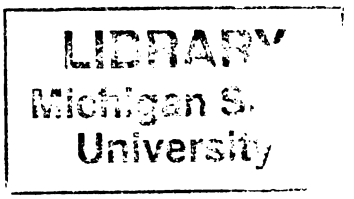

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THE RELATIONSHIP BETWEEN GAP JUNCTIONAL INTERCELLULAR COMMUNICATION AND DIFFERENTIATION IN A HUMAN FETAL CELL LINE ISOLATED FROM THE CENTRAL NERVOUS SYSTEM

presented by

Coleen V. Dowling-Warriner

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Major professor

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THE RELATIONSHIP BETWEEN GAP JUNCTIONAL INTERCELLULAR COMMUNICATION AND DIFFERENTIATION IN A HUMAN FETAL CELL LINE ISOLATED FROM THE CENTRAL NERVOUS SYSTEM

By

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ABSTRACT

THE RELATIONSHIP BETWEEN GAP JUNCTIONAL INTERCELLULAR COMMUNICATION AND DIFFERENTIATION IN A HUMAN FETAL CELL LINE ISOLATED FROM THE CENTRAL NERVOUS SYSTEM

By

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The hypothesis under investigation is that functional gap junctional intercellular communication is a causal element in controlling cellular differentiation. The human neural-glial cell line, SVG, was used as the experimental model to study expression of gap junction proteins, cell-cell communication, and the ability of the cells to differentiate under specified conditions. The SVG cells were found, using western analysis and immunofluorescent techniques, to express the three gap junction proteins commonly found in the central nervous system: connexin 43, connexin 32, and connexin 26. However, fluorescence recovery after photobleaching analysis of 5, 6-carboxyfluorescein loaded cells failed to show significant dye coupling.

Agents that stimulate the adenylyl cyclase/cAMP pathway were then used to induce gap junctional intercellular communication in the SVG cultures. A 24-48 hr treatment of SVG cells with 5μM forskolin + 200 μM 3-isobutyl-1- methylxanthine (IBMX) in serum free medium increased the percentage of dye-coupled cells from 5 to 65%, using the fluorescent recovery after photobleaching method. The increase in dye coupling induced by forskolin + IBMX was inhibited by octanol, which is a known blocker of gap junction mediated cell communication. Western blot analysis of total protein extracts revealed the

appearance of a higher molecular weight connexin43 protein band after treatment of SVG cells with forskolin + IBMX, that was not observed in vehicle treated controls. Alkaline phosphatase digestion demonstrated that the higher molecular weight band was due to a phosphorylation event stimulated by the forskolin + IBMX combination. In addition, treatment of the SVG cells with the forskolin + IBMX increased expression of connexin32 and connexin26 on western blots.

Differentiation was observed in the SVG cells after treatment with forskolin + IBMX in serum free medium as defined by changes in morphology, antigenic expression of specific neuronal proteins, and growth rate. The elevation in cAMP induced outgrowth of neurite-like processes from the cell body which immunostained positive for the connexin protein as well as antigenic protein markers for neurons and oligodendrocytes. Further investigation revealed that the differentiation was not reversible and that there were significant decreases in DNA synthesis and the number of cells present in the cultures after treatment with forskolin + IBMX.

Although there was a strong correlation between the increase in gap junctional communication and the differentiation, these two events could be disassociated using cell culture techniques. Adding serum to the medium did not affect connexin expression or cell-cell communication, but did inhibit the morphological change. Plating the cells at different densities (sparse vs. confluent) could alter one event without influencing the other suggesting that in the SVG cells gap junctions do not causally affect the initiation of differentiation.

This work is dedicated to my husband, Wade Warriner and my sons Wesley and Logan whose love and support are my driving force

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Lastly, but most importantly, I would like to acknowledge my parents John and Mary Dowling for supporting my efforts and encouraging my dreams.

PREFACE

Purpose/Objectives

Gap junctions appear to play an important regulatory role in embryonic development and emerging evidence suggests that gap junctional coupling between closely opposed cells may determine whether the cell will differentiate or proliferate. Control of cell proliferation, cell differentiation and adaptive responses of differentiated cells have been speculated to be biological roles of gap junctions (Trosko et al., 1993). The nervous system contains eight of the thirteen connexin (individual protein units that comprise the gap junction channel) subtypes known, and a vast amount of recent evidence suggests that neural gap junctions may be responsible for regulating complex events during neurogenesis such as, differentiation, neural cell migration, and eventual neural function.

The nervous system is the focus of this dissertation because the relationship between cell-cell communication and differentiation or development of neural function are correlative in that no one until recently has tried to causally link differentiation or function to cell-cell communication through gap junctions. The purpose of this dissertation is to test the hypothesis that gap junctional intercellular communication is an important and causal factor in controlling and maintaining differentiation.

The main objectives of this dissertation were to first find a cell model that did not have functional cell-cell communication and was not differentiated as defined by expression of specific cell lineage markers, morphology, and proliferative potential. The second objective was to therefore find a way to induce or upregulate cell-cell communication in the cell model and characterize the new found gap junction channels as to connexin subtype and level of cell-cell communication. Thirdly, if the hypothesis is

correct there should be observable differentiation, as defined by several criteria, following the newly induced cell-cell communication. The third objective was to characterize the differentiation by examining cell growth, expression of cell type specific markers, morphology, and the reversibility of the differentiation. The fourth objective was to then determine whether there was a causal relationship between these two events by blocking cell-cell communication and characterizing changes, if any, in the differentiation.

Organization

This dissertation is organized into chapters; a chapter designating each new topic that is introduced. The chapters are further broken down into parts and these are divided into sections. In total there are eight chapters in this dissertation.

Chapter 1, or the Introduction, provides a review of the gap junction field-of-research. The review starts out generally describing the structure, channel regulation, function, and importance of the gap junction channel. The focus then narrows to discuss specific attributes of the gap junctions and their functions in the developing and mature nervous system. The chapter concludes with a part describing the experimental design, hypothesis, objectives, and specific aims.

The Introduction is followed by the Experimental Procedures in Chapter 2. Each experimental procedure comprises a part in Chapter 2, which is broken down into sections highlighting specific steps in each experimental protocol. Statistical analysis is also placed in this chapter when statistics were applied to a specific procedure to further describe the data.

Chapters 3-7 include the results, which uses a specific aim as the theme of each chapter. The chapters are further broken down into parts, which include the results from each different experimental procedure used to achieve the specific aim in question. The parts are then divided into sections, if one experimental procedure was used for several different experiments. For example, Western Analysis is a part that includes sections *Expression of Connexin 43*, *Expression of Connexin 32*, and *Expression of Connexin 26*. All of the chapters describing results end with a brief conclusion summarizing the data.

The final chapter is Chapter 8 and this includes the Discussion. In the Discussion the significance of the discoveries are examined on their own merit as well as in light of ideas and concepts from the literature, which may or may not support the ideas brought about by this dissertation.

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KEY TO ABBREVIATIONS

AP ..... Alkaline Phosphatase
ATP ..... Adenosine Triphosphate
BDNF ..... Brain Derived Neurotrophic Factor
BSA ..... Bovine Serum Albumin
cAMP ..... Cyclic Adenosine Monophosphate
CBX ..... Carbenoxolone
CFDA ..... Carboxyfluorescien Diacetate
CGMP ..... Cyclic Guanosine Monophosphate
CMTX ..... X-linked Charcot- Marie-Tooth disease
CNS ..... Central Nervous System
CREB ..... Cyclic AMP Response Element Binding Protein
C-terminal ..... Carboxyl-terminal
CTNF ..... Ciliary Neurotrophic Factor
Cx ..... Connexin
DDT ..... 1,1-bis(*p*-chlorophenyl)-2,2,2-trichloroethane
DMSO ..... dimethylsulfoxide
EGF ..... Epidermal Growth Factor
EMEM ..... Eagles Minimum Essential Medium
ERK ..... Extracellular Signal-Regulated Kinases
FBS ..... Fetal Bovine Serum
FGF ..... Fibroblast Growth Factor
Gap-FRAP ..... Fluorescent Recovery after Photobleaching
GC ..... Galactocerebroside
GFAP ..... Glial Fibrillary acidic Protein
GJIC ..... Gap Junctional Intercellular Communication
GnRH ..... Gonadotropin-releasing Hormone
HVC ..... Caudal Nucleus of the Ventral Neostriatium
IBMX 3-Isobutyl-1-methlxanthine
IP3 ..... Inositol 1,4,5 trisphosphate
K-ATP ..... Potassium-Adenosine triphosphate channel
kDa ..... Kilodalton
LHRH ..... Leutenizing Hormone-Releasing Hormone
MAP2 ..... Microtubule-Associated Protein 2
MAPK ..... Mitogen Activated Protein Kinase
MBP ..... Myelin Basic Protein
MW ..... Molecular Weight
NE-200 ..... Neural Filament 200 kDa
NGF ..... Nerve Growth Factor
NSE ..... Neuron Specific Enolase
PBS ..... phosphate buffered saline
PDGF ..... Platlet Derived Growth Factor
PKA ..... cAMP-Dependent Protein Kinase or Protein kinase A
PKC ..... Protein Kinase C

PMSF ..... Phenylmethylsulfonyl fluoride
RA ..... Retinoic Acid
RT-PCR ..... Reverse Transcriptase-Polymerase Chain Reaction
SD ..... Standard Deviation
SDS ..... Sodium Dodecyl Sulfate
SL/DT ..... Scrape Load/Dye Transfer
SNB ..... Spinal Nucleus of the Bulbocavernosus
SVG ..... Simian Virus 40 Glial
TPA 12-O-Tetradecanoylphorbol-13-Acetate
VAH ..... Visceroatrial Heteroataxia Syndorme

CHAPTER 1 - INTRODUCTION

1.1 - Background

All cellular activities must be coordinated to maintain homeostasis within individual organs and tissues comprising the multicellular organism. Multicellular organisms have evolved multiple strategies to achieve coordination of these cellular activities without compromising the individual and unique functions of the cells, which are necessary for survival. There are two broad categories that serve to mediate cellular interactions, which include long-range signaling utilized by neural or endocrine mechanisms and short-range signaling that includes physical or direct cell-cell contact. The first strategy involves chemical mediated interactions over a relatively long distance where, for example, a neurotransmitter or hormone is released to act at a specific target site either on a cell of the same type or a different cell type. The second strategy involves direct cell-cell transmission of molecules between two or more closely opposed cells, and one way this is achieved is through a specialized cell surface membrane structure called the gap junction.

Gap junction channels function as passageways between two or more cells allowing two-way communication of electrolytes, second messengers and metabolites less than 1kDa by passive diffusion. Gap junctions are responsible for the rapid electrical transmission between groups of cells, which includes passage of inorganic ions such as Na^+, K^+, and Ca^{+2} and a number of small second messengers like cGMP, cAMP, and inositol 1,4,5-triphosphate (IP_3) (Loewenstein 1981; Spray 1984). In mammals the gap junction, or intercellular channels, are expressed in almost all cell types at some point during their development. Intercellular channels are absent, however, in a few terminally

differentiated cells such as, skeletal myocytes, some neurons, most circulating blood cells and spermatozoa (Bruzzone et al. 1996a).

Gap junctions exhibit a hierarchy of assembly (Kumar and Gilula, 1996). The basic unit of the gap junction channel is the connexin (Bruzzone et al. 1996a, b). The connexins comprise a family of homologous proteins whose sequences predict a common topological model with four hydrophobic regions forming the transmembrane domains with two extracellular loops and a cytoplasmic N and C terminus (Figure 1.1). Six individual connexins aggregate to form the connexon. An individual connexon from one cell associates with a corresponding connexon on a neighboring cell, via the extracellular loops, to form a gap junction channel (Figure 1.2). Multiple channels, in turn, cluster in the plane of the membrane to form gap junction plaques (Figure 1.2). Early permeability studies suggested that gap junction channels were rather nonselective and were permeable to most hydrophilic molecules with diameters of 1.5 nm or less (Loewenstein 1981). However, it has become apparent that the gap junction channels are formed by a number of different connexins. Channels formed by different connexins may have different permeabilities allowing regulation of molecules that pass through the channel, based on size. Gap junction channels analyzed by patch clamp studies in different cells exhibit different unitary conductances, supporting the notion that different connexins have different permeabilities. For example, in adult rat hepatocytes (Spray et al., 1984) expressing primarily Cx32 unitary conductances were 140-150 picosiemens (pS) whereas cardiac myocytes (Burt et al., 1988) expressing Cx43 have unitary conductance of 50 to 60 pS. As of 1996 thirteen different connexin genes have been discovered in rodents with many counterparts discovered in other species, such as human, frog, and chicken.

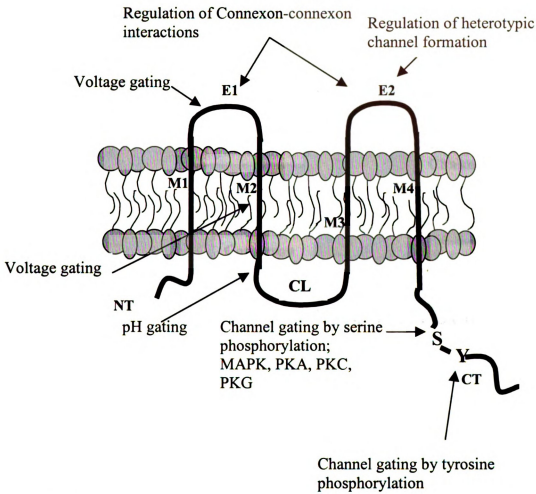


Figure 1.1. Schematic representation and topology of a generic connexin. Hydropathy plots predict four membrane-spanning regions (M1-M4), two extracellular loops (E1-E2), and three cytoplasmic portions, the amino-terminal (NT) and carboxyl-terminal (CT) domains and the central cytoplasmic loop (CL). The variations of the molecular mass among connexins result from the different lengths of the CL and CT sequences. Information taken from Bruzzone et al. (1996a).

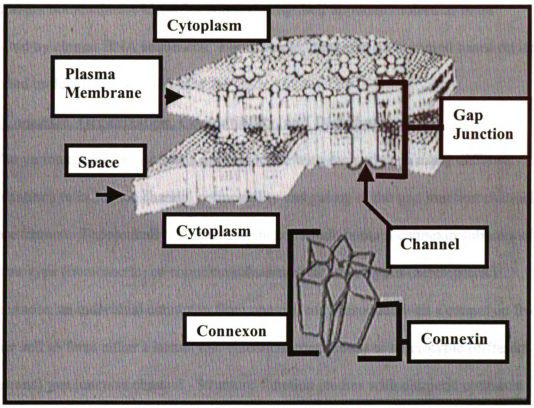


Figure 1.2. Demonstration of the hierarchical assembly of gap junction channels in the membrane of two cells. Six individual connexin subunits aggregate to form the connexon. Connexons are trafficked to the membrane and cluster in the plane of the membrane to form plaques. The connexons from one cell interact with connexons from a closely opposed cell to form the functional gap junction channel, which allows cytoplasmic continuity between the two cells.

The current nomenclature identifies connexins by their apparent molecular mass predicted by cloned DNA sequences. For example, connexin 43 is named based on its predicted molecular mass of 43 kDa.

1.2 - Connexin, Organization, Compatibility, and Distribution

The various connexin isoforms may associate with each other in many different combinations to influence channel permeability and gating of the gap junction channels that are formed. Theoretically, a typical connexon might contain connexin subunits of the same type (homomeric) or connexin subunits of different types (heteromeric). Furthermore, an individual connexon from one cell may associate with a connexon from another cell to form either a homotypic (identical connexons) or heterotypic (different connexons) gap junction channel. Structure-function studies with chimeric connexin constructs performed in a *Xenopus* paired oocyte system show that gap junctions appear to have a code of compatibility where only certain connexins will interact with one another to form functional channels. For example, connexin 31 is restricted to forming homotypic channels, whereas connexin 46 will form heterotypic channels with most other connexins tested (Elfgang et al., 1995; White et al., 1995).

Evolutionary considerations suggest that connexins can be divided into two families α and β (Kumar et al., 1996). By comparing protein or DNA two separate classes of connexins can be differentiated which are more closely related to one another than to members of the other class (Dermietzel 1998). Connexin compatibility, however, is not dependent on family identity; connexin 32 and 26 are in the β family but will form channels with connexins 46 and 50 which are in the α family. Nevertheless, *in vivo* there are few examples demonstrating that connexons can exist in a heteromeric state (Kumar

and Gilula, 1996). Several studies have also demonstrated that adjacent cells programmed to express different connexins have a high probability of not establishing intercellular communication (Elfgang et al., 1995; White et al., 1995; Bruzzone et al., 1993).

The significance of cells expressing so many different connexin isoforms may be for establishing 'communication compartments' to exclude, rather than enhance, cell-cell communication between different tissues or different cell types in the same tissues to aid in organ function or development. One example of the 'communication compartment' idea is the expression of the incompatible connexins Cx43 and Cx40 in the heart. Expression of Cx43 and Cx40 may minimize coupling between the purkinje fibers of the cardiac conduction system and the surrounding ventricular myocardium (Bruzzone et al. 1993, 1996a, b; van Kempen et al., 1995; Elfgang et al., 1995). The separate communication compartments reduce the potential for undesired excitation of myocardial cells along the length of the conducting fibers. The expression of Cx43 and Cx31 in the gastrulating mouse embryo excludes dye transfer between the embryonic cells of the inner cell mass and extraembryonic cells derived from the trophoectoderm providing another example of the 'communication compartment' concept (Dahl et al. 1996).

