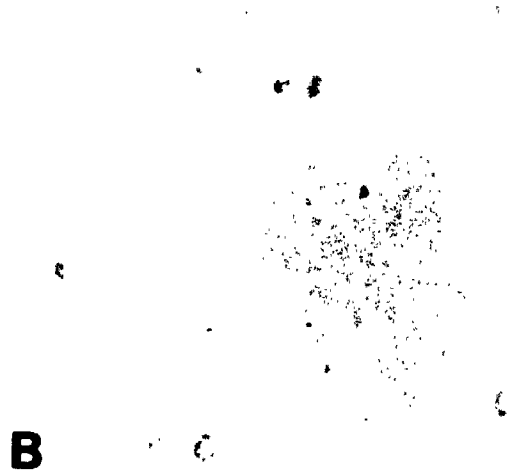
spread GFAP immunostaining agreeing with the homogeneous cx43 mRNA hybridization pattern (data not shown).

3.3.4 Cell isolation

Astrocytes, oligodendrocytes and neurons were isolated from mature rat forebrain to determine connexin expression in these cells. The isolated astrocytes (Fig. 3.12A) were very pure with only a few contaminating neurons and other cell debris. They maintained their astrocytic appearance, extending many processes (Fig. 3.12A). Oligodendrocytes, however, lost their processes during the isolation (Fig. 3.12B). As described by Snyder et al.(1980) they appeared as round, phase-bright cells, the contaminants being capillary fragments, ependymal cells and few neuron cell bodies. The neurons that were isolated maintained short processes attached to their cell bodies (Fig. 3.12C). Neuron-enriched isolations were contaminated by cell fragments, few capillaries and occasional oligodendrocytes. During the isolation of neurons and oligodendrocytes, the RNA collected from the cells was partially degraded which is evident in figure 3.12D. lanes 2-4.

Northern analysis of the RNA isolated from the cell preparations revealed cx43 mRNA in astrocytes (Fig. 3.12D, lane 1) and cx's 32 and 43 in oligodendrocytes (Fig.3.12D, lanes 2, 3). The contaminating cells of the oligodendrocyte isolation have been shown to express cx43 (endothelial and ependymal cells), thus it is probable that the cx43 mRNA signal is derived from

the contaminants and the cx32 mRNA can be attributed to the oligodendrocytes. Although we cannot exclude with certainty the possibility that cx43 is present in oligodendrocytes, such a finding is not supported by the *in situ* hybridization study.

The procedure of Farooq and Norton (1978) describes the isolation of astrocyte- and neuron-enriched fractions. Although the neurons obtained by this procedure looked morphologically similar to those described by Farooq and Norton (1978)(Fig. 3.12C), the resultant RNA was always degraded. As mentioned above, the degradation of RNA is readily apparent and difficult to prevent despite efforts to use RNase free equipment and solutions. It was observed, however, that hybridization with the cx32 cDNA probe resulted in a weak signal at 1.6 kb despite RNA degradation (Fig. 3.12D, lane 4). This suggests the presence of cx32 mRNA in neurons which confirms the patterns of the *in situ* hybridization study described above.

3.4 Discussion

Protein and mRNA for cx32 and cx43 have been shown to be present in the developing and mature brain (Nagy et al. 1988; Dermietzel et al. 1989; Yamamoto et al. 1989a; Yamamoto et al. 1990b; Micevych and Abelson, 1991). In this study the expression of connexin 32 and 43 mRNA was examined in rodent brain and the cellular resolution during development of the rat CNS was determined. This study confirms findings presented in chapter 2 where Northern blotting showed cx43 mRNA being present at all time points examined whereas cx32 was seen beginning at P5-P10 in the hindbrain and P10-P15 in forebrain. Such a signal was present in the medial vestibular nuclei and dorsal paragigantocellular nuclei of the P3 brainstem when hybridized with cx32. Localization of cx43 hybridization was possible at all time points examined including E20, where the signal was abundant in the pial layer. The presence of the signal at this time agrees with observations from other laboratories (Dermietzel et al. 1989). Connexin43 has been reported in leptomeningeal cells (Dermietzel et al. 1989; Yamamoto et al. 1990b; Spray et al. 1991), and the presence of cx43 mRNA in the ependymal cells of the cerebral aqueduct and fourth ventricle agrees with reports of cx43 in these cells (Dermietzel et al. 1989). Cx26 mRNA was also found in leptomeningeal cells confirming observations of cx26 protein in these cells (Dermietzel et al. 1989; Spray et al. 1991). Both

connexin mRNAs reached a plateau at approximately P30 and decreased slightly by adulthood.

3.4.1   Cellular resolution of connexin mRNA

The pattern of hybridization obtained with the two probes implied a differential localization to specific cell populations. Similar hybridization patterns were seen by Micevych and Abelson (1991) and investigators utilizing antibodies (Shiosaka et al. 1989; Yamamoto et al. 1989a; Yamamoto et al. 1990b; Yamamoto et al. 1992). Recent investigations have examined the cellular specificity of gap junction gene expression. Neurons have been shown to contain cx32 protein (Dermietzel et al. 1989; Yamamoto et al. 1989a). Astrocytes contain cx43 mRNA (Dermietzel et al. 1991; Giaume et al. 1991; Naus et al. 1991a), cx43 protein (Dermietzel et al. 19??; Dermietzel et al. 1991; el Aoumari et al. 1990; Yamamoto et al. 1990b; Yamamoto et al. 1992; Naus et al. 1991a), and potentially cx32 (Dudek et al. 1988). Only the cx32 protein has been detected in oligodendrocytes (Dermietzel et al. 1989).

The presence of cx32 mRNA in areas such as the medial vestibular nucleus, paragigantocellular nucleus and layers of the cortex indicates that this mRNA is being expressed in select groups of neurons. By 30 days after birth the pattern of hybridization obtained with the cx32 riboprobe was similar to observations made in the adult by Micevych and Abelson (1991). The results

show that neuron-enriched cell isolation from adult rat forebrain contained cx32 mRNA although degradation of the RNA was problematic, as revealed by Northern analysis. Yamamoto et al. (1989a) detected cx32 protein in vivo in neurons of the hippocampus. Cx32 mRNA has been shown to be in neurons of P2 rat brain (Matsumoto et al. 1991) but cx32 protein was not be detected at this age (Dermietzel et al. 1989). Matsumoto et al. (1991) also reported detectable cx32 mRNA in astrocytes and ependymal cells, observations which were not confirmed by this study.

The pattern of hybridization for cx32 also involved numerous fibre tracts such as the decussation of the superior cerebellar peduncle, spinal trigeminal tract, facial nerve, medial lemniscus, cerebellar white matter, and corpus callosum. These tracts contain axons, some neurons and myelinating oligodendrocytes suggesting these cell types express cx32 mRNA. The expression level of MBP, a protein found in mature oligodendrocytes, is maximal by 15-20 days postnatal (Carson et al. 1983) and is associated with the maturation of oligodendrocytes. The cRNA probe to cx32 hybridized to regions immunostained for MBP in P10 brainstem and cerebellum. This coincident localization of cx32 and MBP was also evident at later postnatal ages. The Northern analysis results from oligodendrocyte isolation showed cx32 and cx43 mRNA being present in oligodendrocyte RNA preparations. The cx43 mRNA detected on these Northerns could possibly be explained by the presence of contaminating ependymal and endothelial cells in the cell isolates, cell types which contain

significant amounts of cx43 (Dermietzel et al. 1989; Dermietzel et al. 1990; Naus et al. 1991a; Micevych and Abelson, 1991). It cannot be ruled out, however, that oligodendrocytes contain cx43, although the results from the *in situ* hybridization do not support such a finding. It was also observed that cultured oligodendrocytes contain cx32 and 43 mRNA and that these cultures contain a 5% astrocyte contamination which likely contributes the cx43 mRNA (unpublished observations).

The hybridization of cx43 mRNA revealed a uniform distribution similar to that reported by Micecych and Abelson (1991). Astrocytes act as support cells and are distributed throughout the CNS. The pattern of hybridization with cx43 would suggest that a large percentage of the signal is present in astrocytes. Immunocytochemistry for GFAP and *in situ* hybridization for cx43 on the same brain section showed that some cells were positive for both the astrocyte specific marker and for cx43 mRNA. Similar double labelling studies showed GFAP and cx43 protein to be localized to similar cells (Yamamoto et al. 1992). The verification of the pattern of hybridization by a separate, non-radioactive *in situ* hybridization paradigm supports these findings. This method demonstrated the presence of cx32 and 43 mRNA in cortical regions such as the piriform cortex in agreement with Micevych and Abelson (1991) and that the mRNA was found in the cytoplasm of the cells. Batter et al. (1992) have shown that astrocytes cultured from different regions of the brain show heterogeneity of expression of cx43 protein and mRNA. Other studies have demonstrated immunocytochemically

that astrocytic expression of cx43 is heterogenous in different regions of the developing and adult brain (Yamamoto et al. 1992). The authors suggest there may be regional specialization of astrocytic coupling and syncytial compartments. These observations are in contrast with this study which shows a more homogenous expression of cx43 mRNA. However, in this developmental study, not all regions of the CNS were examined. It is possible that there is differential regulation of cx43 in subpopulations of astrocytes in regions not examined. Alternatively, culture conditions may influence gene expression resulting in a change in cx43 levels from that of the in vivo situation.

3.4.2 Developmental expression of connexins

Dermietzel et al. (1989) described the expression pattern for cxs 26, 32, and 43 in the developing rat brain using immunocytochemistry. Cx26 and 43 were expressed prenatally with cx26 decreasing in expression with development until it was barely detectable by P6. Connexin43, in contrast, increased in its expression. They also reported that cx32 was detected first at P6 and subsequently increased in expression level. These same patterns of expression for cxs 32 and 43 mRNA were described in the previous chapter. Similarly, cx43 protein has been detected in astrocytes as early as P5 and increasing in abundance to adulthood (Yamamoto et al. 1992). The present study shows that cx43 mRNA was detected in the cerebral cortex of E20 brain and increased in abundance throughout the CNS

during development. No cx43 mRNA was detectable within the telencephalon of earlier embryos by our procedure (data not shown). Hence, the cx43 gene in astrocytes likely turns on in late embryogenesis to begin producing connexin mRNA and protein. Binmöller and Müller (1992) have shown that astrocytes in the developing visual cortex first become dye coupled at P11. Although cx43 mRNA was detected much earlier, it is possible that functional gap junctions are not formed for some time after birth. The expression of cx43 protein in developing rodent brain has been shown to be widely distributed but certain areas expressed 'adult' immunostaining patterns at early postnatal ages (Yamamoto et al. 1992). These areas may contain astrocytes with functional gap junction proteins and, depending on function, cells in certain areas of the brain may 'turn on' their gap junctions at different times.

The cx26 expression was attributed by Dermietzel et al. (1989) to be in leptomeningeal cells and ependyma. Hybridizable cx26 was detected in the leptomeningeal cells of developing brain.

Neuroblasts of the ventricular zone have been found to be coupled during neocortical development (Lo Turco and Kriegstein, 1991). Upon initiation of migration, the neurons uncouple from the clusters to move through the cortex. The number of cells in a cluster (that is, coupled) decrease with development (Lo Turco and Kriegstein, 1991; Yuste et al. 1992; Peinado et al. 1993). Two known connexins fit this pattern of expression; Cx26, as suggested by LoTurco and Kriegstein (1991), and cx37, which has been recently cloned and shown to be

present in rodent brain (Willecke et al. 1991). Willecke et al. (1991) have observed that the expression of cx37 in embryonic brain is 2 to 5 fold higher than adult, making it another candidate for neuronal gap junctions although other neural cells may also express cx37. LoTurco and Kriegstein (1991) also determined that the cell clusters were predominantly composed of neuroblasts and that the radial glial cells did not couple to adjacent cells. Similar observations by other investigators (Connors et al. 1983; Walsh et al. 1989) show that certain neurons decrease their coupling during development. This would suggest that although cx32 may be present in neurons, it would represent a subpopulation of neurons, and that other neurons that expressed cx26 have lost their coupling with maturation. It is also possible that as cx26 protein decreases, cx32 increases in the developing neurons. Results from this study indicate that cultured hippocampal neurons from E18 rats express cx26 protein within 2 days in vitro and that the amount of cx26 decreases as the neurons age (see chapter 4, figure 4.7). These same cultures react with a cx32 antibody when allowed to mature for 3 weeks in culture (chapter 4, figure 4.8).

In summary, this investigation demonstrates a differential expression of mRNAs for cx32 and cx43 in developing rat brain. Cx32 mRNA was localized in developing brain to areas with high concentrations of oligodendrocytes and neurons implicating this connexin in the intercellular communication of these cells. There was an overlap in areas of immunostaining for MBP and hybridization for cx32 further supporting this observation. The cx43 mRNA had a uniform

distribution in a majority of brain areas suggesting a predominantly astrocytic

connexin. The astrocyte specific marker, GFAP, was used to identify these cells,

and they were observed to express cx43 mRNA. Cells isolated from adult brain

confirm the expression of cx32 in oligodendrocytes and neurons and cx43 in

astrocytes. The cellular expression of two gap junction mRNAs within the

developing mammalian brain has been determined and with a clearer

understanding of gap junction expression within neural cells, we can begin to

examine functional roles of CNS connexons.

CHAPTER 4

Analysis of connexin gene expression in cell cultures

4.1 Introduction

Gap junctions are transmembrane channels through which ions and metabolites are transferred from the cytoplasms of adjacent cells (Loewenstein, 1981). In the CNS, gap junctions have been described morphologically between numerous cell types (Sontheimer, 1992; Massa and Mugnaini 1982; Massa and Mugnaini 1985; Brightman and Reese 1969) as well as during development (Mollgard and Moller, 1975; Bennett, 1973). Recent characterization of the gap junction protein family (see Bennett et al. 1991; Dermietzel and Spray, 1993; Willecke et al. 1991 for reviews) has significantly enhanced the complexity of gap junction biology.

In the developing CNS, connexins 26, 32 and 43 (cx26, cx32, cx43) have been detected and shown to be differentially expressed in a temporal and spatial manner (Dermietzel et al. 1989; Yamamoto et al. 1992). The CNS displays a precise development of the various cells found in the mature brain. The cells undergo a series of proliferative and differentiating events during their development into mature neurons and glia (Cameron and Rakic, 1991; Lois and Alvarez-Buylla, 1993). Cell culture techniques allow for close observation of

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developing cells and easier access for the study and manipulation of such systems. In the present study cell culture methods have been used to examine connexin expression in neurons and glia of the developing CNS.

Astrocytes and oligodendroctyes display a unique developmental and differentiation pattern (Dubois-Dalcq and Armstrong, 1992; Cameron and Rakic, 1991; Skoff et al. 1976a; Skoff et al. 1976b). The development of astrocytes is coincident with the expression of cx43 in the CNS (Dermietzel et al. 1989) which has been shown to be expressed in these cells (Dermietzel et al. 1991; Naus et al. 1991a; Giaume et al. 1991). Mature astrocytes demonstrate functional gap junctional intercellular communication as assayed by dye coupling in vivo and in vitro whereas immature (developing) astrocytes are poorly coupled (Binmoller and Muller, 1992; Fischer and Kettenmann, 1985). The postnatal expression of cx32 (Dermietzel et al. 1989) parallels the maturation of oligodendrocytes and their ability to myelinate (Dubois-Dalcq and Armstrong, 1992). Oligodendrocytes are one of the sources of cx32 expression in the adult brain (Micevych and Abelson, 1991) and oligodendrocytes are dye coupled as they mature in culture (Von Blankenfeld et al. 1993).

Oligodendrocytes develop from a bipotential precursor cell, the O-2A progenitor first described in rat optic nerve cultures by Raff and colleques (Raff et al. 1983; Raff, 1989). The O-2A cell is identified by its ability to bind the monoclonal antibody A2B5 (Raff et al. 1983) and differentiate into galactocerebroside positive (Galc+) oligodendrocytes or glial fibrillary acidic acid

positive (GFAP+) type 2 astrocytes (Raff et al. 1983; Raff, 1989). This progenitor

cell has also been identified in cerebral and cerebellar cultures (Behar et al. 1988;

Aloisi et al. 1985) and in other species (Magoski et al. 1992). The type 2 astrocyte

is different from the traditional, protoplasmic type 1 astrocyte in morphology and

biochemical characteristics, including expression of ion channels (Aloisi et al. 1988;

Raff et al. 1983; Beer et al. 1988). Investigations by Sontheimer et al. (1990;

1991b) demonstrated in glial cultures derived from optic nerve that dye coupling

occurred only between A2B5-, type 1 astrocytes but type 2 astrocytes were never

observed to be coupled either to themselves or to type 1 astrocytes.

The objectives of this study were to examine the expression of major

connexin proteins in type 2 astrocytes and to explore the extent of intercellular

communication in these cells. Type 2 astrocytes were compared to primary

cultures of type 1 astrocytes in which the expression of cx43 has been extensively

characterized.

Neurons are thought to arise from bipotential neuron/glia stem cells in the

embryonic ventricular zone (Cameron and Rakic, 1991; Lois and Alvarez-Buylla,

1993). The presence of gap junctional communication in embryonic and early

postnatal neurons has been documented (Lo Turco and Kriegstein, 1991; Peinado

et al. 1993). Cx32 is present in neurons of the developing and adult brain

(Dermietzel et al. 1989; Micevych and Abelson, 1991). The purpose of this study

was to examine connexin expression in primary neuronal cultures. The results

presented in this chapter, although preliminary, demonstrated the presence of cx26

in developing neurons and cx32 in more mature neurons in cultures of

hippocampus and neocortex.

4.2 Materials and Methods

4.2.1 Cell Cultures

a) Primary Astrocytes

Primary astrocyte cultures were prepared using a method similar to McCarthy and de Vellis (1980) and Cole and de Vellis (1989). The cerebral cortices of 24-36 hour old rat neonates were removed following decapitation, dissected free from meninges and placed in Dulbecco's Modified Eagle's Medium (DMEM; Gibco BRL) supplemented with 10% fetal calf serum (FCS; Hyclone, Professional Diagnostics Inc.) and penicillin (50 IU/ml) - streptomycin (50 μg/ml). The pieces of tissue were finely chopped using sterile scissors and triturated through a 10 ml pipet 8-10 times to break up clumps into single cell suspension or small aggregates. The homogenate was passed through an 80μm Nitex mesh and subsequently through a 10μm mesh to remove blood vessels, cellular aggregates and debris. The cell suspension was plated onto 75 mm^2 tissue culture flasks (NUNC), the homogenate from one brain per flask. After 3 days the media containing dead cells and debris was discarded and the cells replenished with fresh DMEM - 10% FCS. The media was changed every 3 days. After 8-10 days, the precursor cells (O-2A cells), which are loosely adherent on top of a sub-confluent layer of protopasmic (Type 1) astrocytes, were removed by vigorously shaking the culture flasks at 200 rpm for 24 hours in order to purify the cultures for type I

astrocytes. The astrocytes were maintained in DMEM - 10% FCS with or without the addition of dibutyryl cyclic adenosine monophosphate (dBcAMP; 0.25 mM; Sigma).

b) O-2A progenitors

When preparing type 2 astrocytes the cultures were allowed to grow for 12-13 days prior to shaking off the precursor cells. The astrocyte cultures were then shaken according to the protocol of Cole and De Vellis (1989). The resultant suspension of cells were plated on poly-D-lysine (10 $\mu g/ml$; Boehringer Mannheim Biochemicals) coated 60 mm^2 and 100 mm^2 tissue culture plates. Precursor cells were maintained in DMEM - 10% FCS for several days during which time they differentiated into type 2 astrocytes. Some cultures were maintained in DMEM - 1% FCS, which promoted the formation of oligodendrocytes.

For immunocytochemical or *in situ* hybridization studies the cells were plated onto 22 mm^2 glass coverslips (Canlab Scientific Products) that were cleaned in concentrated nitric acid (BDH Inc.) for 18 hours followed by 3-2 hour washes in distilled water on a rotating table. The coverslips were autoclaved and coated with poly-D-lysine (10 $\mu g/ml$; 1 ml per coverslip).

c) Neurons

Cortical neuronal cultures were established from E15 rat embryos. The medium used was DMEM supplemented with 10 % FCS and antibiotics. The developing neocortex was removed from each hemisphere and placed in pre-warmed DMEM-FCS. Once all embryos were disected, the fragments of tissue were triturated through a 10 ml pipet 6-8 times. The resultant homogenate was passed through an 80 μm nitex screen and plated onto nitric acid cleaned glass coverslips coated with 10 μg/ml poly D Lysine (Boehringer Mannheim Biochemicals). The nitric acid cleaning aided in the adhesion of neurons to coverslips. The 22mm x 22mm coverslips were spaced apart in metal racks and placed in nitric acid for 18-24 hr and subsequently washed 3 times, 2 hr each, in distilled water. The coverslips were dried in an oven at 80 C for 2 hr, then autoclaved.

The neurons were left to settle and adhere to the coverslips for 20 min after which time the medium and any remaining non-adherent cells were removed by aspiration. The cells were replenished with fresh medium. Three days following plating, the medium was replaced with cytosine arabinoside containing medium at a concentration of 20 μM to reduce the number of proliferating cells. The neurons were maintained for up to 21 days in total after which time they began to degenerate.

Hippocampal neurons were cultured from E18 rat embryos. The hippocampi were collected in Hanks BSS buffered with 10 mM HEPES and trypsinized in 0.25% trypsin for 15 min at 37 C. Trypsinization was stopped by adding Eagles MEM (MEM; Gibco-BRL) supplemented with 10 % Horse serum (HS; Hyclone, Professional Diagnostics Inc.) and Penicillin/Streptomycin. The cells were further dissociated by triturating 3-5 times with a 10 ml pipet. The cells were transferred to poly D Lysine coated, nitric acid treated coverslips and allowed to attach for 4 hr. The coverslips were then removed from the dishes and transferred to culture dishes containing a confluent bed of primary astrocytes which had time to condition the medium for 48 hr. The astrocytes were prepared as described above except that the medium was MEM-HS containing N2 supplements (Bottenstein, 1985); all components from Sigma: tranferrin (100 $\mu g/ml$), insulin (5 $\mu g/ml$), progesterone (20 ηM), putrescine (100 μM), selenium dioxide (30 η M)). The neurons were maintained in these cultures for up to 21 days, one third of the medium being changed every three days.

4.2.2 Immunocytochemistry

a) Cell specific markers

To examine the purity of the cultures, the cells were incubated with cell specific antibodies to cytoskeletal or surface proteins. All procedures were performed on cells that were cultured for at least 6 days. Immunocytochemistry

for surface antigens was performed on live cells. The cells (grown on coverslips) were first incubated with the mouse monoclonal antibodies A2B5 (diluted 1:5; Hybridoma cell line; American Type Culture Collection, Rockville, Maryland; Eisenbarth et al. 1979; Raff et al. 1983), O4 (diluted 1:10; Schachner et al. 1981; Sommer and Schachner, 1981), or galactocerebroside (Galc, diluted 1:50; Boehringer Mannheim Biochemicals) for 1 hour at 4° C in DMEM - 10% FCS buffered with 15 mM HEPES. For the determination of neurons, antibodies to Thy-1 were used (Hybridoma cell line; American Type Culture Collection, Rockville, Maryland) at a dilution of 1:20. The coverslips were rinsed for 2 min in medium with HEPES, fixed with acid-alcohol (5% glacial acetic acid/95% ethanol) for 20 minutes, and washed 3 x 5 min in PBS (120 mM $NaH_2PO_4 \cdot H_2O$, 95 mM NaOH, 70 mM NaCl, pH 7.4). Non-specific sites were blocked with 10% normal goat serum (Dimension Laboratories), 1% BSA (BDH Inc.) for 30 minutes and rinsed briefly. A rhodamine conjugated goat-anti-mouse secondary antibody (Cedarlane Labs) was used to detect the primary antibodies. After 1 hr in the presence of secondary antibodies, the cells were again washed with 3 changes of PBS, 10 min each. The coverslips were mounted in a drop of 50% glycerol (BDH) in PBS containing 0.1% para-phenylenediamine (Valines and Brandtzaeg, 1985) (Sigma) and viewed under a Zeiss Axiophot microscope.

Some cells were fixed prior to immunocytochemistry. These coverslips were fixed as described above, and incubated for 1 hour with an antibody to GFAP. Both monoclonal (diluted 1:20; Boehringer Mannheim Biochemicals) and

polyclonal (diluted 1:100; Chemicon Inc.) antibodies to GFAP were used.

Fluorescein conjugated goat-anti-rabbit (Dimension Laboratories) or rhodamine

conjugated goat anti mouse secondary antibodies were applied to the cells for 1

hour. The coverslips were subsequently treated as described above.

b) Gap junction proteins

Double labelling immunocytochemistry was used to determine the gap

junction protein expression of cultured astrocytes or O-2A cells. Following

fixation and treatment for immunocytochemistry for surface markers, the cells

were incubated for 1 hour with one of three connexin antibodies; cx26 (directed

against a.a. 101-119, diluted 1:25; Nicholson et al. 1987), cx32 (directed against a.a.

223-244, diluted 1:25; Nicholson et al. 1987), or cx43 (directed against a.a. 302-319,

diluted 1:100; Kadle et al. 1991). The remainder of the procedure was the same

as described above. To confirm the specificity of connexin antibodies, fresh-frozen

sections of liver and heart were incubated with appropriate antibodies or pre-

immune sera, following the procedure described above.

4.2.3 Scrape Loading

The cells were examined for their ability to dye couple by scraping them

with a scapel blade and observing the passage of fluorescent dyes, a procedure

known as scrape loading (El-Fouly et al. 1987). Sixty mm^2 dishes of cultured

astrocytes of both types were rinsed with PBS and a 2.5 cm^2 area was marked by removing the cells with a pasteur pipet attached to a vacuum line. This area was filled with 75 μl of a solution containing 0.05% carboxyfluoroscein (MW = 365) and dextran-rhodamine (MW = 4 000; both from Molecular Probe Inc.) in DMEM - 10% FCS and scraped using a scapel blade. The dye solution was left on the cells for 2 min and then removed with successive washes of culture medium. The dishes were placed onto the stage of a Zeiss axiophot microscope fitted with a 16X power neofluor immersion lens. All photographs were taken 10 min after scraping to maintain consistent time for spreading of the dyes.

4.2.4 Northern Blot Analysis

Total cytoplasmic RNA was extracted from cultured astrocytes or O-2A progenitors. The cells were washed once with RNase free PBS (10mM Na$_2$HPO$_4$, 150 mM NaCl, pH 7.2) followed by a wash with cell lysis buffer (20mM Tris-HCl [pH 8.8], 200 mM NaCl, 20 mM MgCl$_2$). The cells were then covered with 1 ml lysis buffer containing 0.5% NP-40 (BDH) for 5 min. Using a rubber policeman, the cells were scraped from the culture dish, pipeted to a 1.5 ml eppendorf tube and centrifuged at 5 000 rpm for 10 min at 4C to remove cell debris. The supernatant was then added to a second buffer (2% SDS, 40 mM EDTA, 100 mM NaCl, pH 8.0) at room temperature for 20 min prior to extraction with phenol-chloroform-isoamyl alcohol (Schibler et al. 1980).

Ten µg aliquots of RNA, as determined by optical density measurements at 260 nm, were prepared for gel electrophoresis and transfer to nitrocellulose as described in chapter 2.2.2. The cx43 and cx32 cDNAs were labeled by random priming using a megaprime kit (Amersham Inc.) and hybridized overnight at 42C. Following hybridization, each blot was washed at 42C for 30 min in decreasing saline concentrations. The first wash consisted of 2X standard saline citrate (SSC; 20X = 3M NaCl, 0.3 M Na$_3$C$_6$H$_5$O$_7$·2H$_2$O, pH 7.0), the second 0.5X SSC and the third 0.15X SSC. Each wash contained 0.2% SDS. Autoradiographs were obtained by exposing Kodak XAR film to the hybridized nitrocellulose.

4.2.5 *In Situ* Hybridization

The cultures were examined for the expression of connexin mRNAs by the technique of *in situ* hybridization. Riboprobes were produced with a Promega RNA transcription kit and labelled according to the procedure describe in chapter 3.2.2. Approximately 500 000 cpm of probe in hybridization buffer was added to each coverslip. The procedure on coverslips was very similar to that described in chapter 3.2.3 except the chloroform step was omitted. Following the last dehydration after post-hybridization washes, the coverslips were fastened to microscope slides for easier handling during emulsion autoradiography. NTB-2 emulsion (Kodak) was used diluted 1:1 with distilled water. The exposure times ranged from 14-21 days.

4.3 Results

4.3.1 Specificity of connexin antibodies

The antibodies used in this study were provided by Dr. B. Nicholson (State University of New York, Buffalo NY). Liver sections reacted positively with cx26 and cx32 antibodies as shown in figure 4.1A and C respectively. Heart sections react with antibodies against the cx43 protein (Fig. 4.1B) at the intercalated discs. Figure 4.1D-E are sections of heart or liver incubated with pre-immune sera and are completely free of any immunoreactivity. When reacted with an antibody to cx32, heart does not show any signal (Fig. 4.1F). These results demonstrate the specificity of each antibody used in this study to unique connexin proteins.

4.3.2 Immunocytochemistry on O2-A progenitors

Using cell specific markers we examined the developmental fate of O-2A progenitor cells after culturing in low and high serum containing medium. The cell-specific expression of various immunocytochemical markers has been well documented (Raff et al. 1983; Dubois-Dalcq and Armstrong, 1992). In low serum, A2B5+, O4+, Galc+ and GFAP+ cells were observed. There was almost 100% overlap between A2B5+/GFAP+ cells. Some cells were O4+/GFAP+, amounting to approximately 50% of the O4+ cells. Approximately 20% of cells

FIGURE 4.1 Specificity of connexin antibodies

Fluorescence microscopy of sections of liver and heart examined for their expression of connexin proteins to determine the specificity of immunoreactivity. Liver sections immunoreacted with cx32 (A) or cx26 (C) and heart sections immunoreacted with cx43 (B) are positive for these connexin proteins. Heart (D) and liver (E) tissue reacted with preimmune sera to cx43 or cx32, respectively, do not contain any signal. Heart sections incubated with an antibody to cx32 did not contain any immunopositive signal (F).
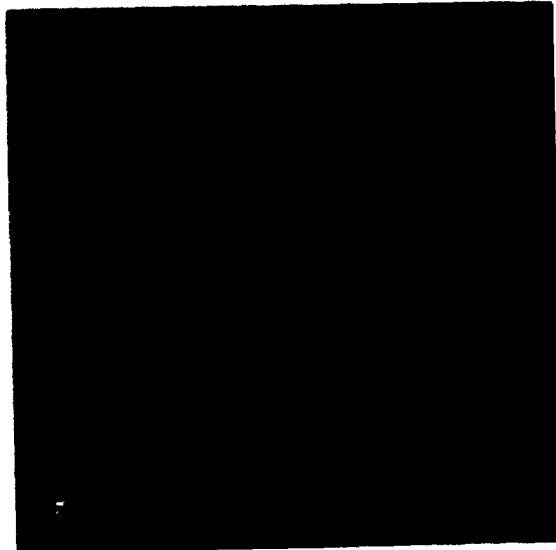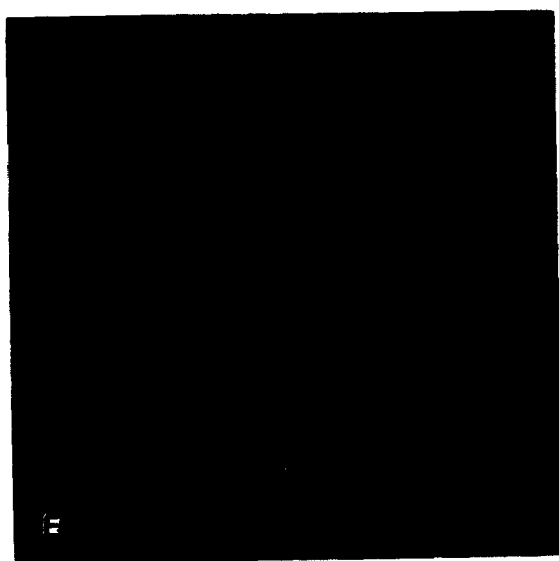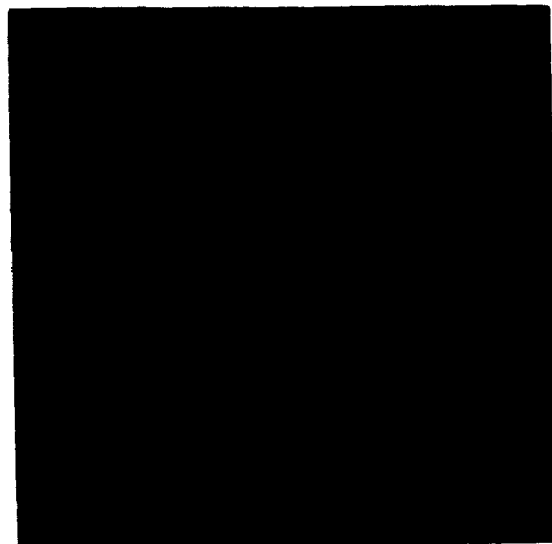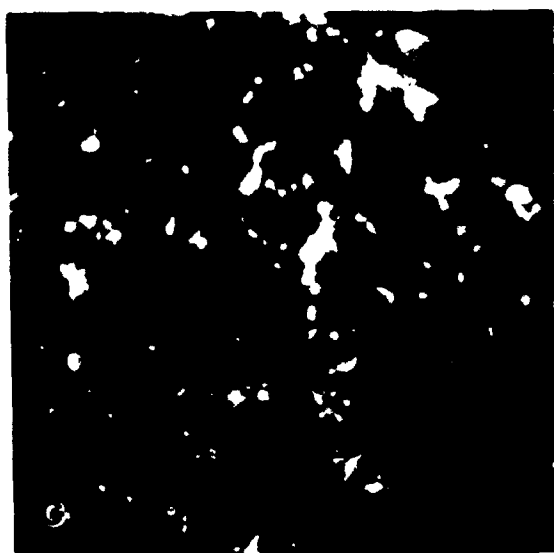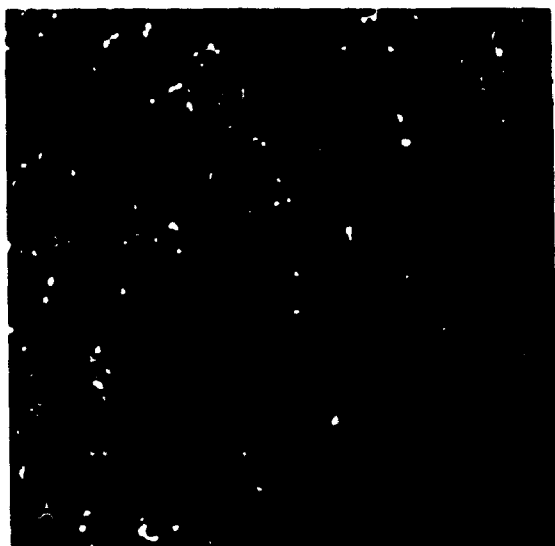
were Galc+/GFAP-. These cells were assumed to be mature oligodendrocytes. In high serum containing medium, the number of A2B5+/GFAP+ cells was much higher. As well, there were a significant number (approximately 20%) of A2B5-/GFAP+ cells in the culture. These were considered type 1 astrocytes. O4+ immunoreactivity was approximately the same in high and low serum cultures.