Connexins are also expressed in overlapping patterns, with most tissues expressing more than one connexin type, and certain combinations of connexins are frequently found in the same organ, although their level of expression may differ. For example, in hepatocytes, connexin 32 and 26 are often expressed together. During development, a number of different connexins may be expressed in a complex and developmentally regulated pattern that varies with the function or differentiated state of the cells. One

example of this phenomenon is the skin where five different connexins are utilized as the cells differentiate into kerotinocytes (Risek et al. 1992; Butterweck. et al. 1994; Goliger and Paul 1994, 1995). The level of connexin expression may also vary as a function of physiological state. For example, just prior to parturition, at a time when myometrial cells need to acquire a coordinated contractility, there is a dramatic increase in both the number of gap junctions and Cx43 mRNA and protein levels (Risek et al. 1990; Lye et al. 1995; Lay et al. 1991). This regulation is tissue specific since myocardial Cx43 is not modulated during labor (Risek et al. 1990; Lang et al. 1990).

1.3 – Gap Junction Channel Gating

The extent of cell-cell communication can also be regulated dynamically by gating, a non-covalent or covalent modification of the channel structure. And like conventional ion channels (e.g. sodium, potassium, calcium) the conductance of gap junction channels is affected by a difference in potential between coupled cells and in some cases between the cytoplasm and extracellular medium (Bruzzone et al., 1996a). Most vertebrate gap junctions display voltage-dependence between coupled cells where there are symmetric changes in junctional currents with equal polarizations on either side. However, heterotypic gap junction channels, formed by different connexons, tend to display asymmetric voltage dependence or rectification. In fact a classical study by Furshpan and Potter (1959) showed that gap junctions between the giant axons of the crayfish nerve cord exhibited asymmetrical voltage-dependence.

There appears to be connexin-specific regulation of channel conductance whether the gating is chemical (e.g. cations) or biochemical (e.g. phosphorylation) with the various connexin subtypes displaying a broad range of sensitivities. In the case of chemical

gating by pH it appears that the C-terminal cytoplasmic loops are responsible for pH-dependent uncoupling with a particle-receptor interaction similar to the original ball-and-chain hypothesis of voltage inactivation.

Takens-Kwak and Jongsma (1992) were the first to sugggest the activation of kinases coincided with a shift in the unitary conductance of single channels recorded between neonatal cardiac myocytes. Examination of dye coupling and single channel conductance of Cx26, Cx43 and Cx45 suggests the existence of a connexin-specific regulation under similar phosphorylating treatments and of a positive correlation between frequency of lower conductance states and decreased permeability (Kwak et al., 1995). In some instances phosphorylation can mean an increase in junctional conductance exemplified by PKA activation and increased junctional conductance through Cx32 channels. In other cases a decrease in junctional conductance is observed under similar phosphorylating conditions, as occurs between AII amacrine cells after cAMP elevation.

1.3.a. – Connexin gating; Phosphorylation

Protein phosphorylation of serine, threonine, and tyrosine is a general mechanism by which extracellular signal transduction systems gate ion channels, including the connexins. The threonine and serine residues on the carboxyl-terminal tail of the connexins are thought to be the target for phosphorylation events, particularly for connexins 32, 43, 40, 45, and 46. The activation of protein kinases causes phosphorylation that is kinase and connexin specific depending on the cell type and particular complement of connexins that are expressed. Phosphorylation events may regulate connexon-connexon interactions as well as the biophysical properties of the intercellular channels.

Neurotransmitters that activate second messenger cascades can alter gap junctional conductances, in particular through phosphorylation in neuronal cell populations (Perez-Velazquez et al., 1997). It has been found that the dopaminergic and cholinergic agonists can modulate gap junctional communication in the CA1 area of the adult rat hippocampus resulting in decreased dye coupling in pyramidal neurons (Perez-Velazquez et al., 1997). However in other brain areas, dopamine can increase the incidence of dye coupling; for example, it increases dye coupling in caudal portions of the nucleus accumbens shell but not the anterior regions (O'Donnell and Grace, 1993). Dopaminergic transmission may uncouple neurons through cAMP-dependent phosphorylation of particular connexins (Lasater, 1987; De Vries and Schwartz, 1989; McMahon, 1994). The differential effects of the neurotransmitters could reflect the expression of different connexins that are differentially regulated by protein kinase phosphorylation, since protein kinases can phosphorylate particular connexins but not others (Kwak et al., 1995).

1.3.b. – Connexin 32 and Connexin 26

The serine at position 233 is the primary site for Cx32 phosphorylation by the cAMP-dependent protein kinase (PKA) while protein kinase C (PKC) phosphorylates the same residue as well as several others (Sáez et al., 1990). The increase in phosphorylation of Cx32 by PKA, in hepatocytes, correlates with an increase in junctional conductance supporting the idea that PKA phosphorylation has a role in the dynamic regulation of cell-cell communication. However, site-directed mutagenesis of both Ser 233 and Ser 240 does not affect the ability of mutant Cx32 to form channels (Werner et al., 1991), suggesting that constitutive phosphorylation is not necessary for Cx32 to acquire functional competence.

Of the connexins that have been examined only connexin 26 has neither consensus sequences for kinases nor is phosphorylated by PKA, PKC or Ca^{+2}/calmodulin dependent protein kinase II (Sáez et al. 1990).

1.3.c. – Connexin 43

On the other hand connexin 43 is differentially phosphorylated in a tissue specific manner (Kadle et al. 1991) and this constitutive phosphorylation has been correlated to the communication competence of some cell lines (Musil et al. 1990 a, b; Oh et al. 1993; Oyamade et al. 1994). Specifically connexin 43 is post-translationally phosphorylated in brain, heart, and kidney (Hossain et al. 1994, Laird et al., 1991, Musil et al., 1990a, b and 1991). In cell lines lacking significant dye coupling, treatment with cAMP analogues induces phosphorylation of connexin 43, presumably through the PKA adenylyl cyclase/cAMP-dependent pathway, which is associated with a rapid increase in cell-cell communication, taking only minutes (Traub et al., 1987; Saez et al., 1986). Unlike Cx32, however, sequencing of phosphorylated peptides has shown that seryl residues at positions 368 and 372 are indeed phosphorylated by PKC but PKA, Ca^{2+}/calmodulin-dependent protein kinase, or cGMP-dependent protein kinase do not directly phosphorylate Cx43 (Saez et al., 1993).

Nevertheless phosphorylation appears to be an important regulator of connexin 43 permeability and connexin 43 can exist in several tissue-specific as well as cell cycle specific forms (Xie et al. 1997). Immunoblot analysis of Cx43 protein demonstrates the existence of up to four different phosphorylation states of Cx43 depending on the tissue examined. Clone 9 cells, derived from normal rat liver, express four forms of Cx43 under control conditions (Saez et al., 1990). The unphosphorylated form of CX43 is

designated NP and corresponds to approximately 40-41 kDa. The other three forms all correspond to phosphorylation events; P' and P1 ~ 42 kDa, P2 ~ 43-47 kDa depending on the cell type and other postranslational modifications (Saez et al., 1993). Laird et al. (1991) demonstrated that Cx43, in cardiac myocytes, exists in three forms 44, 42, and 40 kDa. The 44 and 42 kDa forms are both alkaline phosphatase (AP) sensitive and will reduce to 40kda after AP treatment, demonstrating a role for phosphorylation in the regulation of Cx43. Hossain et al. (1994) have shown that Cx43 (in the brain) can exist in two states as well, a 41kDa inactive form and the active phosphorylated 43 kDa form. They speculate that a brain phosphatase may be involved in modulating Cx43 permeability and GJIC. The inactive or non-phosphorylated form of connexin 43 has been detected in the cytoplasm by immunofluorescence suggesting that phosphorylation creates a signal necessary for processing and/or functional activation of this channel forming protein (Willecke et al., 1991).

1.3.d. - Connexin gating; pH & Calcium

pH and changes in calcium can also regulate gap junction channels. In fact some of the first chemical inhibitors of junctional coupling to be described were pH and calcium changes (Turin and Warner; 1977; Rose and Loewenstein 1975). Using pairs of *Xenopus* oocytes expressing different connexins it was found that all connexins are sensitive to acidification to some degree although some connexin subtypes are more sensitive than others (Werner et al., 1991; Liu et al., 1993; Ek et al., 1994; White et al., 1994).

A number of independent studies have demonstrated that the middle and C-terminal cytoplasmic loops are major determinants of chemical gating (Bruzzone et al., 1996a).

Rat Cx32 is weakly sensitive to acidification whereas Cx38 rapidly closes with decreasing intracellular pH (Werner et al., 1991; Liu et al., 1993). The greater sensitivity of *Xenopus* Cx38 has been shown, through domain swapping experiments, to result from the middle cytoplasmic loop of Cx38. A chimera in which the middle cytoplasmic loop of rat Cx32 is replaced with the corresponding region of Cx38 makes Cx32 highly pH sensitive (Wang et al., 1996). Furthermore, truncation of Cx43 C-terminal tail, which is highly pH sensitive, generates a mutant with reduced pH sensitivity similar to Cx32 (Liu et al., 1993). In guinea pig hippocampal brain sclices cytoplasmic acidification using propionate significantly decreases dye-coupling to 6% compared to 28% in control solution (MacVicar et al., 1985).

Alkalosis in turn can enhance gap junctional coupling. This has been shown in rat hippocampal CA1 pyramidal neurons. Exposure of the CA1 neurons to high-pH medium increases the incidence and extent of dye coupling (Church and Baimbridge, 1991).

Calcium has been shown to be a regulator of gap junctional communication in cardiac myocytes, Novikoff hepatoma cells and bovine lentoids (Dahl and Isenberg, 1980; Lazrak and Perrachia, 1993; Crow et al., 1994). The principal connexin expressed in these cells is connexin 43, suggesting that calcium-sensitivity of GJIC is connexin dependent. The calcium-receptor protein calmodulin is thought to mediate the calcium sensitivity of connexins due to its ability to bind Cx32 and the fact that connexins do not have consensus sequences for calcium-binding sites (Hertzberg and Van Eldeh, 1987).

Gap junctions of bilaterally perfused cell pairs are insensitive to calcium, but recover sensitivity when calmodulin is added to the perfusate (Johnston et al., 1981; Arellano et al., 1988). It is not clear whether calmodulin can bind other connexins, however, or

whether all connexins require a cytoplasmic factor to mediate calcium sensitivity. It has been suggested further that phosphorylation events (Arellano et al., 1990) and changes in open/closed state of the K-ATP channel (Granda et al., 1998) may also regulate calcium sensitivity.

A synergistic action has been suggested between changes in pH and calcium. In cardiac cells, the sensitivity to low pH is greatly reduced if the intracellular calcium concentration is decreased concomitantly (Burt, 1987; White et al., 1990). Vera et al. (1996) has found a reduction in GJIC after depleting cellular ATP levels, in astrocytes, with antimycin (mitochondrial respiratory chain inhibitor). The inhibition of GJIC was dose-dependent and reversible by incubating cells with EGTA, suggesting the inhibition was via a calcium-dependent mechanism. As is the case with H^+ (pH) it is unknown whether calcium interacts with gap junctions directly, indirectly or both.

1.4. – cAMP and Connexin Regulation

The second messenger cAMP produces diverse effects on gap junction expression and function in different tissues and on different connexins, although the most commonly observed effect is an increase in gap junctional communication. Many investigators have observed changes in GJIC in a number of cell types treated with cAMP agonists or analogues along with increases in Cx43, Cx32, and Cx26 protein and/or plaque formation (Banoub et al., 1996; Sáez et al., 1986 and 1989; Traub et al., 1987; Takeda et al., 1987; Wiener and Loewenstein 1983). However, in uterine smooth muscle cells, gap junctional communication is reduced after treatment with cAMP derivatives (Cole et al., 1986).

The turnover of Cx26, Cx32, and Cx43 protein is relatively rapid occurring within 1-5 hours (Traub et al. 1989; Crow et al., 1990; Musil et al., 1990a, b; Fallon et al., 1981;

Laird et al.,1991; Traub et al., 1987). Activation of the adenyly cyclase/cAMP pathway by treatment with cAMP agonists can induce not only phosphorylation of connexins, in this relatively short time frame, but can also enhance connexin aggregation and connexon trafficking to the cell membrane along with delaying the disappearance of gap junction plaques between pairs of coupled cells (Wang and Rose, 1995; Saez et al., 1986, 1989, 1990). Inhibitors of protein synthesis can prevent the induction of junctional communication by cAMP in several systems (Azarnia et al., 1981; Weiner et al., 1983; Kessler et al., 1984). It has also been shown that cAMP increases the amount of metabolically labeled Cx32 in primary cell cultures of fetal hepatocytes (Traub et al., 1987). Atkinson et al. (1995) found that prolonged treatment of mouse mammary tumor cells with cAMP increases intercellular communication by modifying the cellular distribution of Cx43, such that a greater portion is available for channel formation. Matesic et al. (1996) also found that cAMP-inducers could upregulate cell-cell communication in gonadotropin-releasing hormone neurons by enhancing plaque formation of connexin 26 on the membrane. Treatment of the neurons, with cAMP-inducers, resulted in increased GJIC and connexin plaque formation with no increase in Cx26 protein or phosphorylation.

Cyclic AMP can also enhance cell-cell communication by increasing the rate of transcription of connexin mRNA or increasing stability of the message. Blockers of mRNA synthesis can prevent cAMP-induced junctional communication (Kessler et al., 1985). Cyclic AMP stimulates the rate of transcription of Cx43 mRNA in Morris hepatoma cells (Mehta et al.,1990), and in CL-1D cells, a mouse fibroblast cell line (Stagg et al., 1990). In addition it has been shown that the Cx32 gene contains consensus

cAMP response elements near the transcription start site (Miller et al., 1988). Yet, membrane permanent cAMP derivatives increase levels of Cx32 and its mRNA to a similar extent in assays using Cx32 promoter constructs that contain or lack putative cAMP responses. Accordingly, it has been reported that cAMP increases the stability but does not measurably alter the transcription rate of connexin 32 mRNA in cultured adult rat hepatocytes or Cx43 mRNA in the 3T3 mouse fibroblast cell line (Saez et al., 1989; Stagg et al., 1990).

1.5. – Inhibition of Cell-Cell Communication

A decrease in cellular energy, i.e. decreases in ATP levels, will inhibit cell-cell communication. Hossian et al. (1994) have shown that in rat brain tissue a decrease in cellular energy, due to death, causes a dephosphorylation of Cx43. Dephosphorylation of connexin 43 is typically associated with a reduction in cell-cell communication.

Just as phosphorylation events can upregulate GJIC, they can also down regulate cell-cell communication as well. Tyrosine phosphorylation is an important mechanism by which Cx43 channel function can be inhibited. A number of investigators have found that over expression of the *v*-src oncogene or $pp60^{c\text{-}src}$ protooncogene can cause rapid decreases in cell-cell communication due to direct tyrosine phosphorylation of the phenylalanine on the carboxyl-terminus of Cx43 (Atkinson et al., 1981; Chang et al., 1985; Azarin et al., 1988; Jou et al., 1995). However, phosphorylation by src appears to be connexin specific because channels composed of Cx32 are not affected by *v*-src phosphorylation.

A similar reduction in coupling has also been observed when certain cell lines are treated with growth factors that bind receptors with tyrosine kinase activity such as EGF

or PDGF. Inhibition of communication by PDGF or Hepatocyte growth/scatter factor is also dependent on tyrosine phosphorylation (Pelletier and Boynton, 1994; Moorby et al., 1995). Unlike *v-src* or PDGF, EGF stimulation induces a specific phosphorylation of Cx43 only on serine residues, mediated by mitogen activated protein kinase (MAPK) (Lau et al., 1992; Kanemitsu and Lau, 1993; Warn-Kramer et al., 1996). Serine residues on the C-terminus of Cx43 are directly phosphorylated by MAPK resulting in a transient decrease in GJIC. PKC is also involved in inhibition of GJIC, via connexin phosphorylation, when activated by tumor promoting phorbol esters like 12-O-tetradecanoylphorbol-13-acetate (TPA). Studies using TPA result in reduced GJIC which is correlated to changes in the phosphorylation state of Cx43 possibly due to hyperphosphorylation by PKC (Berthound et al., 1992b and 1993; Brissett et al., 1991; Oh et al., 1991). Again, reduced GJIC by TPA is dependent on the connexin expressed, cell type, and in some cases the phosphorylation state of the connexin. In cells where the dephosphorylated form of Cx43 (P0) predominates under basal conditions, stimulation with TPA leads to rapid cell uncoupling. In contrast, a cell where the predominant form of Cx43 phosphorylated TPA promotes junctional communication without detectable changes in the phosphorylation state (Sáez et al., 1990).

1.6 - Functions of Gap Junctions

The passage of important regulatory signals through the gap junction channel has been implicated in regulating events between cells during embryogenesis (Warner et al., 1984), cell proliferation and secretion, and coordination of synchronous activity between excitable cells such as electrical conduction in the heart. It has been postulated that gap junction channels are responsible for maintaining and regulating important cellular

functions due to the diversity of connexin genes expressed and their appearance in nearly every animal tissue from the early cleavage stages of the embryo to adulthood. Perhaps the clearest examples of how connexin expression and functional gap junctional intercellular communication are involved in many necessary cellular processes comes from connexin 43 knockout mice, human diseases, and developmental studies.

1.6 .a - Connexin 43 Knockout Mice and Visceroatrial Heteroataxia Syndrome

The importance of a regulated expression of specific connexin genes in cardiac embryogenesis has been demonstrated by generation of mice lacking connexin 43 (Reaume et al., 1995). The mice homozygous for ablation of Cx43 survive to term but die shortly after birth due to heart malformation. Specifically there is a gross enlargement of the conus overlying the right ventricular outflow tract of the heart. Reaume et al. (1995) have found that the conus is filled with intraventricular septae leading to interconnected or blind-ended chambers. Neonatal death is a consequence of obstruction of the pulmonary artery. Surprisingly, abnormal morphogenesis only occurs in the heart although Cx43 is expressed, in mice, from the start of zygotic transcription and widely distributed in many organs well into adulthood, including the brain. The cardiac defect in Cx43 knockout mice is similar to a human syndrome called visceroatrial heteroataxia syndrome (VAH), a genetically heterogeneous syndrome characterized by complex defects of laterality and/or transposition of the great arteries. Britz-Cunningham et al. (1995) have found that patients with VAH have several mutations in the carboxyl-terminal tail of Cx43 clustered in a region containing several phosphorylation consensus sequences. Serine phosphorylation of Cx43, as already discussed in "Regulation of

Connexins", is extremely important for regulation of connexon assembly, docking, and channel permeability.

1.6. b - X-Linked Charcot-Marie-Tooth Disease

Another human disease that clearly demonstrates the importance of connexins in maintaining homeostasis, by allowing diffusion of important regulatory signals between cells, is the X-linked form of Charcot-Marie-Tooth disease (CMTX). Charcot-Marie-Tooth disease is the most common inherited peripheral neuropathy, associated with demylination and decreased nerve conduction velocity (Bruzzone et al., 1996b). The proper functioning of connexin 32 is critical for the homeostatic maintenance of the schwann cell, the myelinating cell of the peripheral nervous system. A total of 42 different Cx32 mutations have been reported in families having a high incidence of CMTX (Deschênes, 1997). Mutations appear throughout the Cx32 molecule, with all domains effected except the fourth transmembrane domain (Bergoffen et al., 1993).

Initially expression of the human Cx32 mutants in the paired oocyte system indicated a complete loss of channel activity (Bruzzone et al,. 1994). However, it was demonstrated that some Cx32 mutants, associated with CMTX, have a C-terminal deletion that does not affect the ability to induce junctional currents when expressed in the paired *Xenopus* oocyte systems (Rabadan-Diehl et al., 1994). The C-terminal deletion may, however, disrupt channel gating, permeability or pore size. More recently, Cx32 knockout mice have been developed, however, the Cx32-null mice are initially only effected by a mild phenotype and develop normal myelin (Nelles et al., 1996; Anzini et al., 1997). After about four months, the Cx32-null mice begin to show signs of a late-onset demyelinating neuropathy similar to what is observed in patients with CMTX

(Anzini et al., 1997). Interestingly, diffusion of low molecular weight dyes, across the myelin sheath, was not blocked in schwann cells from Cx32-null mice, indicating that other connexins participate in forming gap junctions in these cells (Baleice-Gordon et al., 1998). Together these findings suggest that although mutations in Cx32 may inhibit functional communication, the blockage of GJIC is not as important as the interference of diffusion of critical messenger molecules and nutrients in the schwann cell, that perhaps only Cx32 can transfer (Bergoffen et al., 1993; Bruzzone et al., 1995; Deschênes et al., 1997; Paul 1995a). The interference of the diffusion of messenger molecules may alter the ability of schwann cells to respond to normal glial-neuron interactions, which are critical for maintenance of myelin sheaths (Doyle and Colomann 1993).

Supporting the idea that the message transferred by the gap junctions is more important than cell-cell communication between two or more individual Schwann cells is the observation that Cx32 protein does not appear between areas of schwann cell-cell contact, but rather at the paranodal regions and schmidt-lantermann incisures (Bergoffen et al., 1993; Scherer et al., 1996). This distribution of Cx32 is incompatible with the formation of gap junctions between two or more individual schwann cells, but shows that Cx32 forms channels within a single schwann cell between the turns of myelin. The formation of gap junctions between the myelin sheaths of a single schwann cell would constitute a faster diffusion pathway for the transfer of important ions, nutrients, and molecules from the perinuclear to the periaxonal region of the compact myelin wraps.

As with the connexin 43 knockout mice, the Cx32 mutations are associated with a restricted phenotype in the schwann cell, although Cx32 is widely distributed in many other cell types including oligodendrocytes, the myelin-forming cells in the central

nervous system (Paul et al., 1993; Spray and Dermietzel 1995). In the schwann cell, the expression of Cx32, like that of Cx43 in the heart, is developmentally regulated in parallel to that of other myelin related genes, suggesting the presence of common transcriptional mechanisms (Scherer et al., 1996). It may be that during development there is defective transcriptional regulation of Cx32 as well as other myelin associated genes so that the combination of Cx32 mutations along with mutations in other myelin related genes is additive in producing the CMTX phenotype. In other cell types expressing Cx32, another connexin may compensate for the loss of GJIC or Cx32 may not be as intertwined in the development of the cell's function as it is in the schwann cell. One recent example supporting this notion is the finding that P0, or protein zero, a myelin associated protein involved in myelin compaction, can induce a severe early onset-demyelination in mice completely devoid of P0, but containing wildtype Cx32 (Neuberg et al., 1998). Neuberg et al. (1998) found, as already discussed, mice devoid of Cx32 only present a mild form of demyelination. However, mice that lack Cx32 and have only one allele of P0 have an accelerated destabilization of myelin and present an early-onset demyelination suggesting that the phenotype of mutant myelinating schwann cells in $Cx32^{0/0}/P0^{+0}$ mice is additive (Neuberg et al., 1998).

1.6.c - Carcinogenesis

Loewenstein (1981) first proposed the hypothesis that blocked or decreased communication could lead to uncontrolled cell growth due to the presumptive decrease in growth regulatory signals transmitted through gap junctions. Supporting this hypothesis are multiple lines of evidence showing that tumor promoters, oncogenes, and growth factors modulate channel permeability and loss of intercellular communication is a

common feature of transformed cells (Bruzzone et al., 1996a). Lee et al. (1992) have shown that in human mammary epithelial tumor cell lines, connexin 26 and connexin 43 are reversibly down regulated at the transcriptional level. Sato et al. (1997) have shown that the functional form of Cx43 (P2) is not detectable in the majority of atypical and anaplastic meningiomas, suggesting that reduced cell-cell communication may be associated with more rapid growth of the meningiomas. Wilgenbus et al. (1992) was also able to show that there was a significant decrease in the gap junction proteins in breast cancer, renal cancer, and sarcomas as opposed to normal tissue. Furthermore, restoration of cellular coupling is associated with a decreased incidence of tumorigenesis (Yamasaki, 1990; Rogers et al., 1990; Lau et al., 1992; Loewenstein and Rose, 1992; Hossain et al., 1993; Trosko et al., 1993). For example, transfection of connexin 43 and 32 into connexin-devoid human mammary carcinoma cells suppresses the cancer phenotype by slowing their growth rate and restoring their differentiation capacity (Hirschi et al., 1996). In turn, Chen et al. (1995) and Jou et al. (1993) have shown that transfection of rat connexin 43 into a phenotypically transformed dog kidney cell line and rat liver cell line respectively, restored GJIC, decreased tumorgenicity, and normalized cell growth. In glioma cells lacking functional cell-cell coupling, transfection with the connexin 43 gene induced increases in cell-cell contacts and organization of actin filaments into stress fibers along with decreased proliferation (Naus et al., 1991). Conversely nontransfected cells have reduced cell coupling and little actin filament organization (Naus et al., 1991). Naus et al. (1997) found that astrocytes cultured from Cx43-null mice also had an altered growth rate compared to normal wildtype mouse astrocytes. In this case, however, the

growth of homozygous Cx43 devoid astrocytes was significantly decreased along with saturation density (Naus et al., 1997).

However, not all studies have supported the idea that gap junctional communication controls growth rate. In fact, there appears to be a negative correlation between tumor growth rate and cell coupling (Eghbali et al., 1991; Zhu et al., 1991; Mehta et al., 1991; Naus et al., 1992; Rose et al., 1993). For example, blockade of normal cell-cell communication in the BALB/c3T3 cells, using antisense oligonucleotides, did not have any affect on cell growth rate (Ruch et al., 1995). What was found, however, was that blocking normal GJIC, in BALB/c3T3 cells, increased saturation density threefold and allowed these cells to become contact-independent. An established feature of transformed cells is their lack of contact-dependent growth inhibition (Bruzzone et al., 1996a). Mesnil et al. (1995) have also found evidence of connexin specificity in the restoration of normal cell growth *in vivo* suggesting that some connexins may be important in the maintenance of cell growth while others are not. For example, Hela cells do not normally express connexin protein, however, when transfected with different connexins they respond differently to tumor-promoters and anti-promoting chemicals (Mazzoleni et al., 1996). When these connexin-transfected Hela cells are injected into nude mice the tumorigenicity of the transfected cells is dependent on the connexin species expressed. Transfection with Cx26, but not with Cx40 or Cx43, markedly inhibited cellular growth in spite of similar intercellular communication (Mesnil et al., 1995).

Furthermore, cell-cell communication can still occur between tumor cells and tumor cells and normal cells, suggesting again that it isn't the cell-cell coupling that is

necessarily important but the message that is transferred through the channel. Selective lack, reduction, and differential regulation of the coupling between transformed and control cells have been demonstrated (Enomoto and Yamasaki, 1984; Mehta et al., 1986; Mesnil et al., 1987, 1993; Mehta and Loewenstein, 1991; Krutovskikh et al., 1994). El-Sabban and Pauli (1991) have shown that communication between metastatic tumor cells and vascular endothelium may not be beneficial, but actually help tumor cells cross the endothelium and invade other organs possibly due to aberrant transfer of messenger molecules through the established gap junctions. Further, Wilgenbus et al. (1992) found that some tumor cells do express distinct gap junction proteins that are not observed in the normal tissue. For example, Cx43, which is not found in normal adult hepatocytes, was detected, by immunofluorescence, in human hepatocellular carcinoma. Connexin 26 is not found in normal adult skin, but was detected in samples of basal-cell carcinoma (Wilgenbus et al., 1992).

1.6.d - Gap Junctions in Development

Perhaps the least understood functional role for gap-junctional intercellular communication is its involvement in development. 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, 1987).

There is a large body of correlative evidence suggesting that gap junctions play a significant role in regulating cellular activities during development, such as symmetry, pattern formation, and differentiation. In fact, three connexin genes are already expressed at the four-cell stage mouse embryo, and three more accumulate by the eight

cell-stage (Davies et al., 1996). Blockade of GJIC in the developing embryo has demonstrated a role for functional cell-cell coupling in regulating symmetry and pattern formation (Fraser et al., 1987; Lee et al., 1987; Warner et al., 1986; Bevilacqua et al., 1989; Paul et al., 1995b; Olsen et al., 1991). Warner et al. (1986) and Lee et al. (1987) have shown that blockade of the 27kDa (Cx32) gap junction protein, using antibodies, disrupts brain formation and left/right symmetry in amphibian and mouse embryos respectively.

During development a number of connexins are expressed in a complex and overlapping pattern in many different tissues including skin, liver, and brain (Risek et al., 1992; Rosenberg et al., 1996; Dermetzial and Spray et al., 1993) that is not clearly understood. It seems however, that during development the connexins expressed appear to be most suited for the state of the tissue at the present time and their expression correlates with development of adult organ function. In the heart the developmental pattern of appearance of immunostained Cx43 correlates with the reported developmental increase in conduction velocity (Van Kempen et al., 1991). In the liver, the adult pattern of connexin distribution is attained at a time when the metabolic zonation and maturity of hepatic acini seen in adults is reached (Berthound et al., 1992a).

One explanation for the regulated expression of different connexins during development would be to establish gradients or boundaries of communication (as discussed in "Regulation of Cell-Cell Communication") between populations of coupled cells that would result in distinct signals being sensed within the group of cells. Gene expression programs could, therefore, be regulated within a specific group of coupled cells without an anatomical boundary. Again, the separate expression of incompatible

connexins would be a way to achieve compartmentalization without affecting cell-cell contact. This compartmentalization could then allow differentiation to proceed in certain coupled cell populations while proliferation occurs in others (Trosko et al., 1993). The nervous system expresses many different connexins; of the thirteen that are known at least 8 are found in the CNS. Therefore, the nervous system can be considered as a series of communicating compartments, the strength or lack of coupling within and between compartments is then due, in part, to the pattern of the connexin complement expressed by each compartment. For example, compartmental interfaces where coupling between compartments might occur is between Cx32/Cx45 expressing oligodendrocytes, and Cx43/Cx45/Cx40/Cx30 expressing astrocytes or Cx32/Cx43 expressing neurons (Dermietzel, 1998). However, coupling is rarely seen between the astrocytic compartment and the neuronal compartment.

The vertebrate brain offers one example of the dynamically regulated expression of connexins during development, where many different connexin subtypes are expressed in overlapping patterns depending on the cell type and the differentiation-state of the cell. In the developing brain connexin mRNA and protein expression is differentially regulated (Belleveau 1991; Dermietzel 1989). Specifically Cx43 mRNA is detectable, in the mouse and rat forebrain and hindbrain, at embryonic day 18 and increases, with development, from birth until postnatal day 30 (Belliveau et al., 1991). Connexin 32 mRNA is not detected at embryonic day 18 but is seen at postnatal day 16 in the forebrain and postnatal day 10 in the hindbrain and decreases in the adult (Belliveau et al., 1991). Connexin 26 appears to be regulated complementarily to connexin 32 (Dermietzel and Spray 1993). The appearance of the different connexins may correlate

with the appearance of different cell types in the brain. This has been shown in cultures of cells induced to differentiate with retinoic acid to produce different neuronal cell types. In P19 mouse embryonal carcinoma cells, differentiation with retinoic acid produces astrocytes and neurons. The P19 cells initially express both connexin 43 and connexin 26 protein although their pattern of expression differs e.g. connexin 43 is expressed homogeneously while connexin 26 distribution is more variable (Belliveau et al., 1997). Upon differentiation connexin 43 is localized in only the astrocyte population while connexin 26 remains in the neuronal population. NT2/D1 is a human teratocarcinoma cell line that can also be induced to differentiate into neurons when stimulated with retinoic acid (Andrews et al., 1987). The NT2/D1 cells only express connexin 43; when treated with retinoic acid they lose connexin 43 expression and functional dye-coupling as differentiation proceeds (Naus et al., 1997).

During neocortical development, there appears to be a high degree of intercellular coupling. In slices of postnatal day 4 rat neocortex, as many as 80% of the neurons examined were dye coupled, declining to 20% in the adult (Conners et al., 1983). In the neonatal neocortex, a form of endogenous coordinated activity is present as locally restricted intercellular calcium waves that are observed within small, discrete, and often columnar regions of developing cortex. The localized calcium waves or domains, exhibit simultaneous increases in intracellular free calcium in the complete absence of sodium action potentials and chemical synaptic transmission (Yuste et al., 1992, 1995). Gap junction proteins expressed during neuronal development are thought to be important in grouping neurons into these local assemblies or domains to synchronize electrical or biochemical activity among the neuronal neighbors (Peinado et al., 1993a; Kandler and

Katz, 1998a). The synchronization of neuronal activity may be important for the functional specification of brain areas and the formation of synaptic circuits (Kandler and Katz, 1998b; Sharz and Stryker, 1988; Weliky and Katz, 1997). The gap junctions are found almost entirely at dendrodendritic and dendrosomatic contacts and the coupling appears to be selective occurring primarily in cells of the same type (Peinado et al., 1993a). For example, in the cortex, nonpyramidal neurons couple selectively to other nonpyramidal neurons and do not pass dye to pyramidal neurons and never to glial cells (Pienado et al., 1993; Kandler 1997). Many investigators have also found that neuronal coupling via gap junctions is prevalent during the first two weeks after birth, in the rat neocortex and visual system, but then steadily decreases after day thirteen-fourteen (Conners et al., 1983; Peinado et al., 1993b; Kandler and Katz, 1998b). In the ferret developing visual system, however, coupling is not seen at birth but increases at postnatal day five and declines after postnatal day fifteen (Kandler and Katz, 1998b). Nevertheless, in each case, dye coupling became most pronounced at the same time when synaptic activity increased most dramatically (Kandler and Katz, 1998b; Peinado et al., 1993a, 1993b). Furthermore, the peak in coupling correlated with the time when thalamocortical fibers begin to invade the cortical plate (Allendoerfer et al., 1994; Herrmann et al., 1994; Kandler et al., 1997).

It was postulated that gap junctions might be responsible for coordinating the electrical activity of neurons in the neocortex. Kandler and Katz (1998a), however, have recently demonstrated that intercellular coupling appears to be too weak to synchronize neuronal electrical activity during generation of the spontaneous calcium waves associated with a neuronal domain (Conners et al., 1983; Peinado et al., 1993b). Kandler

and Katz (1998a) found that neuronal domains were initiated by stimulation of metabotropic glutamate receptors and by intercellular increases in the second-messenger molecule inositol triphosphate (IP_3) that releases calcium from internal stores. In addition, IP_3 is the intercellular signal molecule that diffuses between coupled cells and underlies the propagation of neuronal calcium waves. Therefore, gap junctions appear to coordinate neuronal biochemical activity rather than electrical activity. Synchronization of biochemical activity across large cell assemblies is likely to influence cortical development before or during early stages of synapse formation, but not after the emergence of circuits. Yet, although there is much correlative evidence to implicate gap junctional communication in providing the scaffolding on which synapses are formed, there is no causal demonstration that this is indeed what happens.

1.7 - Gap Junctions and Functions in the Mature Central Nervous System

In the mature vertebrate nervous system, gap junctions appear to be restricted to specific areas and cell types, being identified in astrocytes, neurons, ependymal cells, and oligodendrocytes (Dermietzal et al., 1978, 1989; MacVicar et al., 1982; Nagy et al., 1988; El Aoumari et al., 1990).