Figure 4.2 (A and B) shows the morphology of typical cells cultured in high serum. Small cell bodies extend numerous processes which intertwine with processes from other cells. Immunocytochemistry for A2B5 and O4 was performed and demonstrated that these cells were positive for both surface antigens (Fig. 4.2C and D). Double labeling for cx43 and 26 did not detect either of these gap junction proteins in these cells (Fig. 4.2E and F) although there were sites of overlap between processes allowing for the possibility of cell-cell interaction. Type 1 astrocytes can be seen in figure 4.2B mixed among the type 2 astrocytes and they do not express cx26 either (Fig. 4.2D and F). In similar cultures containing a mixture of type 1 and 2 astrocytes (Fig. 4.3A), cx43 immunoreactivity could be detected in the flat protoplasmic type 1 astrocytes (Fig. 4.3B) whereas stellate type 2 astrocytes were negative for cx43 (Fig. 4.3B). Figure 4.3 also shows examples of single type 1 astrocytes in contact with type 2 astrocytes. In these cases no cx43 protein can be detected by immunocytochemistry. Immunocytochemistry for cx32 did not result in any signal in cultures of type 2 astrocytes (data not shown).

FIGURE 4.2 Immunocytochemistry on type 2 astrocytes for cell specific markers and connexin proteins

Immunocytochemistry on type 2 astrocyte cultures to detect cell-specific markers and gap junction proteins. Phase contrast photomicrographs (A and B) reveal that numerous type 2 astrocytes are in contact with each other. A2B5 strongly reacts with the cells (C). The type 2 astrocytes are also O4 positive (D). Immunocytochemistry for gap junctions reveals that the cells are cx43 (E) and cx26 (F) negative. Magnification x360.
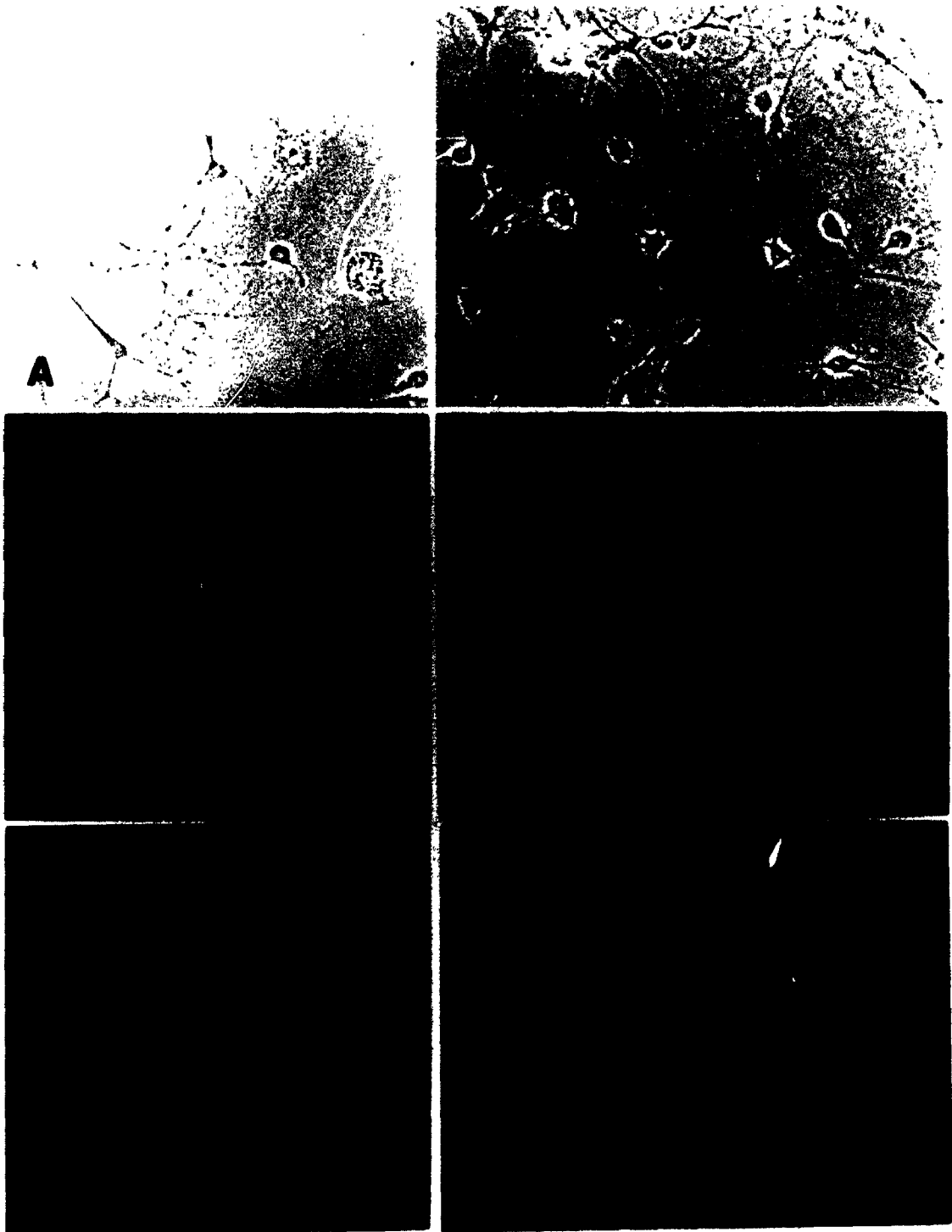
FIGURE 4.3 Mixed cultures of astrocytes showing cx43 in type 1 astrocytes only

Flat, protoplasmic, type 1 astroctyes contaminate cultures of O-2A progenitor cells as seen in the upper portion of the phase contrast photomicrograph in A. These astrocytes are positive for cx43 immunocytochemistry (B), whereas the type 2 astrocytes found in the lower half of the figure (A, arrowheads) do not react with antibodies to cx43 (B). Single type 1 astrocytes seen in contact with type 2 astrocytes (A) are also negative for cx43 (B, asterisks). Magnification x360.
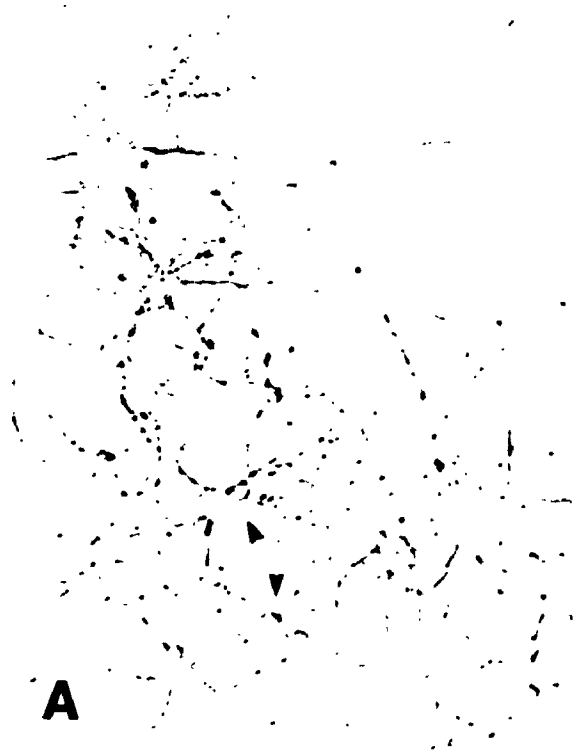
A
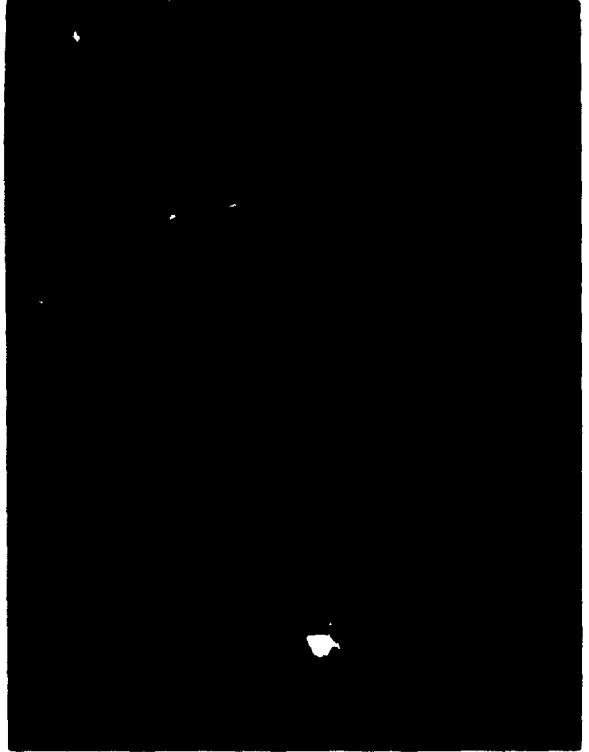
4.3.3 Gap junctional intercelluar communication

As seen in figure 4.3B, type 1 astrocytes express abundant cx43 protein. Confluent cultures of type 1 astroctyes (Fig. 4.4A) show very high levels of dye coupling (Fig. 4.4B) when compared to the passage of dextran rhodamine which cannot pass through gap junctions (Fig. 4.4C). This suggests that the cx43 protein is present in the membrane as functional gap junction channels. The extent of dye coupling agrees with dye injection experiments as previously shown by our laboratory (Naus et al. 1991a). The transfer of dextran rhodamine appears to spread to more than one order of cells (Fig. 4.4C). This is due to the presence of many astrocytic processes and comparisons of figure 4.4B and C clearly shows that passage of carboxyfluoroscein extends to 5th and 6th order cells.

Cultured type 2 astrocytes were also examined for their coupling ability by the scrape loading method. A population consisting of many cells possessing fine processes was scraped through (Fig. 4.5A) as was a group of type 1 astrocytes (as determined from their flat, protoplasmic morphology) found in the same culture dish (Fig. 4.5B). Carboxyfluoroscein entered the injured cells but did not pass to any of the contacting neighbor type 2 astrocytes (Fig. 4.5C). The type 1 astrocytes did pass carboxyfluoroscein to their neighbors as seen in figure 4.5D. Comparison of the dextran rhodamine (Fig. 4.5E and F) confirms that type 2 astrocytes did not pass dye through gap junctions to uninjured cells (Fig. 4.5E) and that type 1 astrocytes did indeed transfer dye to several cells not containing dextran

FIGURE 4.4 Scrape loading of type 1 astrocytes showing abundant dye spread

Scrape loading of confluent primary astrocyte cultures (A) leads to the spread of carboxyfluoroscein to 5th and 6th order cells from the scrape line (B) essentially spreading throughout the whole field of view. Dextran rhodamine does not pass through gap junctions and can be seen in the injured cells which take up both dyes (C). Some cells contain dextran rhodamine even when not near the scrape line. The long processes extending from the astrocytes cross the area of the scrape line and likely have taken up dye when the processes were scraped. Magnification x144.
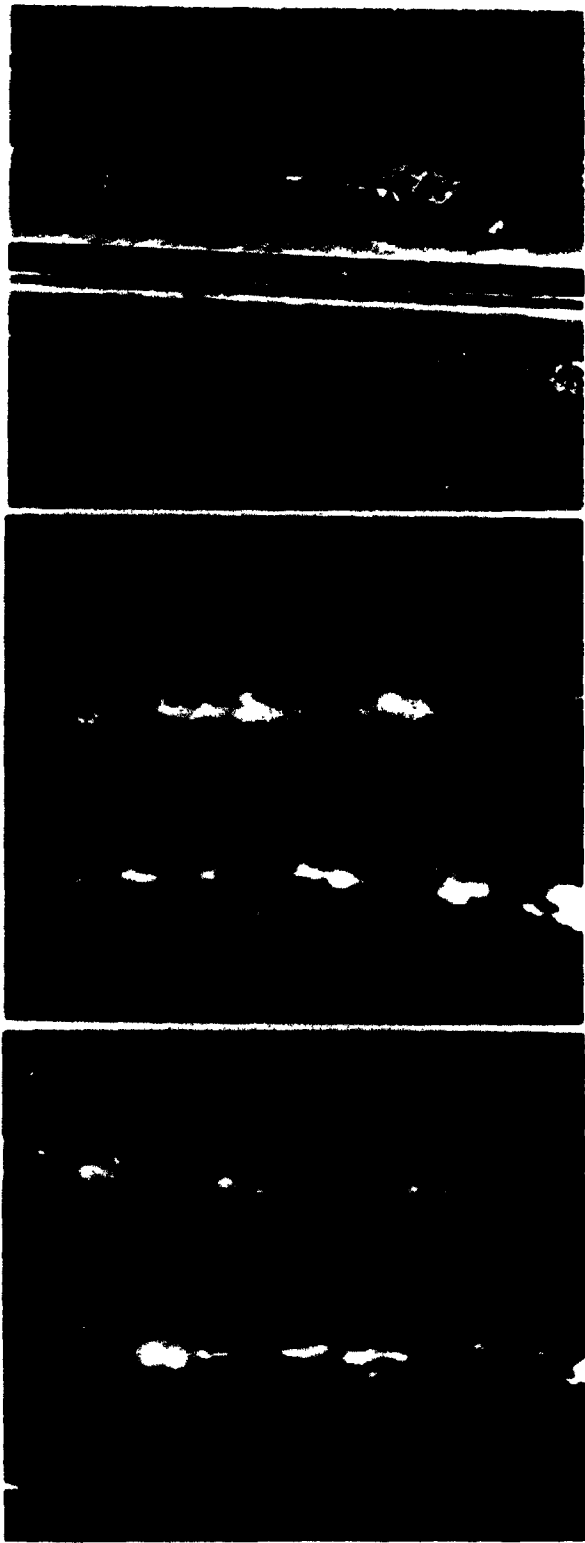
FIGURE 4.5 Scrape loading of type 2 astrocytes shows them to be poorly coupled

Scrape loading of type 2 astrocyte cultures. Some areas of the culture dish contained dense populations of type 2 astrocytes (A) and other areas contain groups of type 1 astrocytes (B). Carboxyfluoroscein entered injured type 2 astrocytes at the scrape line but did not move to nearby contacting cells (C). Type 1 astrocytes, however, did pass carboxyfluoroscein to their neighbors (D). Comparing the spread of carboxyfluoroscein with dextran rhodamine confirms that only injured type 2 astrocytes contain both dyes (E). Dextran rhodamine cannot be found in some type 1 astrocytes (F, asterisks) which contain carboxyfluoroscein (D) indicating dye transfer has taken place. The white lines across the field of view and in the upper right hand corner in panels D and F indicate areas where scrapes were made. Magnification x144.
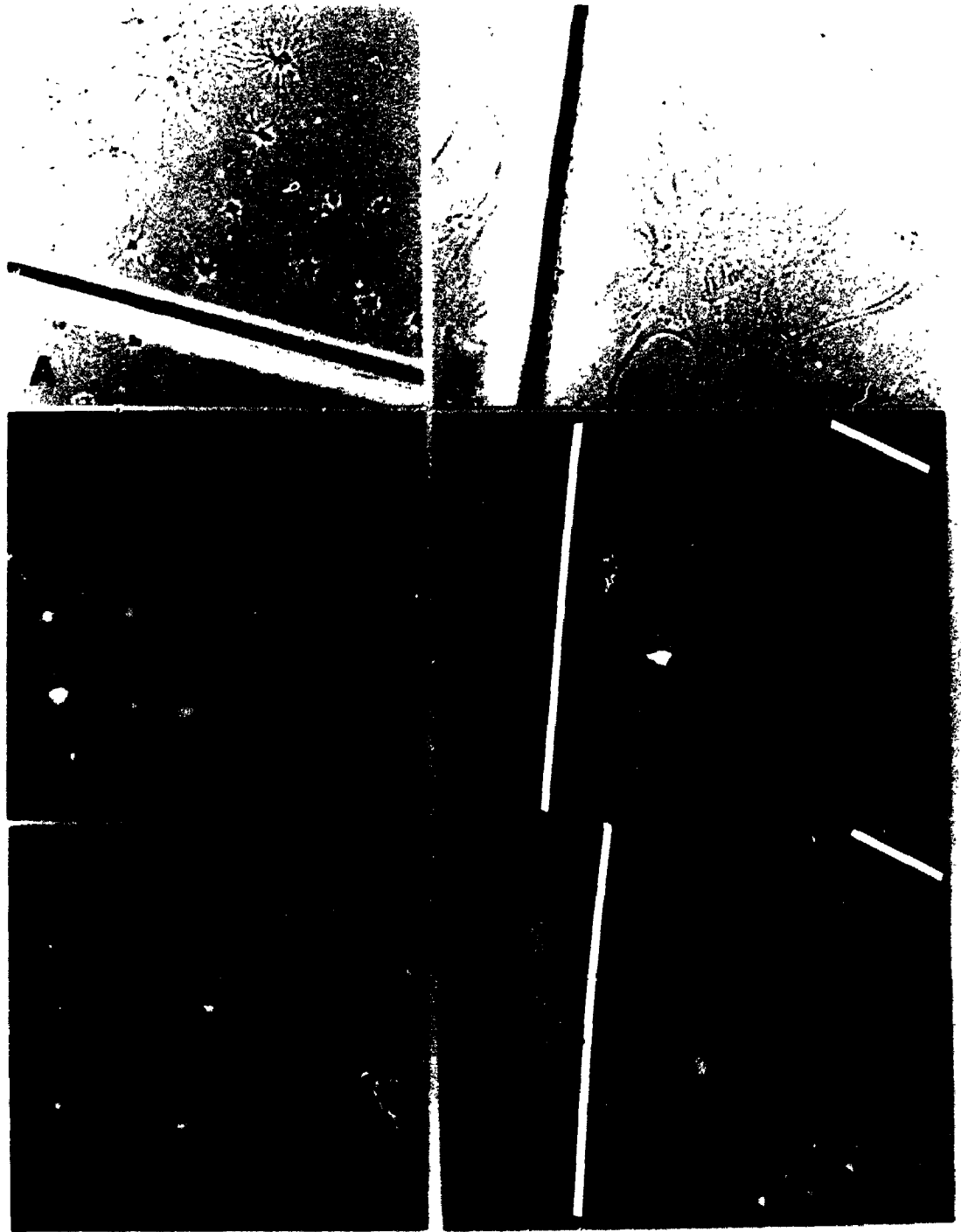
rhodamine (Fig. 4.5F). The more direct method of dye injection was performed on type 2 astrocyte cultures. After three minutes of dye injection into a type 2 astrocyte, no transfer could be seen to any adjacent cell (data not shown). Injections into type 1 astrocytes yielded immediate transfer of dye to neighboring type 1 astrocytes (data not shown).

4.3.4   Connexin mRNA expression in O2-A progenitors

Northern blot analysis confirmed that type 1 astrocytes express cx43 mRNA (Fig. 4.6A). The addition of dbcAMP which causes a pronounced change in morphology of astrocytes from a protoplasmic to a stellate shape did not alter the expression of cx43 mRNA (Fig. 4.6A). This suggests that the morphological difference seen between type 1 and 2 astrocytes does not attribute to the different gap junction expression. Cultures of O-2A cells treated in low serum develop into oligodendrocytes. Figure 4.6A shows that, in addition to cx43 mRNA, cultures treated with low serum express cx32 mRNA. It is likely that the cx43 mRNA is from astrocytes that contaminate these cultures as approximately 5% of the cells are protoplasmic type 1 astrocytes. Oligodendrocytes contain cx32 mRNA as shown in chapter 3. Figure 4.6A demonstrates that cultured O-2A cells promoted to develop along the oligodendrocyte lineage also expresses cx32 mRNA.

In situ hybridization was performed in mixed astrocyte cultures. The purity of the cultures was determined by GFAP immunostaining. Figure 4.6B shows

**FIGURE 4.6 mRNA analysis of type 1 and 2 astrocytes by Northern blotting and
in situ hybridization**

Northern blot analysis and in situ hybridization on glial cell cultures. Northern
blotting of RNA isolated from primary astrocytes show high levels of cx43 mRNA
(A) in cultures treated or not treated with dbcAMP. Oligodendrocyte cultures
show cx43 (upper band) and cx32 (lower band) mRNA hybridization.
Oligodendrocyte cultures contain approximately 5% contaminating astrocytes that
express abundant cx43 and are likely the reason for the cx43 signal. Astrocytes
have not been shown to express cx32, hence the cx32 mRNA is likely expressed in
oligodendrocytes. GFAP positive astrocytes (B) in culture shows two distinct cell
types, protoplasmic (Type 1, 1) or fibrous (Type 2, 2). In similar cultures, phase
constrast microscopy revealed a mixed astrocyte culture (C). *In situ* hybridization
reveals that the cx43 riboprobe strongly hybridizes to the type 1 astrocytes (D;
asterisks indicate nuclei of same cells in photos C and D) but there is no signal in
the cytoplasm of type 2 astrocytes (D, arrows). Magnification B-D x360.

astrocytes
astrocytes+dbcAMP
oligodendrocytes

A

C
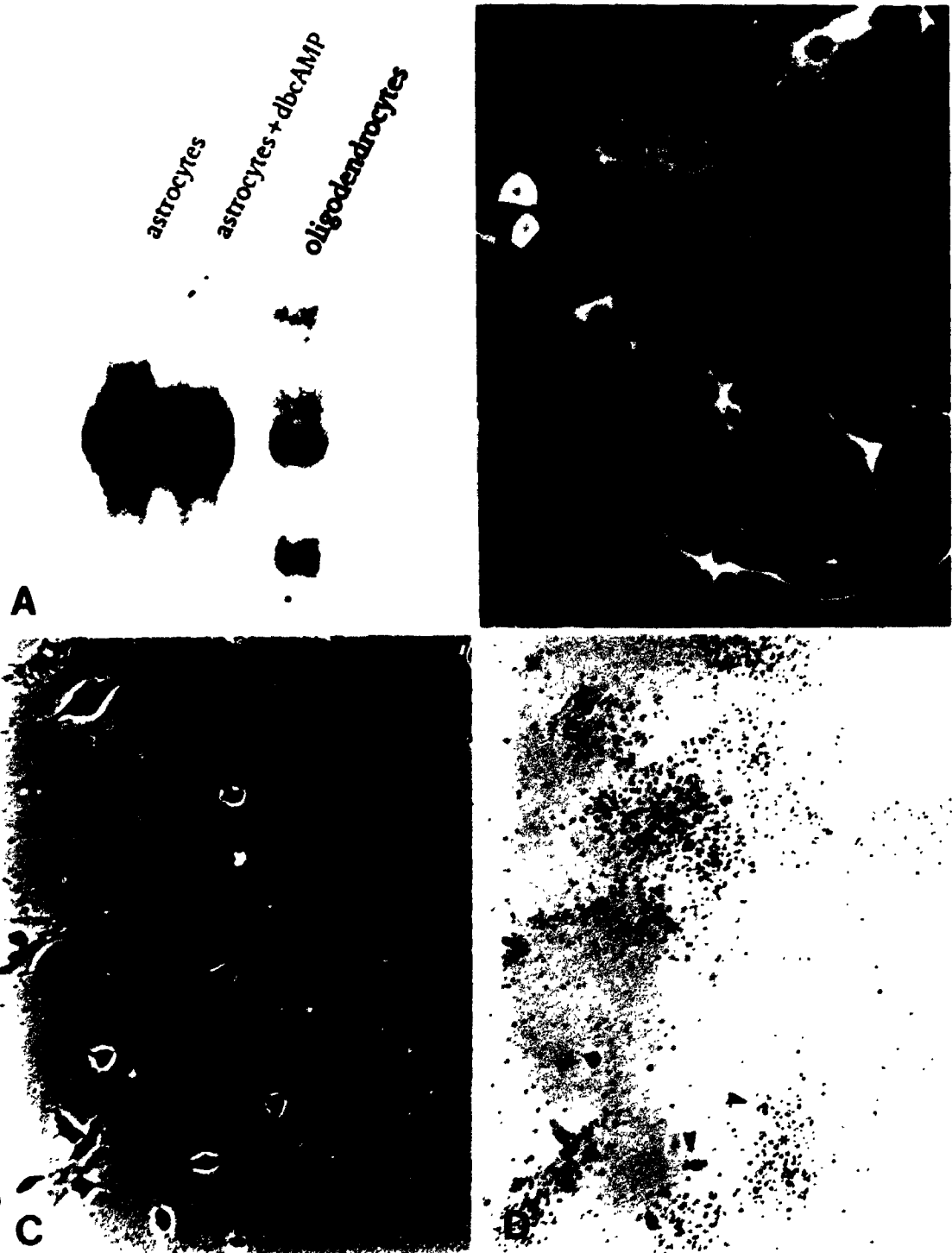
GFAP in types 1 and 2 astrocytes. It can be seen that they are morphologically distinct. The mixed cultures (Fig. 4.6C) when used for *in situ* hybridization showed cx43 mRNA in the type 1 astrocytes (Fig. 4.6D). Connexin43 mRNA could not be detected in t..e phase dark cell bodies of type 2 astrocytes (Fig. 4.6D). Cx32 or cx26 mRNA could not be detected in type 1 or 2 astrocytes by *in situ* hybridization (data not shown).

4.3.5 Expression of connexin proteins in neurons

Within 2 days in vitro (DIV), cells with multiple processes were observed in cultures on cortical neurons (Fig. 4.7A). These cells did not stain with Thy-1 likely due to their age (Morris, 1985) but their appearance is that of immature neurons. The neuronal cultures were positive for cx26 at sites of neuron cell body contact and on some processes (Fig. 4.7B). After 5 DIV, Thy-1 immunocytochemistry was detected on the surface of process bearing cells (Fig. 4.7C). These neuron-like cells were positive for cx26 although subpopulations of neurons did not express detectable levels of this protein. Hippocampal neurons cultured for 2 or 4 days also display cx26 immunoreactivity (Figs. 4.7E, 4.8A) and the neurons are positive for Thy-1 (Figs. 4.7F, 4.8A). Cultures of hippocampal neurons maintained for 21 DIV extended numerous process as seen in figure 4.8B. These particular cells expressed high levels of cx32 protein (Fig. 4.8B).

FIGURE 4.7 Analysis of connexin26 expression in primary cultures of rat neocortical and hippocampal neurons

Neurons from embryonic day 15 rat cultured for 48 hr (A) shows signs of extending processes as shown in phase contrast and immunocytochemistry for cx26 revealed the presence of the protein at sites of contact between cell bodies and on neurites (B). In similar cultures, 5 days in vitro (C and D), Thy-1 is expressed on the surface of neuron-like cells in the culture (C). The same field examined with the fluoroscein filter shows cx26 immunoreactivity on neurons although not all neurons demonstrate cx26 immunoreactivity (D). Hippocampal neurons cultured from embryonic day 18 rats and cultured 4 days also display some cx26 immunoreactivity (E) and are weakly positive for Thy-1 (F). Magnification x360.

A
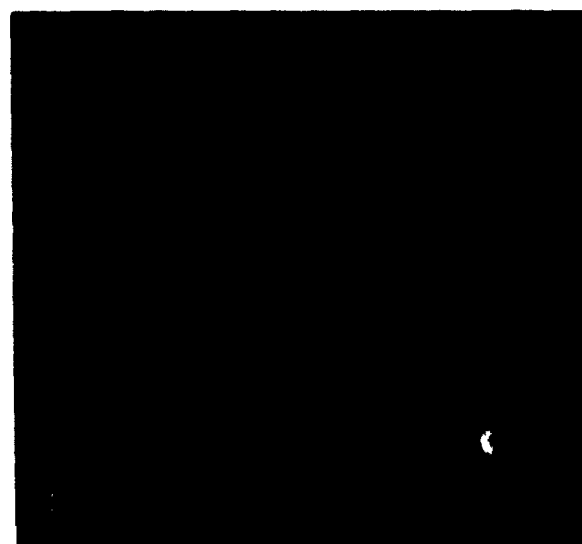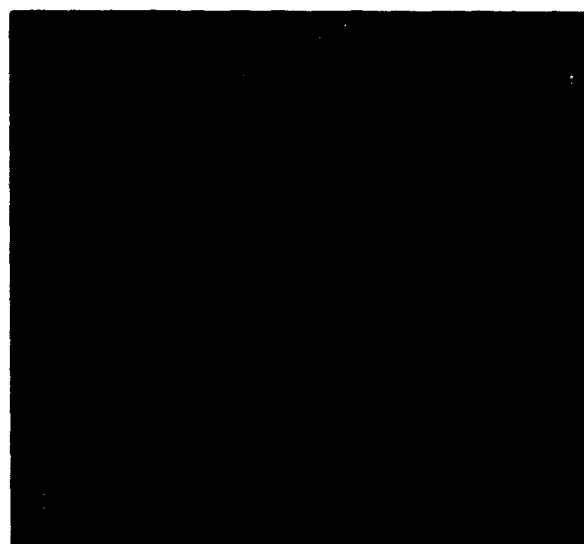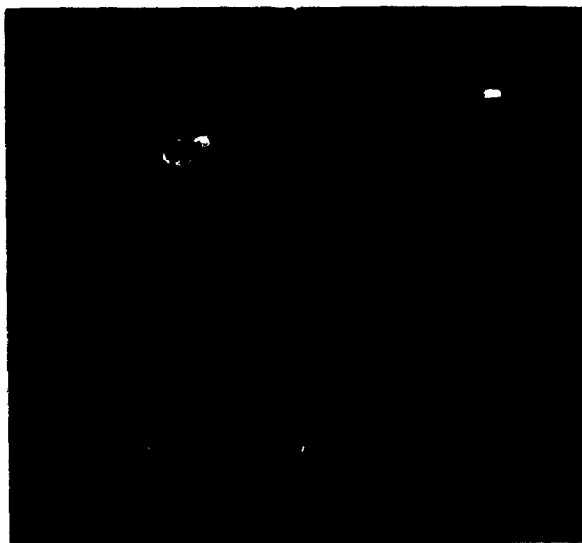
FIGURE 4.8 Analysis of connexin32 protein expression in cultured hippocampal neurons