1.7.a. - Gap Junctions in Astrocytes

Astrocytes are the cells in the CNS most extensively coupled by gap junctions and they primarily express Cx43 (Dermietzel et al 1991; Giaume et al., 1991) but more recently have been shown to co-express Cx30 (Dahl et al., 1996). It has been suggested that *in vivo* coupled astrocytes form a syncytium (Mugnaini, 1986). The astrocytic syncytium may be important in maintaining neuronal homeostasis by buffering potassium ions, in the generation of slow electrical fields associated with neural activity, and in the

propagation of calcium waves following stimulation by glutamate (Batter et al., 1992; Yamomoto et al., 1991). Astrocytes are actively involved in several brain functions, such as neuronal differentiation and migration, control of signaling processes, and control of local ion and amine/amino acid concentrations along with regulation of brain metabolism (Giaume et al., 1991; Giaume and McCarthy, 1996; Tabernero et al, 1996; Bruzzone et al., 1997). Yamomoto et al. (1990), Batter et al. (1992), and Nagy et al. (1992) have found heterogeneity in the expression of connexin 43 protein and mRNA among astrocytes in various regions of the CNS, such as the hypothalamus and striatum. The heterogeneity in expression of Cx43 may lead to variations in astrocyte phenotype and demand for gap junctional coupling within the astrocytic functional syncytia leading to establishment of separate synctial compartments and region-specific function (Yamomoto et al., 1990; Batter et al., 1991). Blanc et al. (1998) have shown that astrocytic gap junctional communication decreases neuronal vulnerability to oxidative injury by stabilizing cellular calcium homeostasis. Co-cultures of neurons and astrocytes revealed extensive astrocyte-astrocyte coupling. When co-cultures were treated with the gap junction blocker 18-α-glycyrrhetinic acid there was a marked enhancement in the generation of intercellular peroxides, impairment of mitochondrial function, and cell death in the neurons, but not astrocytes, following exposure to oxidative insults (Blanc et al., 1998).

Astrocytic gap junctions composed of Cx43 in the adult have the ability to propagate coordinated calcium waves similar to what is observed during neocortical development in the form of domains (Cornell-Bell et al., 1990; Charles et al., 1991; Dani et al., 1992). The calcium waves appear to depend on connexin expression and involve the selective

permeability of a given connexin to specific messengers. Studies *in vitro* with clonal cell lines have shown the importance of specific connexin expression in the propagation of calcium waves. For example, two clones of immortalized neurons show Ca^{2+} oscillations, but only one allows the propagation of intercellular waves, a feature correlated with the abundance of Cx26 mRNA (Bruzzone and Ressot, 1997). In addition, it has been shown that C6 glioma cells transfected with Cx32 allow very little intercellular spread of Ca^{2+} signals, whereas overexpression of Cx43 in the same cell line results in the propagation of Ca^{2+} waves (Bruzzone and Ressot, 1997). In astrocytes cultured from embryonic mice with a null mutation in the Cx43 gene (Reaume et al., 1995), Naus et al. (1997) have examined the propagation of intercellular Ca^{2+} waves. Calcium signaling was significantly reduced (but not eliminated) in astrocytes cultured from Cx43-null mice compared to the wildtype control (Naus et al., 1997).

Schemes et al. (1998) have also examined calcium signaling in Cx43-null astrocytes. Schemes et al. (1998) concluded that the propagation of Ca^{2+} waves between astrocytes from Cx43 knockout mice is not so greatly affected as anticipated by deletion of the major gap junction protein form these cells. It appears that the other connexins present in astrocytes (Cx40, Cx45, and Cx46) are capable of performing long-range Ca^{2+} signaling even though there is a substantial decrease in junctional conductance (Schemes et al., 1998).

As with the neuronal domains in the developing neocortex, the intercellular calcium waves seen in astrocyte syncytium are produced by activation of phospholipase C which in turn generates a rise in IP_3 and release of IP_3-sensitive internal calcium stores.

1.7.b. - Gap Junctions in Neurons

Neurons express several different connexin subtypes, including Cx32, Cx26, Cx43, Cx36, and Cx37. Connexin 43 mRNA is ubiquitously expressed in a variety of neurons. By *in situ* mRNA hybridization a number of cortical, hippocampal and cerebellar neurons, including Purkinje cells, were found to be positive for this connexin (Dermietzel, 1998; Simburger et al., 1997). The gonadotropin-releasing hormone neurons (also called leutinizing hormone releasing-hormone neurons) of the hypothalamus are known to express Cx26 and Cx32 (Matesic et al., 1993, 1996; Hosney and Jennes, 1997). Of the connexins that have been identified in neurons, only Cx36 has been shown to be expressed exclusively in neuronal cells; at least in the rat (Condorelli et al., 1998). Connexin 36 mRNA has been localized by *in situ* hybridization and RT-PCR in the inferior olive, retina, pineal gland, olfactory bulb and hippocampus (Condorelli et al., 1998).

Much less is known about neuronal gap junction function in mammals, although they are the morphological correlate of electrotonic coupling (transmission of current via ion flow), which occurs in various regions of the brain, including the inferior olivary complex, hippocampus, pienal gland, and retina. Gap junctions of the olivary neurons are involved in the generation of patterns of highly synchronous neuronal activity important for dynamic organization of motor control within the olivocerebelar system (Welsh et al., 1995). In the hippocampus, pyramidal neurons are coupled by gap junctions as evidenced by the fast small amplitude depolarizations, termed spiklets, in whole cell recordings that seem to correlate with dye coupling and have a characteristics of electrotonic potentials (Perez-Velazquez et al., 1997).

It is still debated whether there is a specific function for neuronal gap junctions and electrotonic coupling in the mature mammalian brain, however, in invertebrates, such as electrical fish as well as other fish, electrotonic coupling mediated by gap junctions is used for escape responses and electrical discharge (Bennett, 1997). In these instances electrical transmission between neurons is required because of its speed. Furthermore, experiments performed on Mauthner cells of teleosts provide evidence for coordination of neuronal activity via mixed synapses. Mixed synapses are established at the large myelinated club endings of the posterior eighth nerve fibers with the lateral dendrites of the Mauthner cell in teleosts. Pereda et al. (1994, 1996) have shown that sharing the same receptor repertoire and second messenger pathways can modify chemical and electrotonic transmission. Both modalities of transmission can be modulated by dopamine and NMDA-receptor activation.

It has been proposed that, *in vivo*, mammalian gap junctions in neurons, may underlie neuronal activity associated with alkalosis or acidosis in the CNS, are involved in signal flow in the retina, and may help synchronize neuronal firing and coordinate responses such as pulsatile release of hormones and hormonal plasticity.

The mammalian CNS is highly sensitive to pH and intracellular acidosis and alkalosis, will decrease and increase respectively, the incidence and extent of anatomical coupling. Extracellular alkalosis is associated with the development of synchronization of neuronal populations, during epileptogenesis. The synchronous seizure activity is thought to be due to electronic interactions mediated through gap junctions (Church and Baimbridge, 1991). Church and Baimbridge (1991) have found that exposure of rat hippocampal CA1 pyramidal neurons to high-pH medium increases the incidence of dye coupling and mean

number of neurons that are dye-coupled. Changes in pH have a profound effect on connexin channel gating. The increase in dye coupling was associated with the ability of CA1 pyramidal neurons to generate bursts of action potentials in response to intracellularly applied depolarizing current. Exposure to low-pH medium reversibly attenuates burst-firing behavior and depresses evoked field potentials (MacVicar et al., 1985).

Dorsal medulla oblongata neurons have been shown to be dye-coupled (Dean et al., 1997). Cell-cell coupling between CO_2 excited-neurons, in the dorsal medullary region, is hypothesized to function in central chemoreception for the cardiorespiratory control systems (Huang et al., 1997). Huang et al. (1997) have provided indirect evidence for the gap junctions in 20% of the neurons in the solitary complex and 10% of neurons in adjoining dorsal medullary nuclei. Gap junctional coupling may attribute to the pH sensitivity of the CNS and may modulate neuronal excitability in the dorsal chemosensitive area of the medulla. It has already been established that the peripheral chemoreceptors, the chemosensitive glomus cells of the carotid body, are electrotonically coupled (Abudara et al., 1994; Monti-Bloch et al., 1993). Huang et al. (1997) have found a high correlation between anatomical coupling, electrotonic coupling activity and CO_2-induced depolarization suggesting that cell-cell coupling is an important electroanatomical feature in CO_2-excited neurons of the solitary complex.

In the vertebrate retina, virtually all cell types are coupled by gap junctions (Vaney, 1994). Recent evidence suggests that gap junctions participate in the orderly formation of retinal circuitry and signal flow from photoreceptors to ganglion cells and rewiring of circuitry in different lighting conditions (Bruzzone and Ressot, 1997). Little is known

about the types of connexins expressed in the retina although most cell types, including ganglion cells, bipolar cells, AII amacrine cells, and rods and cones have been shown to exhibit stereotyped patterns of coupling when injected with small biotinylated tracers like neurobiotin. Further, tracer labeling of different cell types show that coupling is more frequent between cells of the same type than of a different cell type (Vaney et al., 1994). However, rod AII amacrine cells are heterologously coupled to cone bipolar cells (Kolb and Famiglietti, 1974). Stimulation of the rod photoreceptors leads to depolarization of the AII amacrine cells, and this response is transmitted through gap junctions (Vaney et al., 1998). The heterologous communication between the two cell types also allows cone cells, which generally use the excitatory neurotransmitter glutamate, to accumulate the inhibitory transmitter glycine by diffusion from the rod AII amacrine cells (Vaney et al., 1998). The findings by Vaney et al. (1998) are important in light of the fact that there is no experimental evidence that neurotransmitter coupling occurs naturally in the nervous system although it could because most amino acid transmitters are small enough to pass through typical neuronal gap junctions. Recently, studies have identified the presence of Cx43 in the catfish retina (Giblin and Christensen, 1997) and Cx35 in the skate retina (O'Brian et al., 1996) but it is not clear which cells types might express these connexins. Connexin 50 has been found, by Schütte et al. (1998), to be expressed in the macroglia, specifically astrocytes and Müller cells, of the retina.

Gonadotropin-releasing hormone neurons (GnRH) (otherwise known as leutenizing hormone-releasing hormone neurons or LHRH) sparsely localized in the hypothalamus control the pulsatile release of GnRH, which is an absolute requirement for induction and maintenance of reproductive function (Knobil, 1980, 1988; Marshall et al., 1986).

Intercellular signaling mechanisms that mediate the periodic release of GnRH are still not entirely understood. Primary GnRH cultures and isolated *in vitro* preparations of mediobasal hypothalamus continue to release in a periodic fashion suggesting that signaling mechanisms underlying this secretion are localized within the hypothalamus or GnRH neurons (Melrose et al., 1987; Krsmsnovic et al., 1992; Wetsel et al., 1992). Connexin 26 has been shown to be expressed in immortalized GnRH-secreting neuronal cell lines (Goldsmith et al., 1993; Mellon et al., 1990; Matesic et al., 1993, 1996). Hosney and Jennes (1998) have also demonstrated connexin 32 protein and mRNA expression in GnRH-secreting neuronal cell lines.

Immunohistochemical evidence shows that cell membrane contacts exist between GnRH neurons in a variety of mammalian species including monkeys, hamsters, and rats (Matesic et al., 1996). GnRH varicosities in the median eminence are often clustered together in direct plasma membrane contact *in vivo* (Lehman et al., 1988). Further, gap junctions have been detected by freeze fracture and transmission electron microscopy in the hypothalamic arcuate nucleus in female rats (Perez et al., 1990). Gap junctional coupling has also been proposed to underlie the pulsatile release of other hypothalamic steroid hormones (Hatton et al., 1987).

Hormonally induced plasticity by sex steroids has been described in motoneurons of the adult male rats and neurons of adult female canaries (Matsumoto, 1997; Gahr and Garcia-Sequra, 1996). In the case of adult male rats, androgens have been reported to induce growth of neuronal somata and dendrites of motoneurons in the spinal nucleus of the bulbocavernosus (SNB) of adult male rats, and an increase in the number and size of chemical synapses onto these motoneurons. The size and frequency of gap junction

plaques are also regulated by androgen (Matsumoto et al., 1988) in the SNB. Matsumoto (1997) has demonstrated that castration of male rats (to remove androgen) significantly reduces the expression level of gap junction mRNA in SNB motoneurons at the somata and proximal dendrites. Testosterone treatment of castrated rats for two days can prevent decline of gap junction message.

A similar observation has been made with singing in female canaries. Gahr and Garcia-Sequra (1996) found that females treated with testosterone developed a male-like song and had an increased number of neuronal soma-somatic gap junctions in the caudal nucleus of the ventral neostriatium (HVC) compared with the untreated singing females. A chain of interconnected brain areas controls singing in canaries; one of these areas is the (HVC) which is sensitive to androgens and estrogens. In the HVC neurons are grouped into tight clusters having tight contacts with other cells, making it possible that they communicate through electrical synapses (i.e. gap junctions). Freeze fracture analysis show that in the HVC, female canaries have an increase in the number of gap junction plaques compared to untreated controls. It is not certain which connexins form the gap junctions or what types of neurons contain them however. Both the results with the rat spinal motoneurons and female canaries demonstrate that gap junctions may be involved in the plasticity of sex steroid sensitive neural circuits.

1.8 - Relationship between Cell-Cell Communication and Cell Function

Since the first discovery that gap junctions were responsible for the fast excitatory transmission in the crayfish giant fiber system (Furshpan and Potter, 1954), a multitude of data has come forth demonstrating a role for cell-cell communication in most processes important for cellular survival. It has been demonstrated that cell-cell

communication controls cell growth and differentiation and is important for maintenance of a mortal phenotype; and many extracellular and intracellular signal transduction pathways can modulate the individual units, connexins, that comprise the gap junction channel.

Specifically in the area of neurobiology studies, examining neural development and differentiation have implicated gap junctions as having a pivotal role in these two processes as well as functioning of the mature nervous system. However, the putative functions that gap junctions are proposed to fill in neuronal cells and other cell types as well are highly correlative relationships at best. It hasn't been until very recently (1995-1999) that investigators have actually tried to form relationships between the changes in gap junctional communication and relate it causally to their proposed function by blocking the cell-cell coupling in various ways.

1.8.a. – Connexin 43 and Neural Crest Cells

Recent studies have implicated Cx43 as important player in cardiac morphogenesis. The first indication of the importance of Cx43 in heart development came from Cx43 knockout mice which yielded a characteristic phenotype exemplified by obstructions in the right ventricular outflow tract and enlarged conus region (Reaume et al., 1995). However, the Cx43 knockout mice did not provide much insight into the role of gap junctions in cardiac development, as there is little Cx43 expressed in the outflow tract (Van Kempen et al., 1991; Gourdie et al., 1992). Development of the outflow tract is known to be dependent on the activity of neural crest cells (Kirby, 1993; Waldo and Kirby, 1995) and neural crest cells are known to express Cx43 (Lo et al., 1997). Therefore, Sullivan et al. (1998) and Huang et al. (1998) investigated the blockage of gap

junctional communication in neural crest cells expressing Cx43 to determine whether there would be an effect on heart morphogenesis.

Sullivan et al. (1998) generated transgenic mice exhibiting dominant negative inhibition of gap junctional communication in neural crest cells. They found that all transgenic mice were homozygote inviable, dying neonatally and exhibiting heart malformations involving the right ventricular outflow tract, similar to the Cx43 knockout mice generated by Reaume et al. (1995). Histological examination showed abnormalities in the differentiation of the conotruncal myocardium. It appears that a precise level of gap junctional communication in cardiac crest cells is important for right ventricular outflow tract morphogenesis (Sullivan et al., 1998).

Huang et al. (1998) took the analysis of gap junctional communication in neural crest cells one step further and examined several different model systems, including Sullivan's dominant negative mice, to determine the underlying mechanism involved in the cardiac malformations. Huang et al. (1998) used a neural crest outgrowth culture system to examine migration of neural crest cells derived from CMV43 transgenic embryos overexpressing Cx43, Cx43 knockout mice, and the dominant-negative Cx43 expressing transgeneic mice. These studies showed that overexpression of Cx43 in the CMV43 embryos caused an increased migration rate of cardiac crest cells, while a decreased migration rate was seen in the dominant-negative Cx43 crest cells and the Cx43 knockout crest cells. Blocking gap junction communication in non-transgeneic cardiac crest outgrowth cultures, with oleamide, also resulted in a reduction in the rate of crest migration. Huang et al. (1998) examined neural crest migration *in vivo* using the three transgeneic model systems discussed above. Using a lacZ transgene to visualize the

distribution of cardiac neural crest cells in the outflow tract, Huang et al. (1998) found a reduction in lacZ- positive cells in the outflow septum in the Cx43 knockout mice, but an increase in lacZ- positive cells in the CMV43 embryos overexpressing Cx43. There were also changes in cell proliferation in the myocardium-an elevation in the CMV43 mice vs. a reduction in the knockout mice. These findings suggest that gap junction communication mediated by Cx43 plays an important role in cardiac neural crest migration and may modulate the growth and development of non-neural crest-derived tissues (Huang et al., 1998).

1.8.b. – Blockade of Dye-Coupling in Neural Cell Lines Induced to Differentiation

A number of other reports have described the use of glycyrrhetinic acid derivatives to block cell-cell coupling and determine whether there is an effect on cellular differentiation *in vitro,* as well as examining neuronal and glial cells from Cx43 knockout mouse embryos. Results appear to depend on the cell type which connexins are expressed in the cell or tissue and whether the experiment is performed *in vivo* or *in vitro.* Bani-Yaghoub et al. (1999a, b) and Le and Musil (1998) have both used carbenoxolone and 18-β-glycyrrhetinic acid to block cell-cell communication during induced differentiation in neuronal cell cultures and lens cell cultures respectively.