Hippocampal neurons cultured for 2 days in vitro extend processes towards neighboring cells (A), are immunopositive for Thy-1 (rhodamine) and express cx26 protein (fluoroscein). At 21 days in vitro, hippocampal neurons immunoreacted for cx32 (fluoroscein) show a strong signal for this gap junction protein (B). Magnification x360.
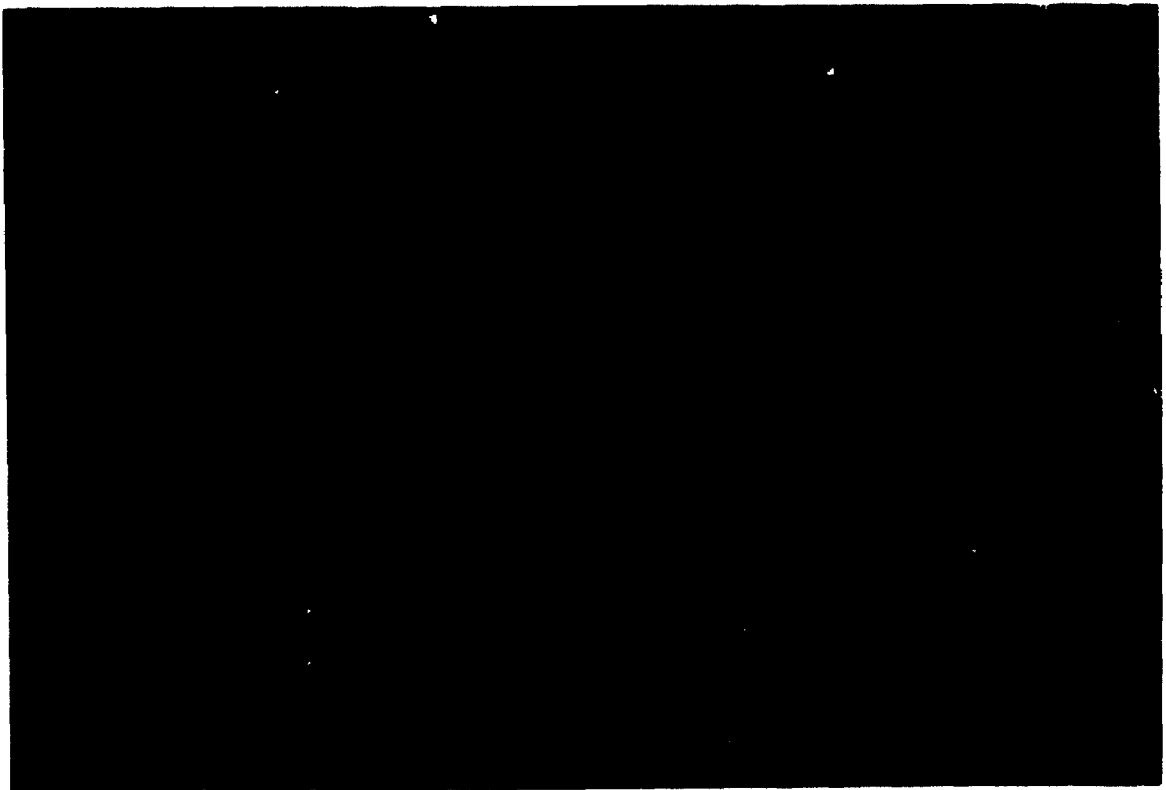
4.4 Discussion

4.4.1 Types 1 and 2 astrocytes

The type 2 astrocyte lineage is derived from a common oligodendrocyte-type 2 astrocyte precursor, the O-2A progenitor, first described in rat optic nerve cultures (Raff et al. 1983; Raff, 1989). It has been shown that type 2 astrocytes of optic nerve are not dye coupled when Lucifer Yellow is injected into a cell (Sontheimer et al. 1990; Sontheimer et al. 1991b). In the present study, cerebral type 2 astrocytes were not coupled to each other or to type 1 astrocytes whereas type 1 astrocytes are strongly coupled to other type 1 astrocytes. Ransom and Kettenmann (1990) have shown that dye coupling may not be sensitive enough to detect gap junctional intercellular communication in mixed oligodendrocyte-type 1 astrocyte cultures. In their study, weak electrical coupling was detected between astrocytes and oligodendrocytes in the absence of dye coupling. Although it is possible that type 2 astrocytes are weakly coupled electrically, immunocytochemistry for the connexin proteins documented to be present in the CNS reveals that type 2 astrocytes do not express cx26, 32 or 43 protein at detectable levels. *In situ* hybridization results demonstrate that type 1 astrocyes express high levels of cx43 mRNA and that type 2 astrocytes do not express this message nor mRNA for cx26 or cx32.

Type 1 astrocytes are known to express the cx43 gap junction protein (Dermietzel et al. 1991; Naus et al. 1991a; Giaume et al. 1991; Micevych and Abelson, 1991; el Aoumari et al. 1990) and have been shown to contain numerous gap junctions on their membranes (Mugnaini, 1986; Massa and Mugnaini, 1982; Massa and Mugnaini, 1985). The type 2 astrocyte is thought to represent the fibrous astrocyte of white matter associated with the nodes of Ranvier (ffrench-Constant and Raff, 1986). These perinodal astrocytes are intimately associated with the nodal gap although they do not form gap junctions with the nodal axon (Black and Waxman, 1988). They do, however, form gap junctions with oligodendrocytes and other perinodal (type 2) astrocytes (Waxman and Black, 1984; Black and Waxman, 1988). ffrench-Constant and Raff (1986) suggest that the O-2A cell lineage may be specialized for myelination as it develops into myelin forming oligodendrocytes and node of Ranvier-associated type 2 astrocytes. Considering that both cell types develop from a common precursor, they may share a similar class of gap junctions, namely members of the β connexins(Risek and Gilula, 1991; Gimlich et al. 1990). Cx32 is considered a member of the β connexin class as is the recently isolated cx37 which has also been shown to be present in the CNS (Willecke et al. 1991). The evidence we present indicates that cx32 is not expressed by type 2 astrocytes. It is possible that the level of expression is below detection. Gap junctions have been detected morphologically, but this does not indicate functional channels. A counter argument is the fact that *in vitro* studies do not necessarily mimic in vivo

conditions. Although oligodendrocytes and type 2 astrocytes develop from O-2A cells in culture as they are thought to do so *in vivo* (Cameron and Rakic, 1991; Raff, 1989; Levison and Goldman, 1993; Goldman and Vaysse, 1991), they differentiate in the absence of neurons and axons or mature myelinating oligodendrocytes. Hence, the lack of intercellular communication could be considered an artifact of the culture system.

There is mounting evidence that suggests type 2 astrocytes have unique properties when compared to type 1 astrocytes. The expression of ion channels, for example, is different (Barres, 1991). Single, type 2 astrocytes display Ca^{2+} signalling in response to histamine (Inagaki et al. 1991) and have been shown to exhibit glutamate and gamma-amino-butyric acid (GABA) activated responses (Magoski et al. 1992; Barres, 1991). O-2A cells also appear to synthesize GABA (Barres, 1991). Type 2 astrocytes also express the "neuronal" form of Na^+ channel (Minturn et al. 1991; Barres, 1991; Sontheimer et al. 1991a; Barres et al. 1990) whereas type 1 astrocytes have a glial type (Barres, 1991). It has been hypothesized that type 2 astrocytes may represent an extraneuronal source of Na^+ channels that can be contributed to the neuronal membrane at the node of Ranvier (Minturn et al. 1991; Gray and Ritchie, 1985). Na^+ channels have been localized to sites adjacent to the node of Ranvier in presumptive perinodal astrocytes by immuno-electron microscopy (Black et al. 1989). Interestingly, Sontheimer et al. (1991) have shown that Na^+ current expression is restricted to uncoupled astrocytes in hippocampal cultures. Coincident with this finding is the

fact that in mixed astrocyte cultures, type 1 astrocytes are highly coupled and type 2 astroctyes are not coupled at all (this study and Sontheimer et al. 1991b; Sontheimer et al. 1990).

The potential role for type 2 astrocytes differs from the major function attributed to type 1 astrocytes. The ability of type 1 astrocytes to form a coupled syncytium (Fischer and Kettenmann, 1985) allows them to act as spacial buffers for excess K^+ ions in the extraneuronal environment (Orkand, 1977; Barres, 1991) and to spread glutamate induced Ca^{2+} waves via their gap junctions (Cornell-Bell et al. 1990; Charles et al. 1991; Charles et al. 1992).

The heterogenous expression of gap junction genes between different types of astrocytes may represent functional specialization of these cells related to their developmental lineage and anatomical localization in the mature CNS. The inability to detect gap junction proteins in this study suggests that type 2 astrocytes are functionally uncoupled and that their function is unrelated to the gap junction - mediated spacial buffering capacity of type 1 astrocytes. It has yet to be determined what the precise role of type 2 astrocytes is in the CNS or if they indeed exist as a unique cell type *in vivo* (Noble, 1991; Skoff and Knapp, 1991), but evidence suggests that they have specific functions which may be reflected in ion channel expression.

4.4.2 Cultured Neurons

In vivo, gap junctions have been identified in neurons by morphological (Brightman and Reese, 1969; Mollgard and Moller, 1975) and molecular criteria (Micevych and Abelson, 1991; Dermietzel et al. 1989; Shiosaka et al. 1989; Yamamoto et al. 1989a). Developing and mature neurons are coupled suggesting the presence of functional channels (Lo Turco and Kriegstein, 1991; Peinado et al. 1993; Connors et al. 1983; Gutnick et al. 1981). In the hippocampus and neocortex, intercellular coupling of neurons has been documented (Sloper and Powell, 1978; Sotelo and Korn, 1978; MacVicar et al. 1982; Kosaka, 1983a; Kosaka, 1983b).

With respect to specific connexin expression, cx32 is present in select populations of neurons as detected by immunocytochemistry (Shiosaka et al. 1989; Yamamoto et al. 1989a) and in situ hybridization (Micevych and Abelson, 1991). Cx32 was detected in isolated neurons of the adult brain (chapter 3.3.4) and during development of the CNS (chapter 3.3.1). Cultured neurons from either the neocortex or hippocampus display cx26 immunoreactivity as early as 2 DIV. Immunocytochemistry for cx26 could not be detected by 7-10 DIV and at 21 DIV, cx32 was detected in hippocampal neurons. The presence of cx26 in embryonic neurons may represent the mechanism of coupling in neurons of the developing neocortex (Lo Turco and Kriegstein, 1991). The reduction in level   this protein coincides with the reduced extent of coupling in the cortex of developing rat brain.

In these preliminary experiments, it cannot be determined if the same neurons are losing cx26 as they begin to express cx32 protein. It is possible that separate neuronal populations are expressing each connexin. The hypothesis of embryonic and mature forms of gap junction channels in developing neurons is an attractive one. These preliminary investigations support such a conclusion.

CHAPTER 5

Connexin expression in neural cells derived from retinoic acid treated P19 cells

5.1 Introduction

The regulation of cell fate and determination during development involves a diverse series of events including gene induction, cell migration and cell interactions (Melton, 1991). Intercellular communication via gap junctions has been demonstrated to be one type of cell-cell interaction that is involved during development (Caveney, 1985; Kidder, 1987; Bennett et al. 1981).

The P19 embryonal carcinoma (EC) cell line (McBurney and Rogers, 1982; McBurney, 1993) represents a pluripotential stem cell that can differentiate along epithelial, muscular, and neural lineages depending on the environment (Jones-Villeneuve et al. 1982; McBurney et al. 1982; Edwards et al. 1983). EC cells appear to differentiate in a manner similar to normal cells of the embryo (McBurney, 1993). Numerous investigators have utilized EC cells as experimental models to study various events that occur during cell lineage development (den Hert. et al. 1991; Schmidt et al. 1992; Tanaka et al. 1992). EC cells have even been shown to contribute to the normal environment of the host when injected into blastocysts (Rossant and McBurney, 1982) or directly into the CNS (Wojcik et al. 1993). Junctional complexes have been detected between P19 cells (Smith et

al. 1987) and gap junctions between embryonal carcinoma cells and blastocysts were detected morphologically in another EC cell line, F9 (Lehtonen et al. 1984). The F9 cell line has been utilized as a model to examine the expression of gap junction genes during early mouse development (Nishi et al. 1991). A cDNA library of F9 cells was used to characterize novel mouse connexin genes, cx31 and cx45 (Hennemann et al. 1992c), adding to the list of cxs 26, 31.1, 32, 37 and 43 already detected in these cells (Willecke et al. 1990; Willecke et al. 1991; Willecke et al. 1991). Differentiation of F9 cells by retinoic acid or dibutyryl cyclic adenosine monophosphate (dbcAMP) alters the level of connexin expression and species of connexin genes present (Nishi et al. 1991; Willecke et al. 1990).

The objective of this investigation was to examine connexin gene expression in P19 cells differentiated with RA. The model of neuronal and glial development demonstrated by P19 cells will be valuable in the study of developmental regulation of connexin expression in the nervous system.

5.2 Materials and Methods

5.2.1 Culture and Differentiation of P19 cells

The P19 cell line (McBurney and Rogers, 1982) was cultured and maintained as described by Rudnicki and McBurney (1987). P19 cells were cultured in α-modified Eagle's minimal essential medium (αMEM; Gibco-BRL) supplemented with 7.5% calf serum/2.5% fetal calf serum (Professional Diagnostics Inc.) and containing 50 IU/ml penicillin-50 μg/ml streptomycin. The cells were maintained at subconfluency by subculturing them every 48 hr onto tissue culture grade dishes (Corning).

In order to differentiate P19 cells, they were removed from the culture dishes by treatment with 0.25% trypsin (Gibco-BRL)/1.0 mM EDTA[T-EDTA] (BDH) in Duelbecco's phosphate buffered saline (dPBS; 2mM KCl, 1 mM KH_2PO_4, 130 mM NaCl, 80 mM Na_2HPO_4, pH 7.4). The cells were plated at a density of $5x10^4$ cells/ml in αMEM containing 0.3 μM retinoic acid (RA; Sigma) in 100mm bacteriological grade petri dishes (Falcon) and allowed to aggregate for 48 hr. At this time the medium was changed by pipetting the cells into a 15 ml conical tube (Falcon) where the aggregates settled to the bottom and the used medium aspirated. The cells were replenished with fresh RA containing medium for another 48 hr and the cell aggregates separated from the spent medium in the same manner. Control cultures were treated identically to experimental cultures

except that RA was omitted from the medium. Following the fourth day of RA treatment, the aggregates were plated onto tissue culture grade dishes in medium without RA and used to analyze the effects of RA on connexin expression or to examine the development of astrocytes. Other aggregates were treated with T-EDTA for 5 min, and passed through a 10 ml pipet to break up the clumps prior to plating. This procedure increased the number of neurons in the culture. In addition to dispersing the aggregates, some cultures were treated with 20 μM cytosine arabinoside (Sigma) after 24 hr to kill proliferating cells and further enrich the neuronal population.

Primary astrocyte cultures were prepared as described in chapter 4.2.1 (a).

5.2.2   Immunocytochemistry

Antibodies to various gap junction proteins or to cell specific markers were used in single and double labeling experiments. The connexin antibodies were gifts from Dr. Bruce Nicholson, State University of New York, Buffalo. Anti-connexin43 (cx43) was used at a dilution of 1:300 and anti-connexin26 (cx26) at 1:25. Preimmune sera were used as controls for the connexin polyclonal antibodies at the same concentration as the primary antibodies. Antibodies to glial fibrillary acidic protein (GFAP; used at 1:10) and fibronectin (used at 1:200) were obtained from Boerhinger Mannheim.

P19 cells were cultured for 36 hr as described above on nitric acid-treated coverslips. The nitric acid treatment aided in the adhesion of P19 cells to the coverslips, in particular when the cells were approaching confluency. The cells were fixed for 20 min at -20C in 95% ethanol -5% glacial acetic acid. Following fixation, the coverslips were washed in 3 changes of PBS (120 mM $NaH_2PO_4 \cdot H_2O$, 95 mM NaOH, 70 mM NaCl, pH 7.4) for 5 min each. Non-specific binding sites were blocked with 10% normal goat serum (Dimension Laboratories) and 1% BSA in PBS for 30 min at room temperature. All subsequent incubations were also performed at room temperature. The blocking solution was rinsed off of the coverslips and a solution of 0.1% BSA in PBS containing the primary antibodies added. In the double labeling experiments, both antibodies were present in the solution. The cells were incubated in the presence of primary antibodies for 1 hr followed by 3 - 10 min washes in PBS. Fluorescein conjugated goat-anti-rabbit (Dimension Laboratories) or rhodamine conjugated goat-anti-mouse (Cedarlane Labs) secondary antibodies were used to detect the primary antibodies. After 1 hr in the presence of secondary antibodies, the cells were again washed with 3 changes of PBS, 10 min each. The coverslips were mounted in a drop of 50% glycerol (BDH) in PBS containing 0.1% para-phenylenediamine (Sigma) and viewed under a Zeiss Axiophot microscope.

5.2.3 RNA Extraction and Northern Blot Analysis

Total cytoplasmic RNA was extracted from cultures of P19 cells at 4 and 7 days post-RA treatment (see above) and from untreated cells. The procedure is described in chapter 4.2.4. The cx43 and 26 cDNAs were gifts from Dr. E. Beyer, Washington State Medical School, and Dr. B. Nicholson, State University of New York, Buffalo, respectively (Beyer et al. 1987; Zhang and Nicholson, 1989). Dr. D. Denhardt, Rutgers University, kindly provided the 18S ribosomal RNA probe which was used to demonstrate that equivalent amounts of RNA was loaded in each lane.