Mouse P19 embryonal carcinoma cells and human Ntera 2/Clone D1 cells offer two well-characterized cell culture systems where treatment with retinoic acid induces differentiation into neuronal cell types (Rudanicki and McBurney, 1987; Andrews, 1984). Bani-Yaghoub et al. (1999b) used the gap junction blocking agent, carbenoxolone (CBX), a synthetic form of glycyrrhetinic acid, to inhibit cell-cell communication in P19 cell aggregates exposed to retinoic acid (RA). Undifferentiated P19 cells were previously

shown to express both Cx43 and Cx26 (Belliveau et al., 1997). Upon differentiation Cx26 is found to co-localize with microtubule-associated protein 2 (MAP2), a neuronal marker, while Cx43 is co-localized with glial fibrillary acidic protein (GFAP), an astrocyte marker (Belliveau et al., 1997). Bani-Yaghoub et al. (1999b) found that treatment of P19 cell aggregates with RA and carbenoxolone resulted in fewer neurons, about 10% total, compared to aggregates treated with RA alone which contained 45% neurons, as determined by counting cells that stained positive for MAP2. Similar results were obtained with cells that differentiated into astrocytes. Cells treated with RA alone showed high levels of immunoreactivity for GFAP whereas those treated with RA + carbenoxolone differentiated into fewer GFAP positive cells (Bani-Yaghoub et al., 1999b). Although cultures containing the carbenoxolone gap junction blocker were treated during the entire course of the experiment some cells still differentiated into neurons and astrocytes. It could be that the role of gap junctions in differentiation might be to promote and maintain the differentiated state but not initiate differentiation. Therefore, the neurons and astrocytes formed in carbenoxolone-treated cultures may be the cells that had the capacity to initiate their own differentiation but were unable to promote the differentiation of adjacent cells (Bani-Yaghoub et al., 1999b).

Bani-Yaghoub et al. (1999a) also found a similar effect of CBX on Ntera2/Clone D1 cells a human teratocarcinoma cell line that can differentiate into neurons when exposed to the appropriate concentration of RA. Ntera2/Clone D1 cells initially express Cx43, however, after treatment with RA there is a dose dependent decrease in Cx43 as differentiation progresses to a neuronal phenotype characterized by expression of MAP2 and a reduction in expression of cytokeratin, vimentin, and nestin (Bani-Yaghoub et al.,

1999a). Blocking the initial cell-cell communication in the Ntera2/Clone D1 cells resulted in a reduction in the number of MAP2 positive neurons to less than 7% of that of control cultures (Bani-Yaghoub et al., 1999a). Together the results obtained with the P19 cells and Ntera2/Clone D1 cells show that cell-cell communication is important for neuronal differentiation *in vitro*.

However, *in vivo* Perez-Velazquez et al. (1996) found that brain slice cultures from Cx43 knockout mouse fetuses contained morphologically and electrophysiologically normal astrocytes and neurons. Contrary to the results with the P19 and NT2/D1 cells there was no decrease in the number of neurons or astrocytes from the Cx43-null fetuses. The only difference between the Cx43-null and Cx43-normal fetuses was in the localization of astrocytes, suggesting that Cx43 may regulate astrocyte migration but not differentiation.

1.8.c. – Blockade of Dye-Coupling in the Lens

Like Perez-Velazquez et al. (1996), Le and Musil (1998) obtained very different results from the studies done by Bani-Yaghoub et al. (1999a, b). Le and Musil (1998) investigated the blockade of gap junctional communication in embryonic chick lens cell cultures using 18-β-glycyrrhetinic acid to determine whether there would be an affect on the differentiation of secondary fibers. In the mature lens there are two cell types, a monolayer of epithelial cells and a core of elongated, crystallin-rich fiber cells (Le and Musil, 1998). These cell layers are functionally linked together by gap junctions. The epithelial cells express Cx43 while fibers cells express high levels of Cx45.6 and Cx56 (Rup et al., 1993; Jiang et al., 1994). Le and Musil (1998) found that 18-β-glycyrrhetinic acid greatly reduced gap junction-mediated intercellular transfer of dye between

embryonic cells, but did not affect the differentiation of these cells into MP28-expressing secondary fibers. They concluded that the high level of gap junctional communication characteristic of the lens equator *in vivo* is not required for secondary fiber formation but is a consequence of lens fiber differentiation that may play a role in lens physiology (Le and Musil, 1998).

1.9 - Experimental Design

1.9.a. Cell Model

The question pertaining to the importance of gap junctional communication in the process of cellular differentiation and development is highly debatable. Recent results trying to link cell-cell communication with differentiation in some cases suggests a causal relationship (Bani-Yaghoub et al. 1999a, b), while other experiments suggest no relationship at all (Le and Musil 1998; Perez-Velazquez et al. 1996). I wanted to directly test whether there was a causal relationship between cell-cell communication and differentiation in a human neuronal system, which thus far has not been done. However, due to ethical concerns and technical difficulty in maintaining primary human cells in culture, *in vitro* investigations with the human progenitor cell systems have been difficult for many investigators. As an alternative to primary human tissue neuronal cell lines, such as PC12 (Greene et al., 1976), NT2/D1 (Andrews, 1984), and P19 (McBurney et al., 1982) have been used to investigate the effect of various mechanisms and factors on neuronal differentiation and growth, including cell-cell communication as discussed in the introduction. However, all of these cell lines are tumorigenic being derived from human teratocarcinoma, mouse embryonic carcinoma, and rat pheochromocytoma respectively. I therefore obtained a human fetal immortalized cell line thought to be a

presumptive progenitor cell of the CNS (provided by Dr. E. O. Major, NINDS, NIH, Bethesda, MD). The SVG cell line appears to be a candidate model system for *in vitro* investigation of gap junctional communication and its role in differentiation for the following reasons: (1) the SVG cells were isolated from human fetal tissue making our findings relevant to human development and differentiation (2) the SVG cells are transfected with the SV40 large T antigen, but not isolated from tumor tissue and do not form tumors in nude mice (personal communication, Dr. E. O. Major, Laboratory of Molecular Medicine and Neuroscience, NINDS, NIH, 36 Convent Drive, Bethesda, MD 20892-41643) (3) morphologically the SVG cells do not appear differentiated.

The SVG line was obtained by dissection of human fetal brain material from a 12 – week old abortus (Major et al., 1985). The cells were characterized as astroglial based on their weak reactivity with a monoclonal antibody to glial fibrillary acid protein (GFAP) and lack of activity to anti-galactocerebroside, anti-neuron specific enolase, and neurofilament (200, 140, and 68 kDa subunits all tested) (Major et al., 1985 and Tornatore et al., 1996). The primary cultures of human fetal brain cells were transformed with plasmid DNA pM146, containing an origin-defective mutant of simian virus 40 (SV40) (Major et al., 1985).

The SVG cells were originally isolated and established to be used as a non-tumorigenic vehicle for investigation of viral replication in the brain and transplantation experiments involving replacement of tyrosine hydroxylase activity to lesioned areas of dopaminergic neurons (Major et al., 1985; Tornatore et al., 1996).

1.9.b. Hypothesis

With these observations in mind, the purpose of this Dissertation is to test the hypothesis that gap junctional communication is a causal element in controlling and maintaining cellular differentiation in the SVG cell line. Preliminary evidence using the adenylyl cyclase inducing agent forskolin along with the phosphodiesterase inhibitor, IBMX, showed the SVG cells could at least morphologically appear differentiated by extending neurite-like processes after 10 hours treatment in serum-free medium (Figure 1.3 c-d) correspondingly there was also an increase in dye transfer between cells after treatment (Figure 1.3 a-b).

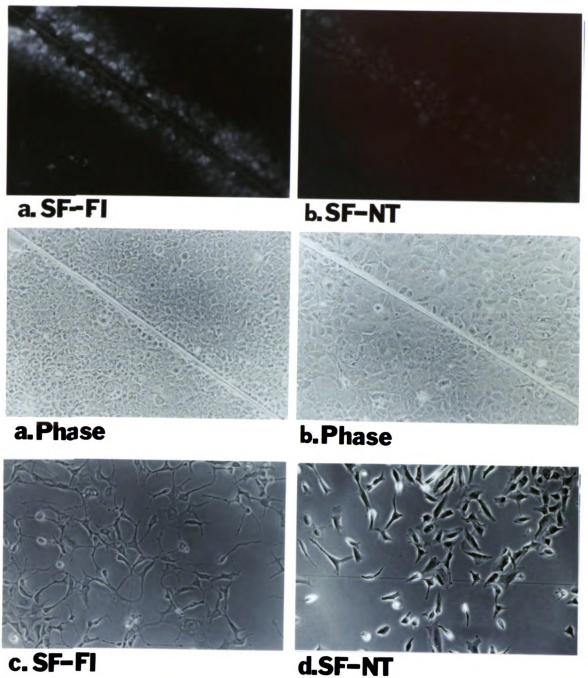


Figure 1.3. Preliminary studies revealed a correlation between dye transfer and morphological differentiation. (a) Scrape load/dye transfer (SL/DT) of cells cultured in serum-free medium containing 5 μM forskolin + 200 μM IBMX for 24 hr (SF-FI). (b) SL/DT of cells cultured in serum-free medium without treatment (SF-NT). (c) Morphology of cells treated with 5 μM forskolin + 200 μM IBMX for 24 hrs compared to untreated controls (d).

I therefore, generated three broad specific aims to test the hypothesis that gap junctions may be causative agents in controlling and maintaining the observed differentiation of SVG cells after treatment with cAMP-inducers.

Hypothesis: Gap junctional intercellular communication is a causative element in controlling and maintaining differentiation in the SVG cell line after treatment with cAMP-inducers.

Objectives:

(1) Characterize the SVG cell line as to its expression of connexins and ability to communicate through gap junction channels

Specific Aims:

i. Functional Cell-Cell coupling

ii. Connexin protein and plaque formation

iii. Connexin mRNA

(2) Characterize the differentiation in this cell line using several criteria as follows

Specific Aims:

i. Morphological change

ii. Expression of protein markers specific for the various cell types of the CNS (Neurons, astrocytes, oligodendrocytes)

iii. Reversibility of the morphological and biochemical changes

iv. Change in growth rate

(3) Utilize Serum and Different Plating Densities to Determine Whether Cell-Cell Communication and Differentiation are Related Causally

Specific Aims:

i. Assess cell communication

i. Changes in morphology

ii. Expression of neuronal-type protein markers

1.10. – Experimental Design to Accomplish Specific Aims

1.10.a. – Conditions to Induce Differentiation and Cell Communication

To better assess the differentiation pathway of the SVG cells they will be grown in defined medium without serum. Placing cells in serum-free medium will allow synchronization of the exponentially-growing cell population, to yield a more homogeneous population available to differentiate. Rudkin et al. (1989) performed similar experiments in PC12 cells exposed to NGF. They found that addition of NGF to serum-starved cells resulted in an accumulation of cells in G1, and NGF action on this population was more robust than on an exponentially-growing culture.

Furthermore, the SVG cells appear to be a candidate progenitor cell population. If this is true then they should behave similarly to embryonic neural progenitors that have been isolated from human and rat brain. Neural progenitors can be induced to proliferate using mitogens like EGF and FGF (Reynolds et al., 1996; Craig et al., 1996; Kuhn et al., 1997; Gensburger 1987; Ray et al., 1993; Gritti et al., 1996; Kilpatrick et al., 1993). Removal of the mitogenic stimulation and plating in serum-free medium with neurotrophic factors induces differentiation (Gage 1998; Shetty et al., 1998; Arsenijeniz et al., 1998).
The same concept is going to be tested with the SVG cells. Mitogenic stimulation by culturing these cells in serum should allow cell proliferation while removal of the SVG cells to serum-free medium with cAMP-inducers or neurotrophic factors should allow differentiation. The increase in cell-cell communication that has been observed to occur

with the morphological differentiation will be tested to determine if it is a necessary factor for the differentiation.

1.10.b. – Measurement of Cell-Cell Communication and Connexin Expression

To determine whether the SVG cells have functional cell-cell communication through gap junctions, two techniques will be employed that use dye-transfer between cells as a measure of gap junction function. These two techniques are Scrape Load / Dye Transfer (SL/DT) and fluorescent recovery after photobleaching (gap-FRAP).

SL/DT is a quick method that can be used to qualitatively measure the ability of cell populations to transfer dye. This technique involves wounding of the cell membrane. Membrane wounding will allow a membrane impermeant dye (Lucifer Yellow) to be taken up by the wounded cell. If the cells have functional gap junctions then dye should transfer from the wounded cell to a subset of other cells around it. This technique requires that cells form a monolayer so that most cells are in cell-cell contact.

Gap-FRAP is a more precise method to examine cell-cell communication in individual cells. This technique utilizes a membrane permeant dye called 5, 6-carboxy fluorescine diacetate (CFDA). Once the 5,6- CFDA has moved inside the cell it is cleaved by phosphodiesterases within the cell which convert the dye to its membrane impermeant form. A laser is then used to photobleach the dye in individual cells. If the cells are dye-coupled by gap junctions then dye will transfer from a neighboring cell that has not been photobleached. This technique does not require a cell monolayer although cells need to be touching at least two or more neighboring cells.

Connexin expression will be examined using Western analysis to observe the type of connexin protein subtypes expressed in SVG cell extracts. The location of gap junction

plaques formed by the connexins is important because it is necessary that they be localized to the cell membrane to have functional gap junctions The locality of gap junction channel formation will be assessed by immunofluorescent techniques using antibodies specific for the different connexin subtypes.

1.10.c. – Criteria for Differentiation

Preliminary studies using phase / contrast microscopy demonstrated that the SVG cells could alter their morphology after treatment with forskolin + IBMX. However, a morphological change may or may not indicate cellular differentiation, by which I mean, cells morphologically look differentiated but also express some genes or proteins that are specific for the cell type with which they can be morphologically identified. In some cases altering the calcium concentration *in vitro* can alter cellular morphology without biochemical or molecular changes in genes specific for that cell type, which would not be considered differentiation. With regards to the nervous system there is a great deal of plasticity and morphological changes can occur under different conditions without changes in cell-type specific antigens Furthermore, if there is a morphological change and changes in cellular genes and protein then it may be reversible or irreversible.

Several criteria have been established to determine whether the SVG cells are truly differentiating. These include examining cells for repeatable morphological changes as well as expression of proteins specific to the different cells found in the CNS, reversibility of these morphological and protein changes, and a decrease in their growth rate.

Examination of a number of protein markers for specific cell types in the CNS will be accomplished using immunofluorescent techniques. In the literature, it is accepted that

identification of differentiated neuronal cell types can be accomplished using antibodies for specific antigens expressed by the differentiated cells (Gage 1998). Glial fibrillary acidic protein (GFAP) is found in astrocytes while cells expressing neurofilaments, neuron specific enolase or β-III-tubulin are considered neurons (Weiss et al., 1996; Kukekov et al., 1997; Reynolds et al., 1996; Kalyani et al., 1997). Oligodendrocytes can be identified by expression of galactocerebroside or myelin basic protein (MBP) (Williams et al., 1991). I have utilized these markers to identify biochemical differentiation in the SVG cells.

Reversibility of the morphological change will be assessed by treating the cells for an extended time period with forskolin + IBMX. The treatment will be removed and the morphological change as well as the expression of protein markers will be evaluated.

Lastly examination of the proliferation rate of the SVG cells will be accomplished using uptake of [H^3]thymidine and cell counting before and after treatment to determine whether treatments are affecting the growth rate of the SVG cells. Typically it has been shown that cells undergoing constant renewal, like stem cells or embryonic progenitor cells, will not differentiate until they decrease their growth rate. For example, embryonic neural progenitors as described previously will continue to proliferate when exposed to growth factors like FGF or EGF. When mitogenic stimulation is removed they differentiate into neurons and glia. This concept is being applied to the SVG cells. If SVG cells are grown in serum containing medium they will continue to proliferate, when they are transferred to the serum-free environment and exposed to the forskolin + IBMX they should differentiate. If the SVG cells are differentiating they should have a decreased growth rate as well.

1.10.c. – Procedures to identify a Relationship between Differentiation and Cell-Cell Communication

To determine whether the differentiation and cell-cell communication are interconnected by a causal relationship blockade of gap junctional communication was going to be accomplished using the established gap junction inhibitors *o,p*-DDT (Ruch et al., 1994), 18-β glycyrrhetinic acid (Goldberg et al., 1996; Davidson et al., 1988), and antisense oligonucleotides (Ruch et al., 1995) to specific connexin subtypes. However, preliminary experiments using all compounds proved ineffective at blocking cell communication (as measured by dye transfer) in the SVG cells after treatment with the cAMP-inducers. The morphological change was not altered either.

There are at least two main reasons for the inability to inhibit dye transfer in the SVG cells. The first reason being that the SVG cells were found to express more than one connexin subtype. The various connexin subtypes are regulated in the cell differently and not all compounds will block cell-cell communication mediated by various connexins in the same way. For example, *o,p*-DDT has been shown to inhibit Cx43 mediated gap junctional communication by removing phosphorylated forms of Cx43 (predominantly the P2 form) from the membrane, but it is not clear if it also affects Cx32 or Cx26 (Ruch et al., 1994). Connexins 32 and 26 do not require phosphorylation to form functional gap junction channels. The second reason is the need to induce cell-cell communication with cAMP-inducers for at least 16-24 hours. Treatment with cAMP-inducers might upregulate the cell-cell communication to such an extent that blockage is not possible. Ruch et al. (1995) found that antisense oligonucleotides do not inhibit intercellular communication if the cells are well coupled by gap junctions. Due to the inability of *o,p*-DDT, 18-β glycyrrhetinic acid, and antisense oligonucleotides to fully block dye-transfer

in the SVG cells, this method was not pursued and the objective was investigated using a different approach.

Going back to the concept that progenitor cells will not differentiate in serum lead to the observation that when the forskolin + IBMX treatment is added to medium containing serum, the morphological change is inhibited. To accomplish this objective, cell culturing techniques using serum and different cellular plating densities were utilized to investigate the relationship between cell-cell communication and differentiation.

With these specific aims in mind, I have studied the expression of the gap junction proteins Cx43, Cx32, and Cx26 and intercellular communication in the SVG cells along with the ability of the cells to differentiate under specified conditions. The expression of connexins, the level of intercellular communication, and the ability of the cells to differentiate have not been previously documented or characterized in the SVG cells.

CHAPTER 2 - EXPERIMENTAL PROCEDURES

2.1 - Cell Culture and Treatment Protocol

The SVG cell line was cultured and maintained as described by Major et al. 1985. Briefly, SVG cells were cultured in Eagles Minimum Essential Medium (EMEM) containing Earles salts and 1% L-glutamine (Gibco-BRL) supplemented with 10% fetal serum bovine (FBS) (Gibco-BRL).

In order to induce dye coupling in the SVG cells, they were removed from 75 cm^2 flasks by treatment with 0.25% trypsin/1.0 mM EDTA. The cells were then plated in 35 mm culture dishes or multiwell glass chamber slides and allowed to reach the desired confluency in EMEM 10% FBS. The EMEM was then poured off, and cells were re-fed with serum-free neurobasal medium (Gibco-BRL) supplemented with 100 μg/ml transferrin, 100 μM putrescine, 30 nM sodium selenium, 20 nM progesterone (Bottenstein et al., 1979) and the treatment of interest. In some instances cells were re-fed with EMEM containing 5% or 10% FBS along with the forskolin/IBMX combination to compare the effects of serum and the different media on cell morphology. Treatments were first diluted in the culture medium before addition to cells to ensure equal distribution of the compounds. SVG cells were treated with 5 μM forskolin or 5 μM forskolin with the addition of 200 μM IBMX for 24-72 hr at which time morphological changes, as well as increased dye-coupling, were observed. The WB rat liver epithelial cell line, used in some instances as a positive control, was cultured and maintained as described previously (Matesic et al., 1994).

2.2 - Immunofluorescent Staining

Antibodies to Cx43, Cx32, Cx26 or to cell-specific markers were used in single labeling experiments. The mouse monoclonal Cx43 antibody (Transduction Labs) was used at a dilution of 1:200. The mouse monoclonal Cx32 antibody (Chemicon) was used at a dilution of 1:200. The rabbit polyclonal Cx26 antibody (Chemicon) was used at a dilution of 1:100. Antibodies to the monocolonal mouse anti-GFAP (used at 1:400) and mouse-anti-neuron specific enolase (1:400) were obtained from Chemicon. Polyclonal rabbit-anti-neural filament 200 (1:400) and rabbit-anti-galactocerebroside (1:400) were purchased from Sigma, and polyclonal rabbit-anti-nerve growth factor was obtained from Santa Cruz Biotechnology. Anti-Myelin basic protein was purchased from Amersham and used at a final dilution of 1:200.

Cells were grown in multiwell chamber slides until they reached the desired confluency. Cells were then gently rinsed three times with PBS and fixed with 1% formalin (NF-200, GalC, Cx32, Cx26), 5% Acetic Acid/100% Methanol (Cx43, GFAP), or 100% ethanol (myelin basic protein) for about 20 minutes. Permeablization of formalin fixed samples was accomplished with 100% cold methanol for 10 minutes.

Cells were rinsed three times with PBS and incubated in blocking buffer (PBS, 10%, Goat Serum, and 0.1% Tween 20 or Super Block, SyTeck) for two hours. Primary antibody was added to cells in PBS containing 1% bovine serum albumin (BSA) and incubated for one hour-overnight. Cyanine2-conjugated goat-antirabbit or goat-antimouse (Jackson Immunoresearch Laboratories) secondary antibodies were used to detect the primary antibodies at 1:1000 dilution. The cells were rinsed three times with PBS for 5min each and incubated with the secondary antibody in 1% BSA for 45 minutes – 1

hour. The cells were again rinsed three times with PBS for 5 min each, then one time with distilled water to prevent crystal formation. Propidium iodide was used in some instances to stain nuclei of cells. Negative controls were prepared by eliminating the addition of primary antibody. The chambers were removed and 1-2 drops of aqueous mounting medium (aqua polymount, Polysciences) was applied to the slide. A coverslip was placed over the mounting medium and the edges sealed with nail polish. Cells were analyzed for fluorescent emission by confocal microscopy with an Ultima (Genomic Solutions, Inc., Ann Arbor, MI USA) or Nikon Eclipse T300 fluorescent microscope (Meager Scientific).

2.3 - Western Analysis

Proteins were isolated in 20% SDS containing 2 mM PMSF and sonicated for 3, 30 second pulses, at 35% maximal power to shear the DNA. The sample protein concentration was measured using the BioRad DC colorimetric protein assay (Bio-Rad) with horse γ-globulin as the standard. Protein samples were solubilized in Laemmli sample buffer (Laemmli 1970). Rat brain lysate was obtained from Transduction Laboratories and used as a positive control for connexin 43 protein expression on western blots. Briefly the rat brain lysate was prepared by lysis and heat denaturation of rat brain in 1% SDS, 1.0 mM sodium vanadate and 10 mM Tris (pH 7.4).

Samples containing 20-25μg protein were resolved on 12.5% SDS-polyacrylamide gels at 200 mV for one hour. A monoclonal Cx43 antibody (Transduction Labs) was used for Western blotting at a dilution of 1:1000. Biotinylated molecular-weight markers from New England Biolabs were used to establish the size of the resultant bands. A monoclonal Cx32 antibody (Chemicon) was used for Western blotting at a dilution of

1:1000. As a positive control for Cx32 protein a mouse hepatocyte cell line designated C3HMLE was used that is known to express Cx32. A polyclonal Cx26 antibody (Chemicon) was used for Western blotting at a concentration of 3μg/ml. Detection of connexin protein was accomplished using horseradish peroxidase (HRP) conjugated antirabbit or HRP-antimouse secondary antibody at a dilution of 1:2000 for 1 hour at room temperature. New England Biolabs lumiglow was used to detect the HRP-linked antibody after incubation for 1 minute at room temperature. Membranes were then exposed to X-ray film to visualize protein bands.

2.4 - Alkaline Phosphatase Treatment

Treatment of proteins with alkaline phosphatase was performed to determine whether the cAMP-inducing compounds were causing phosphorylation of Cx43. Twenty micrograms of protein was diluted fourfold in alkaline phosphatase buffer (50 mM Tris-HCl, 8 mM MgCl, 0.1% β-Metcaptoethanol, 2 mM PMSF). Ten units of calf intestine alkaline phosphatase (15,000U/100μl, Boheringer Mannheim) were added to each sample and samples were incubated at 37^0C for 2 hours. The reaction was quenched with Laemmli sample buffer and proteins were separated on 12.5% acrylamide gels.

2.5 - Measurement of Gap Junctional Intercellular Communication

2.5.a. – Scrape Load/Dye Transfer

Cells are grown in 35mm dishes and allowed to reach 70-90% confluency. Cells will be washed with 2ml of Ca^{+2}/Mg^{+2} PBS then twice with PBS. Lucifer Yellow is added to the dish (1.5ml of 0.5mg/ml) and five to eight scrape lines are made in the dish with a razor blade. The cells are incubated in lucifer yellow for 3 min before washing 2-3 times with PBS and once with Ca^{+2}/Mg^{+2} PBS. The cells are fixed with 1.5 ml of 4%

formaldehyde in PBS for 15 min. Cells are photographed in UV phase to observe dye-transfer. Triplicate samples are run per experiment.

2.5.b. – Fluorescent Recovery after Photobleaching (Gap-FRAP)

Gap junction-mediated intercellular communication was assayed by the gap-FRAP method (Wade et al., 1984) using an Ultima scanning confocal microscope. Treated and untreated cells were grown to 60% confluence on 35 mm plastic dishes, were washed twice with PBS containing 1.25mM $CaCl_2$, 0.5mM $MgCl_2$, and incubated for 15 min with 5,6,Carboxyfluorescien diacetate (7μg/ml in Ca^{2+}/Mg^{2+}-PBS). For blockage of cell-cell communication, 0.7 mM octanol was added during the 15 min incubation with 5,6,-Carboxyfluorescien diacetate and while the assay was taking place (16 min) for a total time frame of approximately 31 minutes. Excess extracellular dye was washed away and 2 ml of Ca^{2+}/Mg^{2+}-PBS added back to cells. Cells that appeared to make contact with at least two other cells were selected under a microscope with a 10 X objective lens. Individual cells were photobleached with a 488-nm beam to 10-30% of their original fluorescence intensity, and recovery of fluorescence intensity monitored at 4-min intervals. The values obtained (% recovery at 16min) were corrected for fluorescence lost in unbleached control cells. Dye coupled cells were defined as those showing greater than 10% recovery per 16 min scan (Wade et al., 1986).

Quadruplicate dishes were run per treatment group and the mean (+/- SD) percent cells dye coupled was calculated. For each dish 16-20 cells were examined for dye-coupling. Data represent a combination of 8-12 independent experiments (n=8-12). Statistical analysis using the Kruskal-Wallace Test followed by Dunn's post-hoc test was used to compare control to treatment groups and perform all pairwise comparisons.

2.6 - RNA Preparation

Total RNA was isolated from treated and untreated SVG cell cultures using the Boehringer Mannheim High Pure RNA Isolation Kit. Cultures were treated for varying time points in 12 well plates and RNA was extracted as follows. Cells were rinsed with PBS and resuspended in 200 µl PBS, lysed with buffer containing 4.5 M guanidin hydrochloride, 50 mM Tris-HCl and 30% Triton® X-100, pH 6.6 and placed in a filter with collection tube and centrifuged for 15 sec at 8,000 X g. The flow through was discarded and DNase buffer (1M NaCl, 20 mM Tris-HCl, 10 mM $MnCl_2$, pH 7.0) and DNase I (10 KU) were added to the filter tube for a 15 min incubation. RNA was washed and centrifuged for 15 sec at 8,000 X g using buffer containing ethanol, 5 M guanidine hydrochloride, and 20 mM Tris-HCl, pH 6.6. RNA was washed a second time in a second buffer containing ethanol, 20 mM NaCl and 2 mM Tris-HCl, pH 7.5 and centrifuged as before; this step was repeated a second time except centrifugation was at 13,000 X g. The filter tube was placed over a 1.5 ml sterile microcentrifuge tube to collect the extracted RNA. RNA was then eluted with nuclease-free sterile bidest·H_2O and centrifuged for 1 min at 8,000 X g. RNA was measured using a Beckman Spectrophotometer at 280 and 260 nanometers.

The purity of the RNA was examined by electrophoresis of the RNA on a formaldehyde gel. A 1.2% agarose gel was prepared by dissolving 1.2 g of agarose in 100 ml of 1 X MOPS, 5.1 ml of formaldehyde was then added from a 37% stock solution. To 5.5 µl of RNA sample 1 µl 10 X MOPS, 3.5 µl formaldehyde, and 10 µl formamide was added to a final volume of 20 microliters. Before loading samples into the gel Ethidium bromide (1mg/ml) was added to each sample as well as 2 µl of formaldehyde

gel loading buffer (stock solution = 1mM EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol FF, 5ml glycerol, 4.93ml water). Samples were vortexed to mix, heated for 15 min at 65 ^{0}C, and placed on ice. The gel was covered with 1 X MOPS and samples were loaded into the wells. The gel was run for 2½ hrs at 100 V.

2.7 – RT-PCR

2.7.a. – Two Step RT-PCR

Two step RT-PCR was carried out as follows. The Clontech Laboratories Advantage™ RT-for-PCR kit was used to synthesize cDNA. In a sterile 0.5 ml microcentrifuge tube, 1μg of total RNA was added to DEPC-treated water for a total volume of 12.5μl. Oligo$(dT)_{18}$ (1μl) was added to the RNA.

The RNA was heated at 70 ^{0}C for 2 min then placed rapidly on ice. On ice, 4μl of 5 X buffer (50 mM Tris-HCl, pH8.3; 75 mM KCl; 3mM $MgCl_2$), 10 mM dNTP mix (1μl), 1 unit/μl recombinant RNase inhibitor (0.5μl), and the MMLV reverse transcriptase 200 units/μg_{RNA} was added for a total volume of 20μl. The reaction was incubated at 42 ^{0}C for 1 hour, and heated at 94 ^{0}C for 5 min to stop the cDNA synthesis reaction and destroy DNase activity. The reaction was diluted to a final volume of 100μl and aliquoted.

For PCR $MgCl_2$ (50 mM) was added to 10μl cDNA for a final concentration of 1.5 mM along with 5μl 10 X PCR buffer (200 mM Tris-HCl, pH 8.4; 500 mM KCl), 1μl of 10mM dNTP, and 2.5nM of both forward and reverse connexin 43 primers (Chemicon).

Taq polymerase was added at 250 U/ml. The PCR reaction (50 µl) was run as follows for a total of 35 cycles:

PCR Amplification	Final Extension
94 ^{0}C for 30 sec	72 ^{0}C 2 min
61 ^{0}C for 30 sec	4 ^{0}C hold
72 ^{0}C for 30 sec	

After PCR, amplified products were separated on a 1.5% agarose gel prepared with 1XTBE, for 1 hour using a 123 bp DNA ladder as a molecular weight standard.

2.7.b. – One Step RT-PCR

RT-PCR for neurotrophic factors (BDNF, CTNF, and NGF) was performed using the Gibco Superscript™ One-Step™ RT-PCR kit. To 1µg RNA, 5 mM $MgSO_4$ was added at a 1.5 mM final concentration along with 2 X buffer (0.4 mM each dNTP, 2.4 mM $MgSO_4$), 1µM of both forward and reverse primers (Promega), and Superscript II RT-Taq. Control GADPH primers were also run under the same conditions as a control for the RT-PCR reaction. cDNA synthesis and PCR were run as follows:

cDNA Synthesis	Pre-denaturation	PCR Amplification	Final Extension
42 ^{0}C 30 min	94 ^{0}C 2 min	(40 Cycles)	68 ^{0}C 7 min
		94 ^{0}C 30 sec	4 ^{0}C hold
		65 ^{0}C 1 min	
		68 ^{0}C 2 min	

A 1.5-2% agarose gel was used to separate the amplified products.

2.8 – Measurement of Cell Proliferation

2.8.a. – Measurement of DNA Synthesis by Uptake of [^{3}H]thymidine

Synchronization of SVG cell cultures was achieved by culturing in serum-free medium for 72 hours. After the 72 hr time period, cells were reintroduced to 10% fetal bovine serum and 50,000 cells were plated per well in 12 well plates. Cells were allowed to attach for 5 hrs before treatment with forskolin and IBMX. Cell plates were divided into the following groups and treated for 3 days accordingly:

(1) 10% FBS + 5μM forskolin + 200μM IBMX

(2) 5% FBS + 5μM forskolin + 200μM IBMX

(3) serum-free medium + 5μM forskolin + 200μM IBMX

(4) 10% FBS + 5μM forskolin

(5) 5% FBS + 5μM forskolin

(6) serum-free medium + 5μM forskolin.

(7) Serum-free medium

(8) EMEM 10% FBS

(9) EMEM 5% FBS

Two independent experiments were performed (n=2) with three replicates per treatment. After treatment for 3 days, cells were given fresh medium containing 0.5 μCi/ml [^{3}H]thymidine. Cells were incubated with the [^{3}H]thymidine for 2 hours. Cells were then washed with Ca^{2+}/ Mg^{2+} PBS and then treated with ice-cold 10% trichloroacetic acid for 10 minutes. Cells were washed in 70% ethanol, and then 95% ethanol, and were allowed to air dry. Cells were lysed with NaOH at 37 ^{0}C for 1 hour then 1 N HCl was added to neutralize the base. The cell lysate (1ml) was added to a

scintillation vial along with 5 ml of scintillation cocktail. Samples were read in counts per minute (cpm). Statistical analysis was performed using a one-way analysis of variance followed by Tukeys' post-hoc test to compare treatments (forskolin or forskolin + IBMX) to controls (serum-free, 5% FBS, 10% FBS) and to compare serum-free experiments to those with serum.

2.8.b – Cell Counting

SVG cells from two separate flasks were seeded at 20,000/well in 12-well plastic culture dishes (n=2). Cells were grouped as described for uptake of [^{3}H]thymidine. Briefly, cells were allowed to attach for 16 hours in Eagles minimum essential medium before treatment with 5μM forskolin, 5μM forskolin + 200μM IBMX, serum-free medium, EMEM 5% FBS, or EMEM 10% FBS. Cells were treated from 1-7 days and then trypsinized and counted using a hemocytometer. Cell counts were taken on days two, three, five, and seven. Three replicates were prepared for each treatment. Cellular cytotoxicity was also tested, using trypan blue exclusion, to ensure that the treatments were not causing undue cell death Breifly, cells were incubated with 100 μl of trypan blue dye for 5 min at 37 ^{0}C before cell counts were performed. Cells that took up the trypan blue dye were counted for each treatment.

Statistical analysis was performed using a one-way-ANOVA followed by Tukeys' post-hoc test to compare treatment groups (serum-free, 10%FBS, 5%FBS with treatments) to control groups (serum-free, 10%FBS, 5%FBS without treatments) and serum-free groups with or without treatments to corresponding groups containing serum.

2.9 – Protein Kinase A Assay

A Protein Kinase A (cAMP-Dependent Protein Kinase) Assay System (GibcoBRL Life Technologies) was used to measure protein kinase A (PKA) at different time points after treatment of SVG cells with 5 μM forskolin + 200 μM IBMX. Cells were plated in 100 mm tissue culture grade dishes and allowed to grow into a monolayer. Cells were treated with forskolin + IBMX for 10 sec, 20 sec, 30 sec, and 5 minutes. A ^{32}P/substrate solution was prepared by adding 25 μCi/ml [γ-^{32}P]ATP (3000Ci/mmol stock solution) to 4 X PKA substrate solution (200 μM Kemptide, 400 μM ATP, 40 mM $MgCl_2$, 1mg/ml BSA, 50 mM Tris, pH 7.5) and placed on ice. Cells were extracted by rinsing with PBS and scraped with extraction buffer containing 5 mM EDTA and 50 mM Tris, pH 7.5. Cells were homogenized using glass dounce homogenizer. Cellular debris was removed by centrifuging for 2 minutes. The supernatant was saved and placed on ice. For each cell extract, 4 assay conditions were set up to measure the final percentage of activated PKA present. For example:

F+I, 10 sec	Extract	Diluent	4 X PKA Inhibitor	4 X PKA Activator
1.	10 μl	20μl	0 μl	0 μl
2.	10 μl	10 μl	10 μl	0 μl
3.	10 μl	10 μl	10 μl	10 μl
4.	10 μl	0 μl	10 μl	10 μl

To each 2 ml microcentrifuge tube, 10 μl of cell extract was added. The cAMP 4 X inhibitor solution (4 mM PKI(6-22) amide, 50 mM Tris pH 7.5) was added to cell extract in tubes b and d as shown above. A 4X PKA activator solution (40 μM cAMP, 50 mM Tris pH 7.5) was added to cell extract in tubes c and d as shown in the above table.