5.2.4 Protein Isolation and Western Blot Analysis

Total cellular protein from P19 cells was examined at 2, 4, 8, and 12 days post-RA treatment and in untreated cultures at the same time points. Two dishes containing the same number of cells were processed at each time. On one dish 1 ml of sample buffer (0.0625 M Tris-HCl [pH6.8], 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.001% bromophenol blue;(Laemmli, 1970) was added and the cells scraped off of the plate. The second dish was treated with 1.0% SDS only and scraped with a rubber policeman. After the cells were scraped off, each cell-containing solution was passed through a 28 and 21 gauge needle 8 times to break

up cell clumps and shear cell membranes. Due to interference of the sample buffer with protein determination, the second plate was used to quantify protein based on the Lowry method (Lowry et al. 1951).

Samples containing 50 μg protein were resolved on 12% SDS polyacrylamide gels (Jacobson, 1991) and immunoblotted as described in chapter 2.2.4. The connexin43 antibody was used at a dilution of 1:1000. A polyclonal GFAP antibody (Chemicon) was used for Western blotting at a dilution of 1:300. Molecular weight markers from Amersham Inc. were used to establish the size of resultant bands.

5.2.5   Scrape Loading

In order to investigate the effects of RA on gap junctional coupling, a n·odified scrape loading technique first described by El-Fouly et al. (El-Fouly et al. 1987) was used. The cultures examined by this method were prepared slightly differently than mentioned above. Subconfluent monolayers of Pi² cells grown in 60 mm dishes (Corning) were treated with 0.3 μM RA or left untreated for 1, 2 or 4 days. RA containing medium was changed after 2 days for the longer experiment. After the desired time, the cells were washed briefly with dPBS and scrape loaded following the procedure outline in chapter 4.2.3. Dye spread analysis by scrape loading was repeated three times. Immunocytochemistry for

cx43 was done on P19 cells cultured in this manner to correlate the level of dye

coupling to connexin expression.

5.3 Results

5.3.1 Connexin Expression in P19 Cells

P19 cells are rapidly proliferating cells that grow in culture as monolayers (Fig. 5.1A). Immunocytochemistry for cx43 on 36 hr cultures reveals abundant reactivity in these cultures (Fig. 5.1B). Cx26, however, is found at much lower levels as seen in figure 5.1C. The pattern of immunoreactivity is characteristic of connexin expression. Most P19 cells are surrounded by punctate immunofluorescence for connexin43 at sites of membrane appositition. Immunocytochemistry for cx32 did not result in any signal suggesting that the protein is not present in these cells or is present at levels below the detection limits of this technique.

5.3.2 Effects of RA of Connexin Expression

Retinoic acid has been shown to modulate connexin expression in many cell types (Wolf, 1992; Pitts et al. 1986; Brummer et al. 1991; Rivedal and Sanner, 1992). The effect of RA on connexin mRNA and protein level was examined, as well as its abilitv to alter gap junctional communication. Northern blot analysis for cx43 revealed a high level of message in untreated P19 cells after being aggregated for

FIGURE 5.1 Connexin expression in untreated P19 cells

P19 cells grown as a monolayer for 36 hr in the absence of RA. Phase contrast
microscopy shows the closely packed arrangement of cultured P19 cells (A).
Cx43 immunoreactivity is abundant in normal P19 cells (B) whereas cx26 is found
at low levels in similar cultures (C; arrows). Magnification x360.
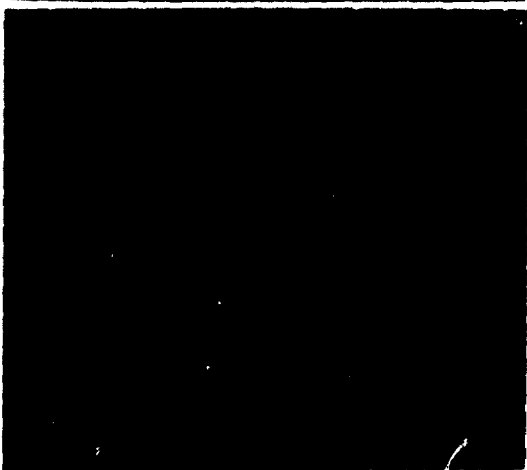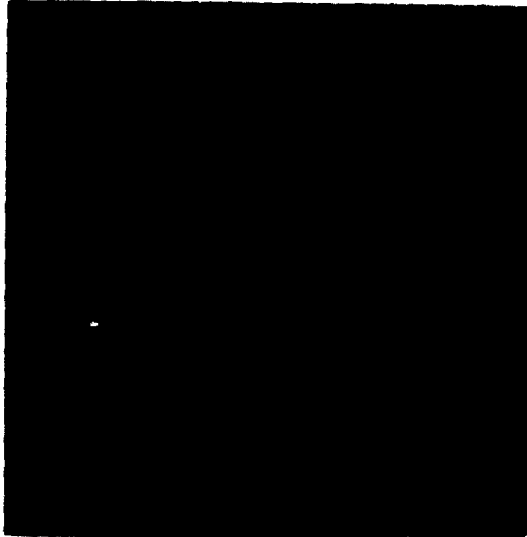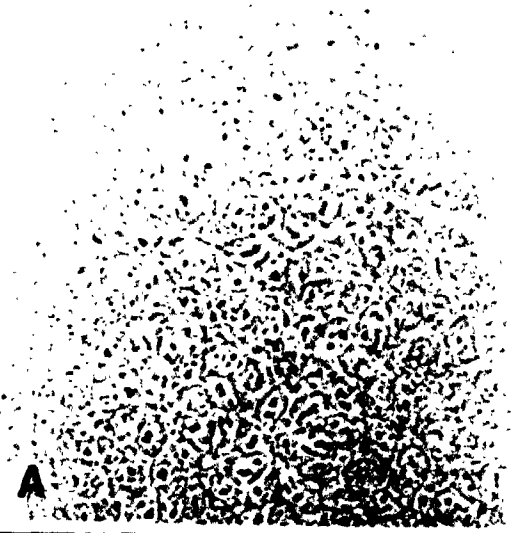
4 days and cultured for an additional 4 or 7 days (Fig. 5.2A). When aggregated in the presence of RA, the level of mRNA for cx43 did not change noticeably (Fig. 5.2A). To ensure equivalent loading in each lane, the same membrane was hybridized with a cDNA to 18S ribosomal RNA (Fig. 5.2B). A second membrane was hybridized with a probe to cx26 and the results show a very low expression of this message (Fig. 5.2C). It appears that although the level of cx26 mRNA is low, it is unaffected by RA treatment.

Western blot analysis for cx43 resulted in the presence of a triplet of bands ranging from 42-45 kD (Fig. 5.3A) characteristic of the phosphoryated states of this protein (Musil and Goodenough, 1990; Musil and Goodenough, 1991). Throughout the length of the experiment, cx43 protein levels were reduced in RA treated cells when compared to untreated controls (Fig. 5.3A). All lanes contained the same amount of protein as indicated in comassie blue stained replica gels (Fig. 5.3B). There was an increase in cx43 protein from the beginning of the experiment up to the end in RA treated cultures (compare Fig. 5.3A, 2 days post-RA to 12 days post-RA). There was also an increase in cx43 protein in untreated cells from 2 to 4 days post-aggregation after which time the level of protein appears to plateau (Fig. 5.3A). Cx26 protein expression was also examined by Western analysis but the protein could not be detected. The antibody available works best for immunocytochemistry. It is possible that the antigen is not preserved in preparation of the protein for Western blotting. In a different series of experiments, cx43 protein was examined immunocytochemically

FIGURE 5.2 Effect of RA on connexin mRNA in P19 cells

Northern blot analysis of P19 cells aggregated in the presence or absence of RA
for 4 days as described in materials and methods then cultured for 4 or 7 days.
Panel A shows the results of hybridization with a probe to cx43. The level of cx43
mRNA (3.0 kb) is unaffected by the RA treatment (+) when compared to
untreated controls (-). To confirm that equal amounts of RNA were loaded in
each lane, the membrane was hybridized with a probe to 18S ribosomal RNA (B).
Cx26 mRNA was detected on a second Northern blot. The probe hybridized to a
2.6 kb band representing cx26 mRNA (C). The signal obtained for cx26 mRNA is
very weak but detectable and does not appear to be affected by RA treatment.
The open arrow indicates position of the 28S ribosomal RNA, the solid arrowhead
indicates 18S ribosomal RNA.

RA

−		+	
4	7	4	7

CX43

A

18 S

B

CX26
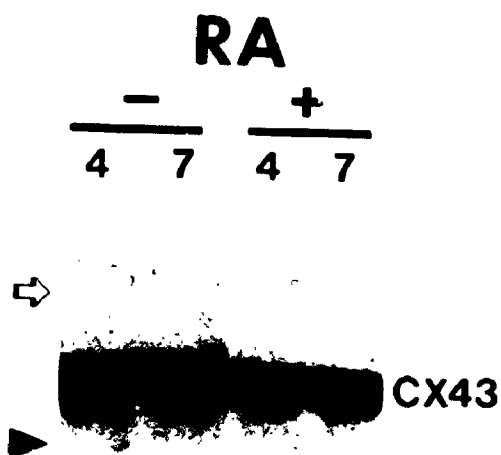
C

FIGURE 5.3 Effect of RA on connexin protein in P19 cells, assayed by Western blotting

Western blotting for cx43 protein following RA treatment. The experiment spanned 2, 4, 8, and 12 days after RA induction of P19 cells. Panel A shows that cx43 protein decreases within 2 days post-RA (+) and remains reduced until day 12 where its level approaches that of untreated cells. Untreated P19 (-) cells express cx43 protein at day 2, the level of which increases to day 4 then reaches a plateau. The antibody detects a triplet of bands ranging from 43-45 kD representing the various phosphorylation states of cx43. The molecular weight markers are ovalbumin (46 kD) and carbonic anhydrase (30 kD). Panel B is a replica gel stained with Coomassie blue to ensure equivalent loading of protein in each lane.
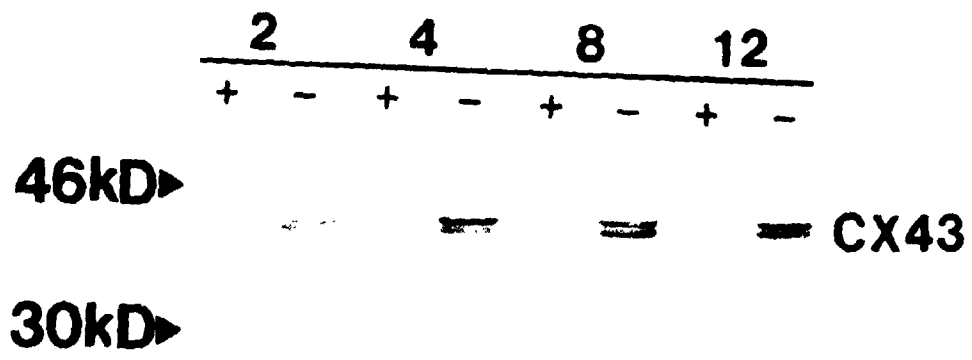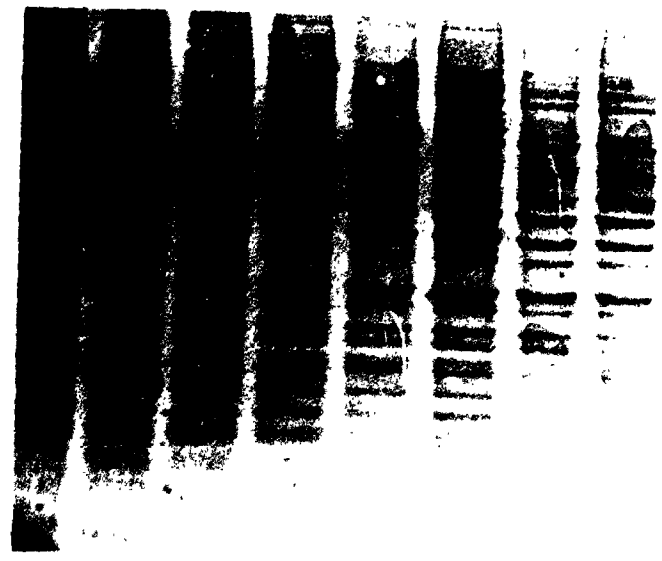
after monolayers of P19 cells were treated with RA. After 24 hours of RA treatment the level of cx43 was notably reduced (Fig. 5.4A) when compared to untreated cells (Fig. 5.4B). The morphology of the P19 cells were not affected by 24 hours treatment with RA (Fig. 5.4C and D). The level of cx43 protein detected by immunocytochemistry remained low after 2 and 4 days (data not shown). Scrape loading was used to examine the extent of gap junctional communication between P19 cells after RA treatment. Phase contrast microscopy shows that monolayers of untreated and treated cells did not differ significantly morphologically (Fig. 5.5A and B). Figure 5.5 (A and B) shows the scrape line through the cells. Scrape loading of untreated cultures resulted in the transfer of carboxyfluoroscein to 3-4 orders of cells from the scrape line where cells were injured (Fig. 5.5C). Similar experiments with RA treated cells resulted in the transfer of dye to 1-2 orders of cells (Fig. 5.5D). In both cases, the passage of dextran rhodamine was restricted to the damaged cells only (Fig. 5.5E and F) as this dye is too large to pass through gap junctions. The inability of cells to transfer dextran rhodamine confirms that carboxyfluoroscein passage is through gap junctions as this dextran rhodamine can pass through cytoplasmic bridges. The same experiment was performed with shorter exposure to RA (5 hours). This length of RA treatment did not result in any observable alteration in connexin expression when examined by scrape loading or immunocytochemistry (data not shown).

FIGURE 5.4 Effect of RA on connexin protein in P19 cells, assayed by immunocytochemistry

Immunocytochemistry for cx43 following a 24 hr treatment with RA reveals a reduction of cx43 protein (A) when compared to untreated cells (B). A phase contrast photomicrograph demonstrates that the cells were not altered by the RA treatment (C and D). Magnification x360.
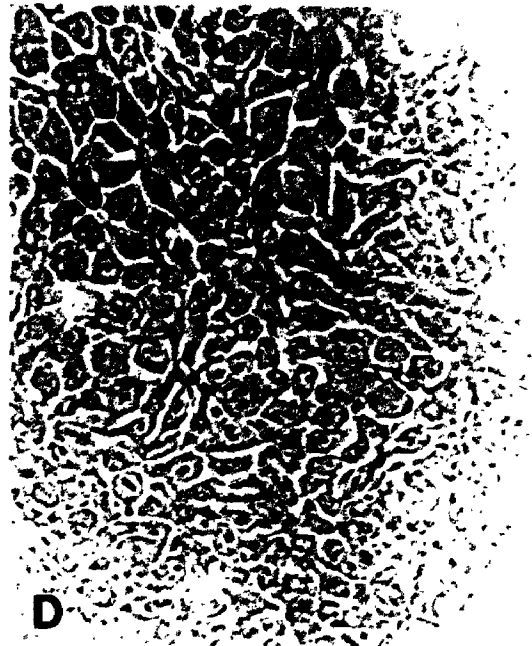
FIGURE 5.5 Effect of RA on functional gap junction channels, assayed by scrape loading

Scrape loading experiment to determine the effect of RA on gap junctional communication. Untreated cells (A, C, E) and those treated with RA (B, D, F) for 24 hr were scraped with a scalpel blade (solid black line running through cultures in A and B). Carboxyfluoroscein (C and D) is small enough to pass through functional gap junctions and demonstrates that untreated cells are coupled to 3-4 orders of cells (C) whereas 24 hr of RA reduces passage of carboxyfluoroscein to 1-2 orders of cells (D). Dextran rhodamine (E and F) is too large to pass through gap junctions but small enough to pass through cytoplasmic bridges. Most of the dextran rhodamine labeling is found in cells damaged by the scrape (E and F), therefore, carboxyfluoroscein must be passing through gap junctions not cytoplasmic bridges. Magnification x144.

A
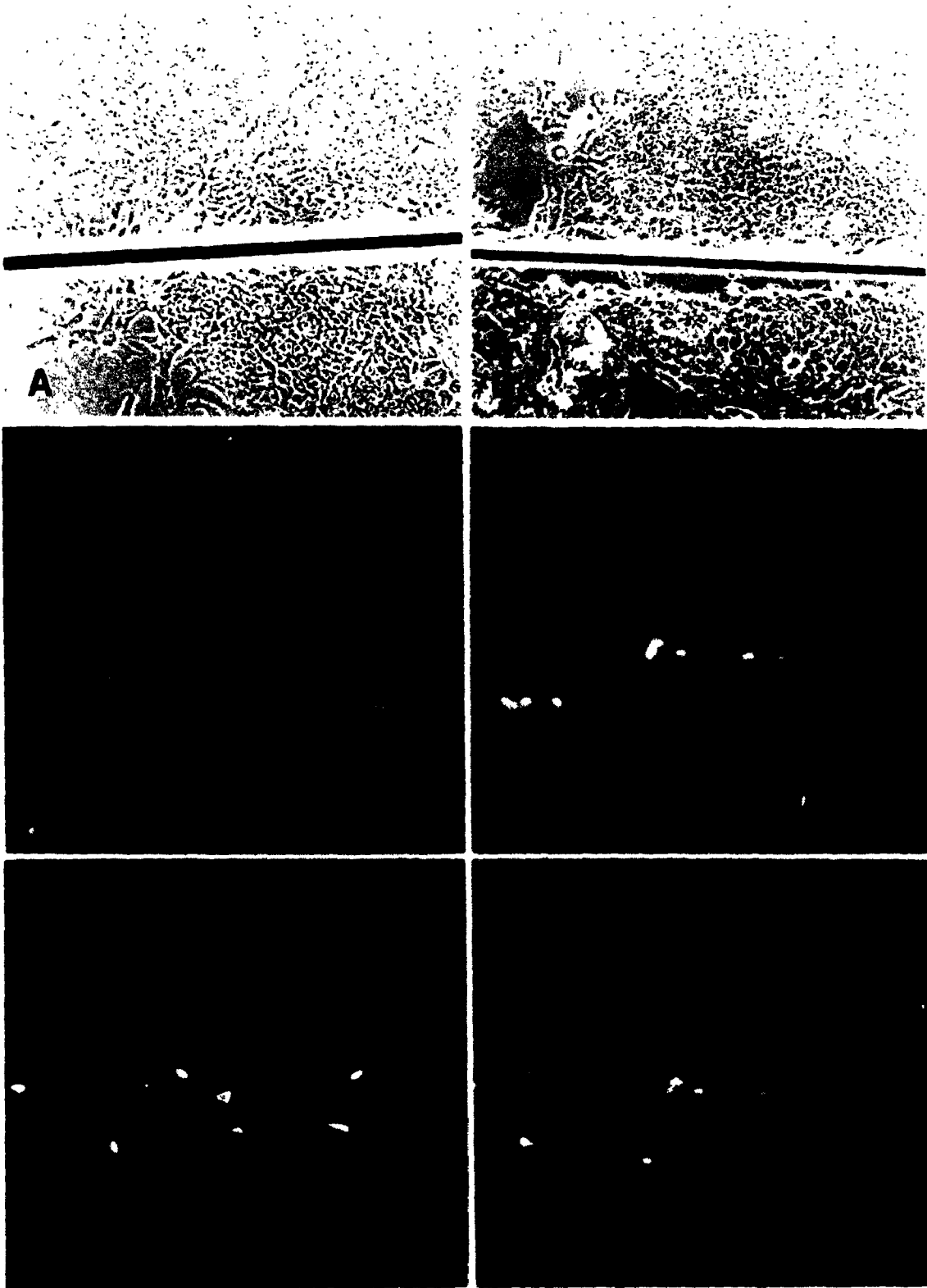
5.3.3 Connexin26 is Expressed in Neurons Derived from P19 Cells

P19 cells differentiate into neural cells following treatment with RA (Jones-Villeneuve et al. 1982; Jones-Villeneuve et al. 1983; Rudnicki and McBurney, 1987). The neuron-like cells, based on morphological criteria, were observed as early as 36 hours post-RA treatment. Figure 5.6A depicts the morphology of P19 derived neurons after 48 hours in culture. The neurons have small, round cell bodies and extend 2-3 neurites, some of which show a beaded appearance (Fig. 5.6A). The neurons can be identified immunocytochemically with the cell surface marker, A2B5 (McBurney et al. 1988; Eisenbarth et al. 1979) as shown in figure 5.6B. Immunocytochemistry for cx26 revealed the presence of this protein at sites of intercellular contact of neurons at 3 days post-RA (Fig. 5.7A). The immunoreactivity is indicated by arrows and compared to the phase contrast photomicrograph of the neurons (Fig. 5.7B). Cx26 immunoreactivity is present at 5 days post-RA (Fig. 5.7C), when more neurons are present (Fig. 5.7D). Without treatment with cytosine arabinoside, the neurons could not be maintained in culture for more that 8 days post-RA. Cytosine arabinoside increased the number of neurons and their survival time. With this treatment, neurons derived from RA treated P19 cells survived for approximately 12 days and still contained cx26 (data not shown). Similar cultures of neurons were examined for their expression of other connexins, namely, cx43 and cx32. No immunoreactivity could be detected for cx43 (Fig. 5.8A) although neurons were

FIGURE 5.6 Surface marker for neurons derived from RA treated P19 cells

Phase contrast photomicrograph of P19 derived neurons 48 hr following RA treatment. Typical feature of P19 derived neurons can be seen; small, round cell bodies and 2-3 neurites per cell (A). Immunocytochemistry for A2B5, a cell surface marker, labels the cell bodies and neurites of each neuron (B). Magnification x 360.

A
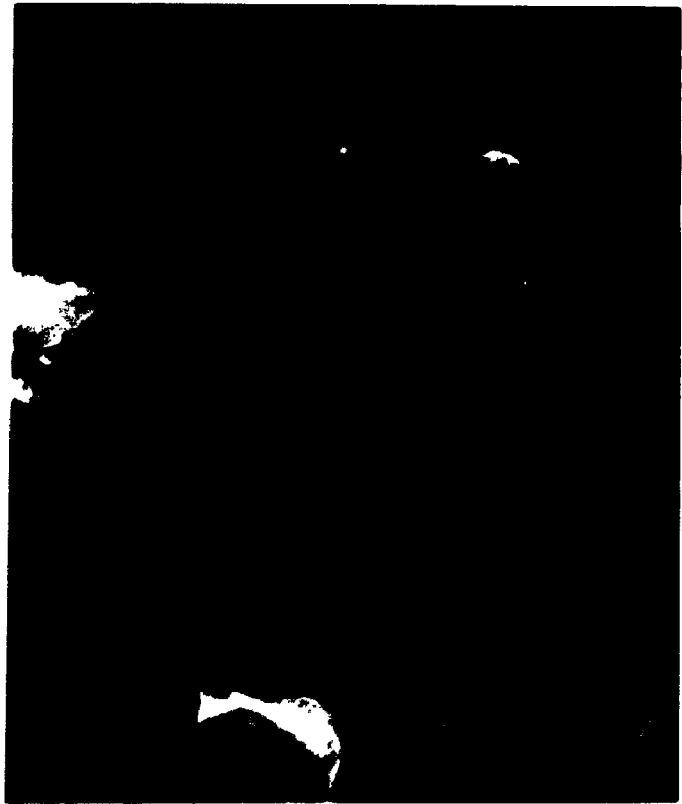
FIGURE 5.7 Connexin26 in neurons derived from RA treated P19 cells

Neurons derived from RA treated P19 cells. After 3 days in culture the neurons expressed cx26 protein as detected by immunocytochemistry (A). The neurons can be morphologically identified because of their fine processes and small round cell bodies (B). In panel C, the neurons were cultured for 5 days and still expressed cx26 immunoreactivity. Phase contrast indicates that more neurons are present at day 5 (D) when compared to day 3 (B). The arrowheads in A-B and C-D indicate sites of cx26 protein expression; areas of cell-cell and cell-neurite contact. Magnification x360.
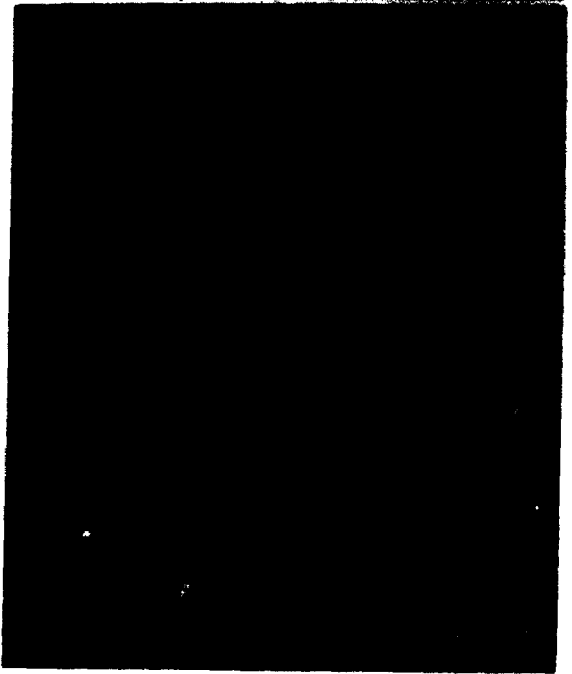
D.

definitely present (Fig. 5.8B). Cx32 was not present in these neurons either (Fig. 5.8C). Phase contrast reveals that the neurons appear identical to those that express cx26 (Fig. 5.8D).

5.3.4 Astrocytes Derived from P19 Cells Express cx43

Astrocytes have been shown to develop from RA treated P19 cells following the development of neurons (Rudnicki and McBurney, 1987). Cultures of P19 cells were examined for the astrocyte-specific intermediate filament, glial fibrillary acidic protein (GFAP). Results from Western blotting showed that RA treated P19 cells express GFAP beginning at 8 days post-RA and continuing to the end of the 12 day experiment (Fig. 5.9). GFAP protein was detected as a single band of approximately 48 kD (Eng, 1980). In culture, P19 cells treated with RA expressed GFAP immunoreactivity after 7 days (Fig. 5.10A). At this time cx43 immunoreactivity could not be detected in GFAP positive cells. At 10 days post-RA, double labelling experiments with cx43 and GFAP showed that GFAP positive cells expressed cx43 at sites of cell-to-cell contact on other GFAP expressing cells. This is demonstrated in figure 5.10B. Although there is coexpression of cx43 and GFAP, not all the cx43 present in RA-treated P19 cells were in astrocytes. The other cells expressing cx43 could be fibroblast-like cells known to develop from RA treated P19 cells (Jones-Villeneuve et al. 1982). Fibronectin was detected in cultures of P19 cells following RA treatment but the

FIGURE 5.8 Lack of connexin43 or 32 in neurons derived from RA treated P19 cells

Immunocytochemistry for cx43 (A) and cx32 (C) did not result in detectable immunoreactivity although neurons were present in the cultures (B and D). Magnification x360.
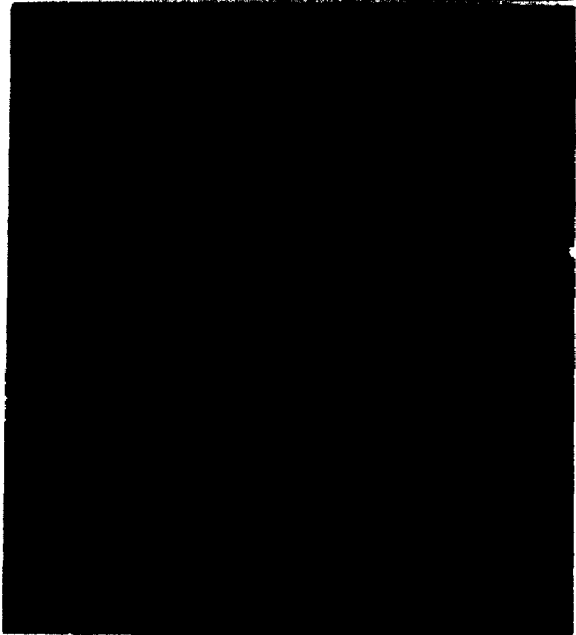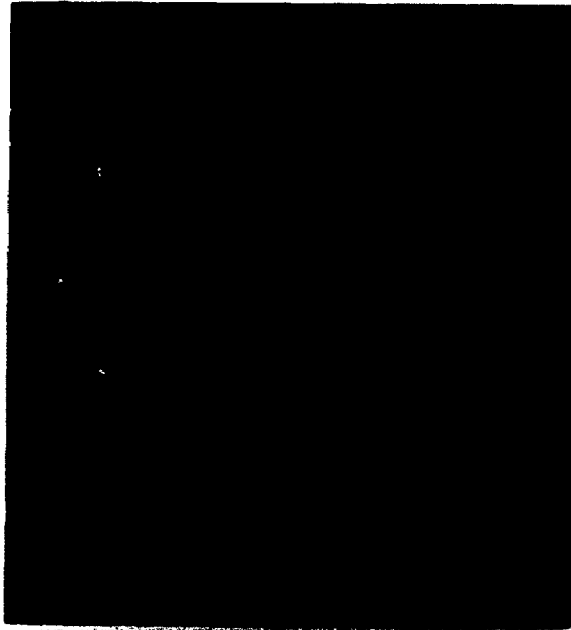
FIGURE 5.9 Western blotting for GFAP in P19 cells

Western blot analysis for GFAP in P19 cells treated with RA. 50 μg of protein
was loaded into each lane from cultures at 2, 4, 8, and 12 days post-RA. A 48 kD
band representing GFAP was detected at 8 and 12 days post-RA. The molecular
weight markers are ovalbumin (46 kD) and carbonic anhydrase (30 kD).
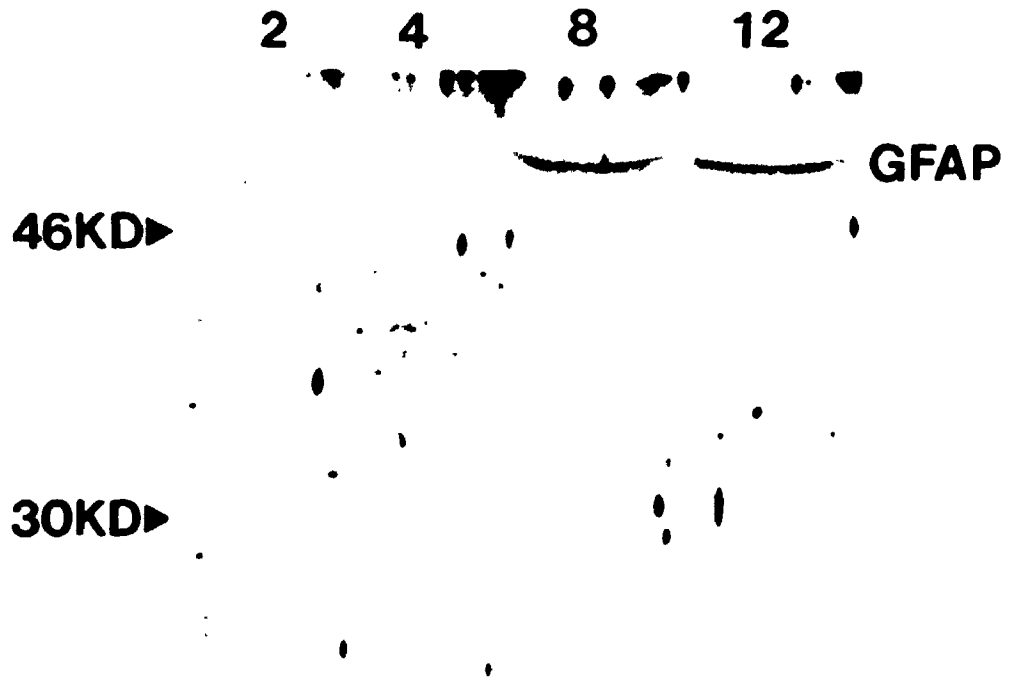
presence of cx43 has not been confirmed in these cells (data not shown). The

astrocytes derived from P19 cells resemble those that develop from primary

cultures of rat brain. The similarities can be seen by comparing figure 5.10B with

5.10C, a photomicrograph of cx43 positive/GFAP positive primary astrocytes. It

appears that the major difference is the size of the resultant astrocytes and their

junctions; P19 derived astrocytes being smaller with smaller gap junction plaques

than primary astrocytes (Fig. 5.10B and C).