Diluent (50 mM Tris ph 7.5) (20µl) was added to tube a while 10 µl was added to tubes b and c. After all assay conditions were set up and solutions added, tubes were incubated at room temperature for 15 min to allow inhibitor to bind. Ten microliters ^{32}P/substrate solution [25 µCi/ml [γ-^{32}P]ATP (3000Ci/mmol stock solution) in a 4 X PKA substrate solution (200 µM Kemptide, 400 µM ATP, 40 mM $MgCl_2$, 1mg/ml BSA, 50 mM Tris, pH 7.5)] was added to each tube and placed at 30 ^0C for 5 minutes. From each tube, 20 µl was removed and spotted onto phosphocellulose discs at 15 second intervals. The phosphocellulose discs were then immersed in 500 ml acid wash (phosphoric acid 1% v/v) for 3 minutes with rocking. This step was repeated. The phosphocellulose discs were then washed twice for 3 min with 500 ml water. Phosphocellulose discs were then placed into scintillation vials, scintillation fluid was added, and peptide-incorporated ^{32}P counted.

2.10 – Measurement of cAMP Concentration

A cyclic AMP immunoassay (R & D Systems) was used for quantitative determination of cyclic AMP in SVG cell culture supernate samples. Cells (35,000/well) were grown to form monolayers in 12 well plates. Cells were then treated for 5 sec – 60 minutes with 1 ml of serum-free medium containing 5 µM forskolin + 200 µM IBMX. Medium was then removed from the cultured cells and pipetted into 2 ml microcentrifuge tubes. All samples were diluted 1:10 using serum-free medium to ensure linearity of the assay. A dilution series was prepared from a 50,000 pmol/ml cAMP standard stock to be used as a set of standards for the assay. Briefly 100 µl of 50,000 pmol/ml standard was added to 900 µl of serum-free medium to produce a 5000 pmol/ml standard. In turn, 100 µl of the 5000 pmol/ml standard was added to 900 µl serum-free medium to produce a

500 pmol/ml standard. Dilutions were continued in this manner until a 0.5 pmol/ml standard was produced. Before the assay all samples and standards were acetylated to allow for measurement of cAMP in the fmol/ml range. To 300 µl of sample, standard, and medium (to be used for the non-specific binding) 15 µl of acetylating reagent (500 µl acetic anhydride, 1ml triethylamine) were added and mixed thoroughly for 2 seconds.

A number of assay conditions were set up as a control to ensure the experiment was working properly. The conditions included measurement of total activity, non-specific binding, maximum binding and substrate blank. To a 96 well microplate coated with goat anti-rabbit polyclonal antibody, 100 µl of standard and sample was added along with 100 µl of serum-free medium to be used as the zero standard or maximum binding (B_0) and 150 µl of serum-free medium to measure non-specific binding. Wells were also reserved for substrate blank and measuring total activity. Next, 50 µl of cAMP conjugate (cAMP conjugated to alkaline phosphatase) and 50 µl of cAMP antibody solution (rabbit polyclonal antibody to cAMP) were added to each well excluding wells reserved for non-specific binding, substrate blank, and total activity. The microplate was then covered and incubated for 2 hours at room temperature on a horizontal orbital microplate shaker. After the 2 hr incubation, all liquid was aspirated from each well and wells were washed with 200 µl, 1 X wash buffer (R & D systems) for a total of three washes. Wash buffer was aspirated following each wash and after the third wash, 5 µl of cAMP conjugate was added to wells reserved for measurement of total activity. Next, 200 µl of pNPP substrate (p-nitrophenyl phosphate) was added to all wells and incubated for 45 min at room temperature on the bench top. Sodium hydroxide (2N) was added to stop the reaction. Optical density was determined using a microplate reader set to 405 nm.

CHAPTER 3 – RESULTS
CELL-CELL COMMUNICATION AND CONNEXIN EXPRESSION

3.1 – Fluorescent Recovery after Photobleaching

Gap junctional intercellular communication measured using gap-FRAP showed that 3% of vehicle-treated (0.1% DMSO) SVG cells were dye coupled (Table 3.1). This level significantly increased to 66% and 57% when cells were treated for 24-48 hr with 5 μM forskolin or 5 μM forskolin + 200 μM IBMX, respectively. The percentage of dye coupling in cells treated with either 5 μM forskolin alone or 5 μM forskolin +200 μM IBMX was not significantly different using a Kruskal-Wallace test followed by Dunn's post-hoc test at $p<0.05$.

Table 3.1. Gap-FRAP analysis of untreated SVG cells versus those treated with 5 μM forskolin or 5 μM forskolin + 200 μM IBMX as indicated.

Treatment	% Cells Dye Coupled
1. Untreated	3% ± 5% (234)
2. Forskolin	66% ± 31% (193)*
3. Forskolin + IBMX	57% ± 9.5% (140)*

*$p<0.05$ vs. control and treated. () Number of cells assayed. Data represent the mean from 8-12 independent experiments. Number of cells assayed per experiment = 20

3.2. – Inhibition of Gap Junction Mediated Cellular Communication

Inhibition of gap junctional communication has been reported using long chain alcohols, like octanol, in glial and neuronal cells (Lee et al., 1994; Matesic et al., 1993). Addition of 0.7 mM octanol for 30 min to SVG cells that had been treated for 24 hr-48 hr with 5 μM forskolin or 5 μM forskolin + 200 μM IBMX significantly reduced the percentage of dye coupled cells assayed by gap-FRAP (Table 3.2). Inhibition of dye-coupling by octanol was reversible when cells were rinsed and refed medium containing 5 μM forskolin or 5 μM forskolin + 200 μM IBMX. Furthermore, cells treated with forskolin, or the forskolin/IBMX combination with the addition of octanol, exhibited dye transfer that was not significantly different from the untreated control cells using a Kruskal-Wallace test followed by Dunn's post-hoc test at $p<0.05$ to compare treatment groups to control (Table 3.2). These results support the concept that the observed upregulation of dye-coupling in SVG cells by treatment with forskolin or forskolin + IBMX involves gap junctions.

Table 3.2. Effect of gap junction inhibitor, octanol, on fluorescent dye transfer in SVG cells, measured by gap-FRAP.

Treatment	% Cells Dye Coupled
1. Control	5% ± 8% (80)
2. Forskolin	66% ± 24.5% (174)
3. Forskolin + IBMX	60% ± 24.7% (202)
4. Forskolin + Octanol	16% ± 21% (94)*
5. Forskolin + IBMX + Octanol	22% ± 23% (105)*

SVG cultures were treated for 24 hr with 5 μM forskolin or 5 μM forskolin + 200 μM IBMX. Octanol (0.7mM) was added for 30min during dye loading and during the duration of the gap-FRAP analysis. *$p<0.05$ vs. treated and treated + octanol. ()Number of cells assayed. Data represent the mean from 4-10 independent experiments. Number of cells assayed per experiment = 20

3.3 – Western Blot Analysis

3.3.a. – Western Blot Analysis of Connexin 43

SVG cell cultures, both treated and untreated, express a protein that reacts with Cx43-specific antibodies on Western blots (Figure 3.1). The connexin 43 immunoreactive protein band from untreated SVG cells (Figure 3.1; NT) migrates as a single weak band at ~ 41 kDa.

Cells treated with forskolin or forskolin + IBMX demonstrate the presence of a triplet of bands characteristic of the phosphorylated forms of connexin 43 (Figure 3.1; F, FI). The Cx43 bands from SVG cells comigrate at the same relative molecular mass as

connexin 43 from rat liver WB-F344 epithelial cells (Figure 3.1; WB) and rat brain (Figure 3.1; RB). Data are representative of at least 4 independent experiments.

3.3.b. – Alkaline Phosphatase Treatment

Treatment with alkaline phosphatase did not alter the migration of connexin 43 immunoreactive bands present in untreated SVG cells (Figure 3.2; NT, NT-AP). The same alkaline phosphatase treatment of connexin 43 protein from cells treated with forskolin, forskolin + IBMX or IBMX converted two protein bands to a single 41 kDa band (Figure 3.2; F-AP, FI-AP, IBMX-AP). Connexin 43 protein extract from rat liver WB-F344 cells was used as a positive control (Figure 3.2; WB). The connexin 43 immunoreactive bands from WB-F344 cells could also be reduced to a single ~ 41 kDa band which comigrated with both the treated and untreated SVG connexin 43 protein exposed to alkaline phosphatase reduction (Figure 3.2; WB-AP). Data are representative of at least 4 independent experiments.

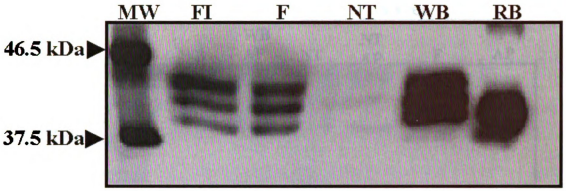


Figure 3.1. Effect of forskolin, IBMX, and forskolin + IBMX on Cx43 protein levels in SVG cells. Western blot analysis, using connexin 43-specific monoclonal antibodies, of total protein extracts isolated from untreated (NT) or cells treated for 24 hr with 5μM forskolin (F) or 5 μM forskolin + 200 μM IBMX (FI). Data represent 4 independent experiments. Total protein loaded per lane = 25 μg. RB = rat brain lysate (positive control), WB = WB F344 rat liver epithelial cell protein (positive control), MW = molecular weight protein markers.

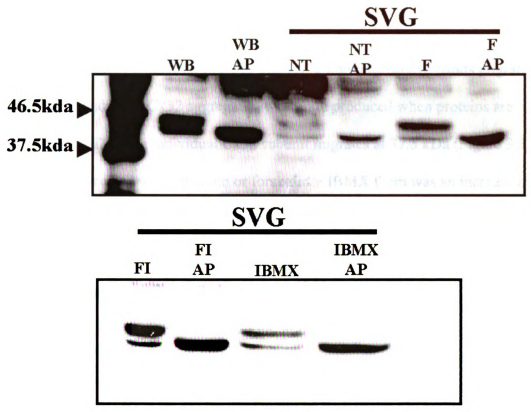


Figure 3.2. Alkaline phosphatase digestion of treated and untreated SVG and WB F344 protein extracts. Western blots incubated with connexin 43-specific antibodies show disappearance of the higher molecular weight protein band (~46kDa) and enhanced immunoreactivity at ~ 41kDa after incubation with alkaline phosphatase (lanes labeled AP) compared to protein extracts without alkaline phosphatase treatment. Note, there is no effect on immunoreactive protein bands present in vehicle treated SVG cell extracts (NT). Data represent at least 4 independent experiments. RB = rat brain lysate (positive control), WB = WB F344 rat liver epithelial cell protein (positive control), MW = molecular weight protein markers, F = 5μM forskolin, FI = 5 μM forskolin + 200 μM IBMX, IBMX = 200μM IBMX

3.3.c. – Western Blot Analysis of Connexin 32

SVG cells, both treated and untreated, express a protein that reacts with Cx32-specific antibodies on Western blots (Figure 3.3). The Cx32 immunoreactive bands migrates at ~ 46 kDa corresponding to Cx32 aggregates, which are produced when proteins are extracted using SDS. The individual Cx32 subunit migrates at 37.5 kDa (Figure 3.3). After cells were treated with forskolin or forskolin + IBMX there was an increase in the amount of the Cx32 protein present (Figure 3.3; F, FI) compared to the control SVG cultures without treatment (Figure 3.3; NT). The mouse hepatocyte cell line designated C3HMLE was used as a positive control, in this instance, and migrates at ~ 27 kDa on a western blot (Figure 3.3; C3HMLE). It is not clear why Cx32, in SVG cell extracts, migrates at a different molecular weight than Cx32 protein extracted from mouse liver hepatocytes (C3HMLE). One explanation might be that, in the SVG cells, there are posttranslational modifications on the Cx32 protein that are not present on the Cx32 protein from the mouse liver hepatocyte cell line (C3HMLE). Data are representative of 4 independent experiments.

3.3.d. - Western Blot Analysis of Connexin 26

SVG cells, both treated and untreated, express a protein that reacts with Cx26-specific antibodies on Western blots (Figure 3.4). The Cx26 bands migrate just below 28 kDa with a weak band also visible at a higher apparent molecular weight. Matesic et al. (1996) also observed a similar doublet of bands when examining Cx26 extracted using 20% SDS in leutenizing hormone-releasing hormone neurons. Matesic et al. (1996) found that this lightly immunoreactive band could not be removed using peptide absorbtion of the Cx26-antibody and is therefore not immunochemically related to Cx26.

As with both the Cx43 and Cx32 protein, there was a noticeable increase in the amount of Cx26 protein after treatment with forskolin or forskolin + IBMX (Figure 3.4; F, FI) compared to control untreated SVG cultures (Figure 3.4; NT). Data are reprasentative of 4 independent experiments.

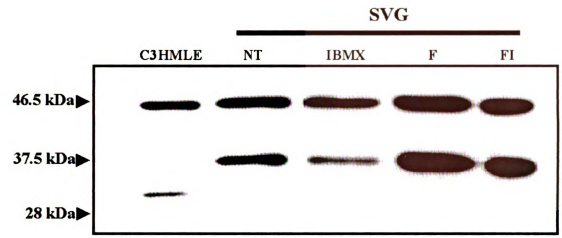


Figure 3.3. Effect of forskolin, IBMX, and forskolin + IBMX on Cx32 protein levels in SVG cells. A. Western blot analysis, using connexin 32-specific monoclonal antibodies, of total protein extracts isolated from untreated (NT) SVG cells or cells treated for 24 hr with 200 μM IBMX (IBMX), 5μM forskolin (F) or 5 μM forskolin + 200 μM IBMX (FI). Total protein loaded per lane = 25 μg. C3HMLE = mouse liver hepatocytes (positive control).

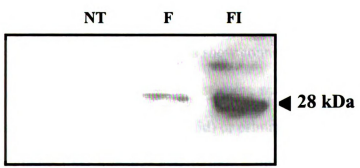


Figure 3.4. Effect of forskolin and forskolin + IBMX on Cx26 protein levels in SVG cells. Western blot analysis, using connexin 26-specific monoclonal antibodies, of total protein extracts isolated from untreated (NT) SVG cells or cells treated for 24 hr with 5μM forskolin (F) or 5 μM forskolin + 200 μM IBMX (FI). Total protein loaded per lane = 25 μg.

3.4 – Immunofluorescent Demonstration of Connexins in Forskolin + IBMX Treated Cells

3.4.a. – Immunofluorescent Demonstration of Connexin 43

Figure 3.5 shows images of immunofluorescently stained untreated SVG cells (Figure 3.5a) or cells treated for 24 hr with forskolin + IBMX (Figure 3.5b) using connexin 43-monoclonal antibodies. A WB rat liver epithelial cell line was used as a positive control for connexin 43 immunofluorescent staining (Figure 3.5c). The untreated SVG cells exhibit few fluorescently labeled connexin 43 punctate plaques compared to cells treated with the forskolin/IBMX combination. Dramatic increases in the number of immunostained cell processes and somas where cell-cell contact was made were observed in the treated cells (Figure 3.5b).

Unlike the WB rat liver cell (Figure 3.5c), which displays a necklace like staining pattern for connexin 43, the treated SVG cells show connexin 43 staining on the neurite-like process and cell body. No punctate Cx43 staining was observed in cells in which peptide competition was employed (Figure 3.5d) or primary antibody was omitted (SVG, Figure 3.5e; WB Figure 3.5f), although cytoplasmic staining can be observed. Data are representative of at least 5-6 independent experiments.

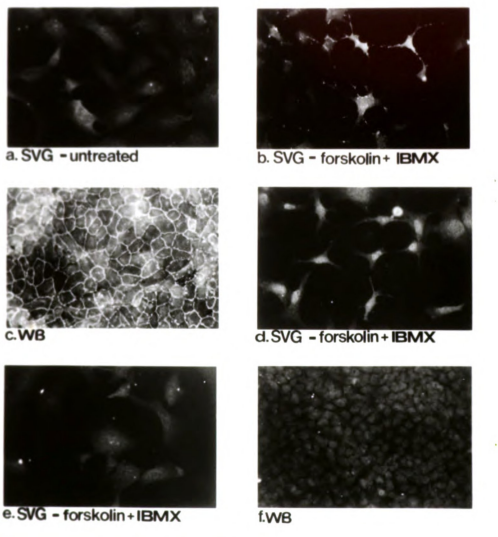


Figure 3.5. Immunofluorescent staining of SVG cells with connexin 43-specific antibodies shows an increased level of punctate staining of cell processes and cell bodies after treatment for 24 hr with 5μM forskolin + 200μM IBMX. Photomicrographs showing untreated SVG cells (a), cells treated for 24 hr with 5 μM forskolin + 200 μM IBMX (b), and WB F344 rat liver cells (positive control) (c). Peptide competition was employed to demonstrate specificity of connexin 43 antibody binding in SVG cells (d). Omission of primary antibody control; SVG (e), WB (f).