FIGURE 5.10   **Double labelling for cx43 and GFAP in astrocytes derived from RA treated P19 cells**

GFAP and cx43 immunocytochemistry on cultures of P19 cells after RA treatment. GFAP immunoreactivity was found in 7 day post-RA cultures of P19 cells (A). The monoclonal GFAP antibody was detected with a rhodamine conjugated goat-anti-mouse secondary antibody. Double-labeling experiments with GFAP and cx43 detected the coexpression of these two proteins in 10 day post-RA cultures (B). GFAP (rhodamine) and cx43 (fluoroscein) were found in the same cells with cx43 at the cell periphery. The localization of cx43 is consistent with the presence of gap junctions at sites of cell-cell contact. Panel C shows the results of double-labeling experiments on primary cultures of rat astrocytes. Cx43 (fluoroscein) is present as punctate regions around the cell membrane of GFAP positive cells. Magnification x144 (A), x565 (B, C).
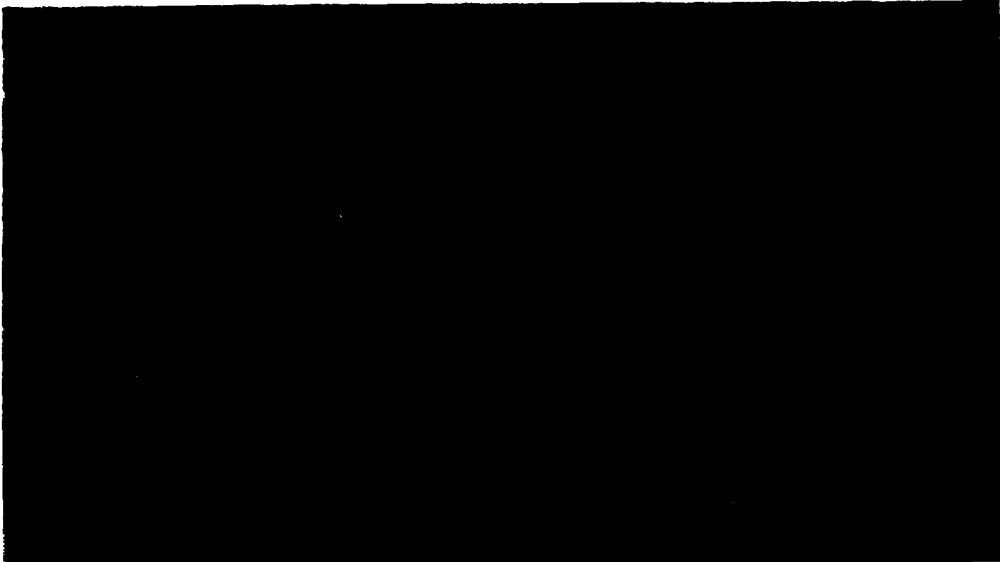
5.4 Discussion

The presence of intercellular junctions, in particular gap junctions, in EC cells has been documented using morphological (Lehtonen et al. 1984; Smith et al. 1987) and molecular criteria (Willecke et al. 1991; Hennemann et al. 1992c; Willecke et al. 1991). The F9 EC cell line has been shown by Willecke and coworkers to express numerous gap junction genes (Willecke et al. 1991; Hennemann et al. 1992c; Willecke et al. 1991; Willecke et al. 1990). They have also used a cDNA library from F9 cells to clone two novel mouse connexins, cx31 and cx45 (Hennemann et al. 1992c). This study shows that the P19 cell line also contains multiple connexins. Northern blotting and protein analysis have revealed the presence of cx43 and cx26 in these cells.

5.4.1 Effects of retinoic acid on gap junctions

Nishi et al. (1991) examined connexin expression in F9 cells to complement their study of developmental regulation of gap junction genes during embryogenesis. Treatment with RA promotes the formation of visceral endoderm cells in cultures of F9 EC cells and dbcAMP promotes parietal endoderm to develop (Nishi et al. 1991). Differentiation of these cells caused an increase in cx26 mRNA (β_2 connexin) and induction of cx32 mRNA (β_1 connexin) in visceral endoderm. Cx43 mRNA (α_1 connexin) was enhanced in both differentiated cell

types (Nishi et al. 1991). Similar observations were made by Willecke et al. (1990).

Modulation of connexin expression was also observed in P19 cells after treatment with RA. This study examined the whole cell population and showed that cx43 protein was decreased within 24 hr, as was gap junctional intercellular communication as assayed by scrape loading. However, notable changes in mRNA level were not detected. There have been many reports examining the effects of RA on cell communication. Non-toxic concentrations of RA have been shown to decrease intercellular communication rapidly and reversibly in various cell types (Rivedal and Sanner, 1992; Brummer et al. 1991; Pitts et al. 1986). RA induces cell-cell communication in fibroblasts lines exposed to carcinogens (Wolf, 1992). Most interestingly, RA can differentially modulate gap junctional communication in the same type of cell depending on the concentration of RA in the medium (Mehta et al. 1989). The effects of RA on heterologous gap junctions, that is between two different cells types, is to decrease coupling whereas as RA increases communication in cultures of similar cells (homologous gap junctions; Mehta and Loewenstein, 1991). Such an effect, if it occurs in vivo, may represent control of communication between cell types thereby establishing communication compartments. The decrease in intercellular communication seen in P19 cells treated with RA may represent such a differentiative signal.

Retinoic acid has been shown to be a morphogen, potentially important in the development of the chick limb bud (Thaller and Eichele, 1987; Melton, 1991).

The actions of RA are mediated by cellular RA binding proteins and nuclear RA receptors (Lohnes et al. 1992). These molecules have been identified in EC cells, including P19 (Wu et al. 1992a; Wei et al. 1989) and are also differentially expressed during mouse embryogenesis suggesting a role for RA during development (Wu et al. 1992b).

5.4.2 Differentiation of P19 cells

Differentiation of P19 cells by RA resulted in the development of neurons, astrocytes and fibroblast-like cells similar to the results reported by previous investigators (Jones-Villeneuve et al. 1982; McBurney et al. 1982; Jones-Villeneuve et al. 1983). Numerous EC cell lines exist that develop into a neuronal phenotype (Cheun and Yeh, 1991; Darmon et al. 1981; Pfeiffer et al. 1981). The neurons that develop maintain many characteristics representative of normal neurons found in the developing animal including expression of neurofilaments (Jones-Villeneuve et al. 1982), neuron specific enolase (Cheun and Yeh, 1991), A2B5 (Morrison, 1991), microtubule associated proteins (Tanaka et al. 1992), and a novel neuron specific nuclear protein (Mullen et al. 1992). The neurons also bind tetanus toxin (Jones-Villeneuve et al. 1983) and contain tetrodotoxin sensitive sodium currents (Cheun and Yeh, 1991). It is thought, however, that the neurons represent embryonic cells as they fail to express markers for mature neurons such

as Thy-1 (Jones-Villeneuve et al. 1983). This investigation determined that the P19 derived neurons express the gap junction protein, cx26. The possibility that cx26 is present in developing (ie. immature) neurons has been suggested (Lo Turco and Kriegstein, 1991; Peinado et al. 1993). This hypothesis is based on observations of clusters of coupled neurons during embryonic and early postnatal development of the neocortex and the fact that cx32 is not found in the CNS at that time (Dermietzel et al. 1989), whereas cx26 is (Dermietzel et al. 1989). The detection of cx26 in these P19 neurons, which are thought to represent normal embryonic neurons would support this hypothesis. Cheun and Yeh (1991) detected the spread of Lucifer Yellow in untreated P19 cells but could not detect dye transfer in injected, differentiated neurons. It is possible that the technique of dye injection was not sensitive enough to detect functional channels. Recent experiments using the tracer, Neurobiotin, have determined that it is much more sensitive and passes through gap junctions more readily (Peinado et al. 1993). Hence, Lucifer Yellow might not be a sensitive enough method of detecting very low levels of coupling. Electrical coupling without the presence of dye coupling has been reported for other neural cells (Ransom and Kettenmann, 1990). It would be very interesting to examine P19 derived neurons after long culture periods to determine if cx32 expression is induced in these cells. The presence of cx32 in select populations of mature neurons has been well documented (Micevych and Abelson, 1991; Yamamoto et al. 1989b; Shiosaka et al. 1989).

The astrocytes which develop from RA treated P19 cells express the astrocyte-specific marker, GFAP (Jones-Villeneuve et al. 1983; Jones-Villeneuve et al. 1982). Mammalian astroglia have been shown to contain cx43 (Dermietzel et al. 1991; Naus et al. 1991a; Giaume et al. 1991). In this study, P19 derived astrocytes expressed the same connexin (cx43) as seen in primary cultures. The physically smaller size of the astrocytes may be related to their immaturity as Jones-Villeneuve et al. (Jones-Villeneuve et al. 1983) have demonstrated that these glia do not express S-100 protein, a marker of mature astrocytes. They also showed that RA treated cells reduce their cell volume by as much as 75% within six days (Jones-Villeneuve et al. 1983), representing another potential reason for decreased size of these cells.

The ability of P19 cells to develop along a second lineage, that of muscle, after treatment with dimethylsulfoxide represents the pluripotential character of these cells (Edwards et al. 1983; Rudnicki et al. 1990; McBurney et al. 1982). Although connexin expression in differentiating skeletal and cardiac muscle was not examined, experiments based on normal tissue would suggest that the developing muscle expresses cx43 (Balogh et al. 1993; Laird et al. 1992). Differentiation of P19 cells appears to signal a divergence of connexin expression in the developing cells. The restricted gap junctional communication caused by RA may represent formation of communication compartments. The expression of specific connexins in neural cells, cx26 in neurons and cx43 in astrocytes, would further enhance any developmental regulation. This study did not attempt to

colocalize cx26 and cx43 to the same P19 cell. Two possibilities present themselves. First, distinct cells express each connexin potentially representing different precursors for each cell type that develops. Second, the same cell can express both connexins and signals to differentiate along certain neural lineages modulate expression of connexin genes. Indeed, cells expressing multiple connexins have been observed (Nicholson et al. 1987; Kuraoka et al. 1993; Spray et al. 1991).

P19 cells offer a model of limited embryonic development that can be readily manipulated by the introduction of exogenous genes. Such experiments have provided evidence for the importance of microtubule associated proteins (Dinsmose and Solomon, 1991), epidermal growth factor receptor (den Hertog et al. 1991) and pp60^{src} (Schmidt et al. 1992) in neuronal differentiation and of cardiac actin in muscle development (Rudnicki et al. 1988). This report has characterized the gap junction expression during P19 cell differentiation into neurons and astrocytes and has shown specific connexin expression in each cell type. P19 cells can be used to further analyze the role of connexin gene expression in differentiation events of developing neural cells.

CHAPTER 6

Conclusions

6.1 Summary of findings

1. Northern and Western blot analysis revealed that cx32 and cx43 are differentially expressed during development of the rat and mouse central nervous system. Cx43 mRNA was detected as early as E18, increasing in abundance to P30 where it remained constant to adulthood. This pattern was seen in both forebrain and hindbrain. Cx32 mRNA, however, was first detected at P5 in hindbrain samples and only at P10 in forebrain samples. The level of cx43 protein increased from P5 to adult and was seen as doublet ranging from 41-43 kD. This likely represents various states of phosphorylation of the cx43 protein. The cx32 protein was also detected as early as P5 and remained through to the adult.

2. The regional distribution of connexin mRNAs, as detected by Northern blot analysis, revealed that cx43 mRNA is homogenously expressed in all regions of the brain examined except olfactory bulb where it was slightly elevated. Cx32 mRNA showed a much more heterogenous distribution being highly

expressed in brainstem, midbrain, pons and medulla and less so in cerebellum and forebrain areas.

3.   The developmental expression of connexin mRNAs was resolved to the cellular level by the use of *in situ* hybridization.   Cx32 mRNA was first observed in the brainstem of P3 animals where the signal was localized to populations of neurons and to the spinal trigeminal tract.   The number of labelled cells increased at P10 where the cerebellar white matter and various fibre tracts of the brain stem were cx32 positive.   The P15 midbrain showed abundant cx32 hybridization in the decussation of the cerebellar peduncles but the central grey matter and lining of the aqueduct were negative.   Cortical layers and hippocampus of the P30 forebrain were positively labelled for cx32 as was the cerebellum.   The presence of cx32 mRNA in fibre tracts and at sites of neuronal populations suggests that this particular connexin is present in oligodendrocytes and neurons.

4.   Cx43 mRNA was present as early as E20 in the leptomeninges of the forebrain and was found at P3 in the developing cortex.   In the hindbrain the signal for cx43 was diffusely located throughout the tissue.   By P10 the cx43 signal was abundant in the brainstem but homogenous in its distribution.   The P15 midbrain when hybridized with cx43 showed a very different pattern from that seen with cx32.   The cx43 mRNA was

distributed evenly in the whole section including the central grey area and the ependymal lining of the aqueduct. Cx43 mRNA is present in cortical layers of the P30 forebrain. The evenly distributed signal for cx43 suggests its presence in astrocytes. This was confirmed by double labelling studies using GFAP immunocytochemistry and cx43 *in situ* hybridization of the same section.

5.   Neural cells were isolated from mature rat forebrain to determine the expression of connexin mRNA in the various cell populations of the CNS. The isolation procedure purified oligodendrocytes, astrocytes and neurons separately although the extent of purification was not 100%. Isolated oligodendrocytes expressed cx43 and cx32 mRNA. Considering that some of the contaminating cells were endothelial and ependymal cells, which are known to express cx43, it is suggested that the cx32 mRNA is from oligodendrocytes and cx43 from contaminants. Astrocytes isolated in a similar manner expressed cx43 mRNA and neurons were shown to express cx32 mRNA.

6.   Cultured astrocytes expressed cx43 protein and mRNA as determined by immunocytochemistry, *in situ* hybridization and Northern blotting. A second type of astrocytes develop from a progenitor cell (O-2A), called type 2 astrocytes, did not express cx26, 32 or 43 when examined by

immunocytochemistry. Dye transfer experiments confirmed that type 1 astrocytes were highly coupled but could not detect any dye coupling in confluent cultures of type 2 astrocytes.

7. Cultured neurons from hippocampus or neocortex develop cx26 immunoreactivity soon after initiation of cultures. The cx26 signal is found on cell processes and cell bodies of neuron like cells positive for Thy-1. The extent of cx26 immunoreactivity decreases over time and neurons cultured for 21 days express the cx32 gap junction protein.

8. A model of neural development is provided in the P19 embryonal carcinoma cell line. These cells when treated with retinoic acid differentiate into neurons and astrocytes. Undifferentiated P19 cells express two connexin proteins, cx26 and cx43.

9. The treatment with retinoic acid does not alter the expression of connexin mRNAs but does significantly reduce the level of cx43 protein as assayed by Western blotting and immunocytochemistry. Functional gap junctional communication is reduced within 24 hours of treatment with retinoic acid as determined by scrape loading.

10. The first neural cell to develop from retinoic acid treated P19 cells are neurons which express the cx26 protein at sites of cell body and cell process contact. The neurons do not express cx32 or cx43 protein. Astroctyes develop second and express the astrocyte marker, glial fibrillary acidic protein. This marker is first seen after 7-8 days in culture and increases in abundance with time in culture. These same cells also express the gap junction protein cx43.

6.2 Synthesis and speculations

The expression of connexin genes in specific cell types and during particular stages of CNS development leads to the conclusion that gap junctions are developmentally regulated in each system of the CNS. The development of the CNS involves many complex cellular interactions. The ability of cells to couple to each other is another important process. The regulation of cx43 in various types of astrocytes, of cx26 and 32 in populations of neurons and of cx32 in oligodendrocytes demonstrates the many systems in which connexins are modulated. The important question arising from these observations is how essential is the regulation of gap junctions for the proper development of CNS components. These results provide the foundation to study the regulation of connexin expression during CNS development. Some potential future studies are described below:

1. Connexin expression in type 2 astrocytes.

Although the type 2 astroctyes were observed not to express the main connexins found in the CNS, morphological studies suggest that there are gap junctions between these cells and their neighbors. Some of the recently cloned gap junction genes have been localized to developing and mature CNS such as cx37, cx40 and cx45. It would be interesting to see if any of these connexins are present in type 2 astrocytes. Do type 2 astrocytes form heterologous gap junctions as has been recently speculated for oligodendrocytes (cx32) and type 1 astrocytes (cx43)?

2. Embryonic versus mature neuronal connexin.

The studies on hippocampal neurons in culture provides evidence for the possibility of an embryonic neuronal connexin (cx26) and a mature neuronal connexin (cx32). Studies using dye coupling show that migrating neurons are coupled in relatively large clusters, these coupled clusters decreasing in cell number with development. The decrease in dye transfer is coincident with the decreased expression of cx26. The expression of cx32, however, parallels neuronal maturation. Using cell cultures and double labelling immunocytochemistry it would be possible to determine if the same neuronal population is losing cx26 protein as it expresses cx32. The other possibility that two populations of neurons

exist, one expressing cx26 during its development and the other not coupled until it matures (by expressing cx32), is equally fascinating.

With the use of transgenic mice expressing neuron specific markers, the issue of cx26 expression in developing neurons could be answered definitively *in vivo*. By beta-galactosidase staining of neurons and double labelling by immunocytochemistry or *in situ* hybridization, the expression of connexins in migrating clusters of neurons can be determined.

3. Altering connexin expression to effect developmental potential

The P19 cell line expresses two connexin proteins which are differentially expressed in the resultant cells following RA treatment. It is possible that altering the expression of one connexin could influence to developmental potential of these cells. Stable transfection of connexin genes in a sense or anti-sense orientation into undifferentiated P19 cells would address the issue of connexin specificity in developing neural cells and the importance of these gap junction proteins during the differentiation process.

4. Altering connexin expression *in vivo*

It is essential to examine the effect of altered gap junction gene expression in vivo. This can be accomplished by expressing promoter driven anti-sense constructs of connexin genes in animals to investigate specific cell systems. The production of transgenic mice lacking a particular connexin (such as knocking out cx32 in developing brain) would address the precise role of that connexin and the importance of its regulation during development.

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