3.4.b. – Immunofluorescent Demonstration of Connexin 32

Figure 3.6 shows SVG cells treated with forskolin + IBMX immunofluorescently labeled with Cx32 specific antibodies. Unlike the CX43 staining punctate Cx32 plaques appear to be localized to cell types having few processes extending from their cell bodies (Figure 3.6a). Untreated cells have few punctate plaques but exhibit much cytoplasmic fluorescence for Cx32 (Figure 3.6b). Figure 3.6c is a mouse liver epithelial cell line used as a positive control for Cx32 staining. Data are representative of 4 independent experiments.

3.4.c. - Immunofluorescent Demonstration of Connexin 26

Figure 3.7 shows SVG cells treated with forskolin + IBMX immunofluorescently label with Cx26-specific antibodies. Connexin 26 staining is less punctate than the Cx43 but is distributed throughout the cell in a similar manner with staining at the ends of neurite-like processes and in the cell body (Figure 3.7a). Untreated cells display no punctate connexin 26 staining, although there is cytoplasmic staining, (Figure 3.7b) and appear similar to cells where peptide competition was employed (Figure 3.7c). Data are representative of 4 independent experiments.

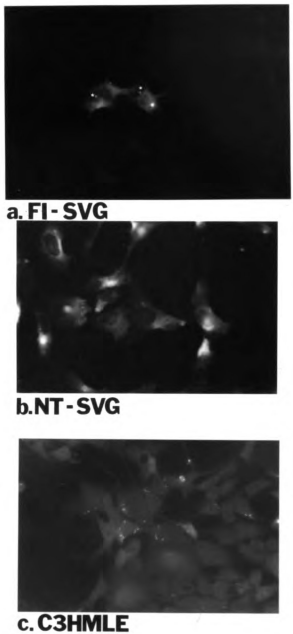
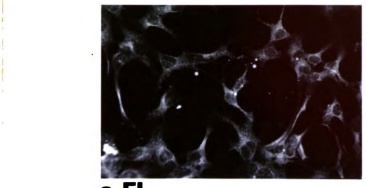
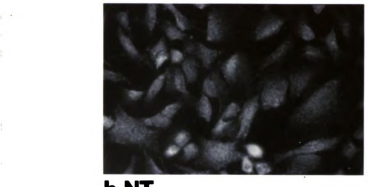


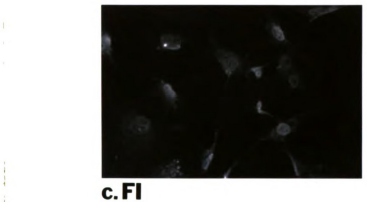
Figure 3.6. Immunofluorescent staining of SVG cells with connexin 32-specific antibodies shows punctate staining of cell processes and cell bodies after treatment for 24 hr with 5μM forskolin + 200μM IBMX. Untreated cells exhibit much cytoplasmic fluorescence for Cx32. Photomicrographs showing cells treated for 24 hr with 5 μM forskolin + 200 μM IBMX (a), untreated SVG cells (b), and C3HMLE mouse liver cells (positive control) (c).



a. FI



b. NT



c. FI

Figure 3.7. Immunofluorescent staining of SVG cells with connexin 26-specific antibodies shows an increased level of punctate staining of cell processes and cell bodies after treatment for 24 hr with 5μM forskolin + 200μM IBMX. Photomicrographs showing cells treated for 24 hr with 5 μM forskolin + 200 μM IBMX (a), untreated SVG cells (b), and cells treated for 24 hr with 5μM forskolin + 200μM IBMX where peptide competition was employed to demonstrate specificity of connexin 26 antibody binding in SVG cells (c).

3.5 - Conclusions

The results from gap-FRAP, western analysis, and immunofluorescent staining clearly demonstrate that the SVG cells have the ability to transfer dye from cell to cell, and express the three main connexin subtypes (Cx43, Cx32, Cx26) found in the central nervous system. Results from gap-FRAP demonstrate that upon treatment with the cAMP-inducers forskolin and forskolin + IBMX there was a significant increase in the number of cells that were dye-coupled. Low expression of the Cx43, Cx32, and Cx26 protein on western blots before treatment with forskolin or forskolin + IBMX, coincides with a very low level of dye coupling between SVG cells. The low level of dye transfer in these cells showed that although there was expression of some connexin protein, the gap junction channels themselves were not dye coupled. After treatment with forskolin or forskolin + IBMX there was a remarkable increase in the levels of Cx43 and Cx26 protein on the membrane, measured by immunofluorescent staining, and to a lesser extent Cx32 protein, as well as a significant increase in the levels of dye coupling in the SVG cells. The increase in dye coupling is due to the increase in the amount of connexin protein observed on Western blots and immunofluorescently stained cell membranes for all connexins examined as well as the presence of the functional phosphorylated form of Cx43 (P2 form).

CHAPTER 4 – RESULTS
THE CHARACTERIZATION OF CELLULAR DIFFERENTIATION

4.1 - Demonstration of Morphological Changes after treatment with forskolin + IBMX [Criterion i]

Treatment of SVG cells for 24 hr with forskolin + IBMX dissolved in the neurobasal defined medium resulted in outgrowth of neurite-like processes and cell neurite contacts (Figure 4.1a, b) compared to cells cultured in neurobasal medium alone (Figure 4.1d), as detected by phase/contrast microscopy. The morphological change could be maintained with forskolin + IBMX treatment in the serum-free environment for up to 7 days after which cells detached from the substrate. Detachment of cells was most likely due to lack of proteins and factors, in the serum-free medium, necessary for cell survival and adhesion to the substrate.

SVG cells treated for 24hr with forskolin dissolved in neurobasal medium exhibited outgrowth of fewer long neurite processes than cells treated with the forskolin + IBMX combination (data represent 10 – 20 independent observations). Treatment of cells with IBMX showed no outgrowth of processes.

4.2 - Immunofluorescent demonstration of neuron-specific and glial-specific proteins after treatment with forskolin + IBMX [Criterion ii]

To determine whether the morphological changes observed after treatment with cAMP-inducers were due to cellular differentiation, antibodies developed against neuron-specific and glial-specific markers were used in immunolabeling experiments. Untreated SVG cells cultured in EMEM/10% /FBS express glial fibrillary acidic protein (GFAP) (Figure 4.2a). However, when cells are treated with forskolin + IBMX, dissolved in neurobasal medium, they lose expression of this astrocyte marker (Figure 4.2c) and begin

expressing proteins of neuronal origin, such as neural filament 200 (Figure 4.2 b). Untreated SVG cells do not express NF-200 (Figure 4.2d). The fluorescent staining in Figure 4.2c and d are nuclei stained with propidium iodide.

Figure 4.3 shows SVG cells treated with forskolin + IBMX intensely stain for neuron specific enolase (Figure 4.3a), nerve growth factor (Figure 4.3b), and galactocerebroside (Figure 4.3c). Untreated cells (Figure 4.3d-f) exhibit very low fluorescent staining for NSE, NGF, and GC and appear similar to the negative control (Figure 4.3g). The fluorescent staining in Figure 4.3 d-g are nuclei stained with propidium iodide. Figure 4.4 shows that SVG cells treated with forskolin + IBMX also express myelin basic protein (MBP), a component of myelin. Untreated cells do not express MBP (Figure 4.4b). From these results it appears that the forskolin/IBMX combination is able to change the expression of protein markers in the SVG cells.

The SVG cells were also tested for three other neuronal specific antigens listed in Table 4.1. The SVG cells did not show expression to two antigens specific to neurons Map-2ab (microtubule-associated protein 2) or neurofilament protein 68 kDa, but did express β III-Tubulin (isoform found in neurons).

Table 4.1. Neuronal specific antigens that were tested for immunofluorescent activity in the SVG cells treated with 5 μM forskolin + 200 μM IBMX.

Antigen	**Immunoactivity**
Map2-ab	-
β III-Tubulin	+
Neurofilament-68 kDa	-

Figure 4.1. Phase-contrast images of SVG cells treated for 24 hrs with 5 μM forskolin + 200 μM IBMX (a), 5μM forskolin (b), 200 μM IBMX (c), or untreated cells (d). SVG cells treated with the forskolin/IBMX combination exhibited extensive outgrowth of neurite-like processes from the cell body. Cells treated with forskolin alone showed fewer neurite-like extensions, while cells treated with IBMX appeared similar to the untreated controls. Data represent 6 independent experiments. Images were detected using a Nikon 20 X objective and Nikon TE300 microscope.

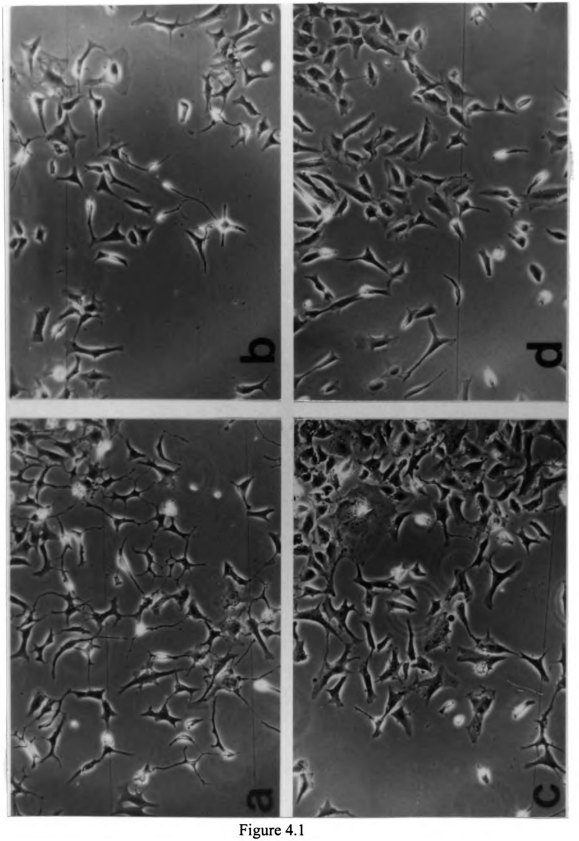


Figure 4.1

Figure 4.2. Untreated cells express immunoreactivity for the astrocyte marker GFAP (a) but lose expression of this protein after 24 hr treatment with 5 μM forskolin + 200 μM IBMX (c). Immunofluorescent staining of NF-200 in cells treated with 5 μM forskolin + 200 μM IBMX (b) compared to untreated cells (d). Nuclear staining was accomplished with propidium iodide (c-d). Data represent 6 independent experiments. Cyanine2-conjugated mouse-specific and rabbit-specific antibodies were detected using an Olympus 60 X oil immersion lens and Ultima scanning confocal microscope.

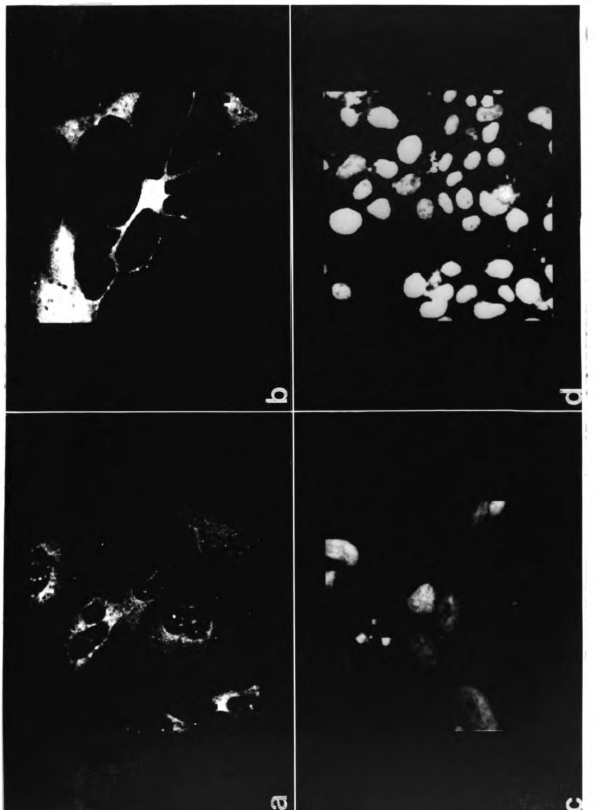


Figure 4.2

Figure 4.3. SVG cells treated with 5 μM forskolin + 200 μM IBMX for 24 hr express immunoreactivity for the neuronal markers NSE, NGF, and the oligodendrocyte marker galactocerebroside (GC). Immunofluorescent staining of NSE, NGF, and GC in cells treated with 5 μM forskolin + 200 μM IBMX (a-c) compared to untreated cells (d-f). Omission of primary antibody control (g). Nuclear staining was accomplished using propidium iodide (a-g). Data represent 6 independent experiments. Cyanine2-conjugated mouse-specific and rabbit-specific antibodies were detected using an Olympus 60 X oil immersion lens and Ultima scanning confocal microscope.

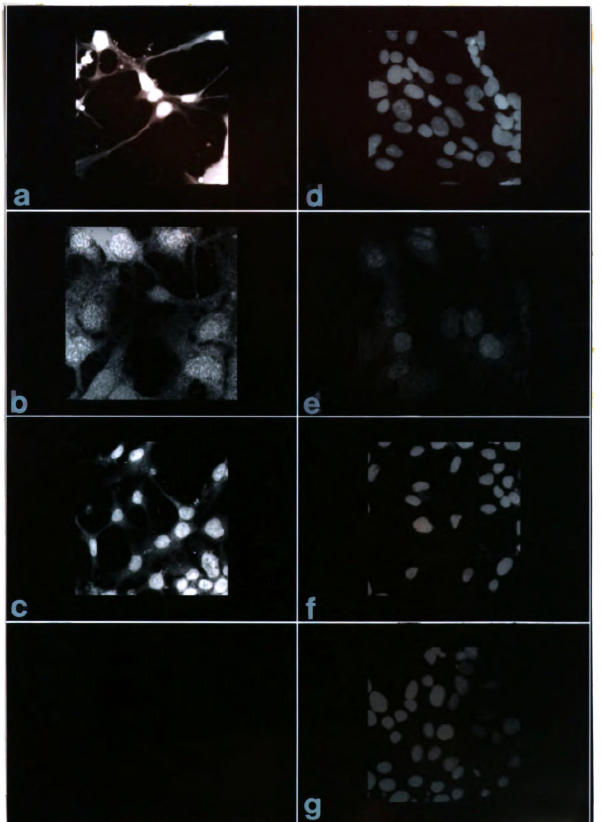
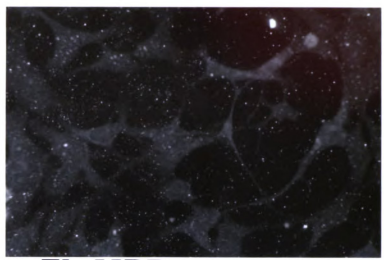
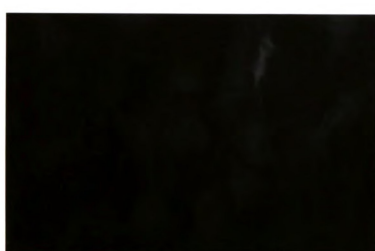


Figure 4.3



a. FI - MBP



b. NT - MBP

Figure 4.4. Expression of myelin basic protein (MBP) in SVG cells treated with forskolin + IBMX. (a) Cells treated with 5 μM + 200 μM IBMX for 24 hr (b) untreated SVG cells. Data represent 3 independent experiments.

4.3– Reversibility of the Differentiation [Criterion iii]

4.3.a. – Reversibility of the Morphological Change

To further show that the morphological change observed after treatment of SVG cells with forskolin + IBMX was due to permanent differentiation, cells were grown in serum-free medium containing 5μM forskolin + 200 μM IBMX for five days. The serum-free medium was then removed and cells were re-fed Eagles minimum essential medium containing 10% FBS for five days. Untreated controls were cultured in the same manner with serum-free medium. Briefly untreated cultures were fed serum-free medium minus the forskolin + IBMX for five days, after which cells were re-fed with Eagles minimum essential medium containing 10% FBS. Figure 4.5a shows that cells treated first with serum-free medium containing forskolin + IBMX, then exposed to minimum essential medium containing 10% FBS, do not reverse the morphological change or 'de-differentiate'. Cells exposed to this double treatment protocol continue to maintain neurite-like processes compared to untreated controls grown in serum-free medium or minimum essential medium with 10% FBS (Figure 4.5b, c). Data are representative of four independent experiments.

4.3.b. – Reversibility of the Biochemical Change

Cells cultured in serum-free medium containing 5 μM forskolin + 200 μM IBMX for three days and then exposed to serum, once again, do not reverse the expression of protein markers of neuronal or oligodendroglial origin. Figure 4.6 shows that cells exposed to the double treatment protocol continue to express NF-200 (Figure 4.6a), GC (Figure 4.6b), and NSE (Figure 4.6c) compared to untreated controls (Figure 4.6e-h). Data are representative of four independent experiments.

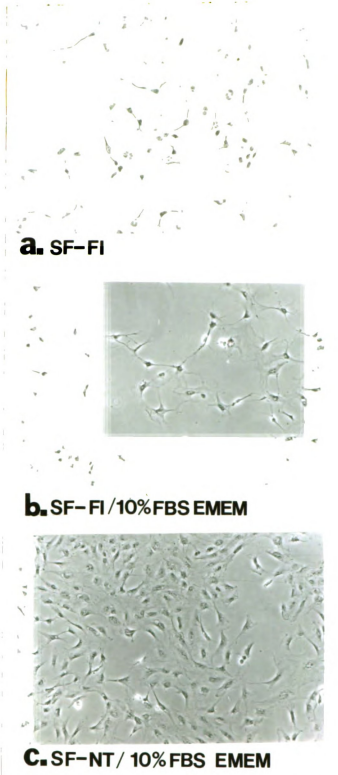


Figure 4.5. The morphological change is not reversible after SVG cell differentiation in serum-free medium with 5 μM forskolin + 200 μM IBMX (FI).
(a) SVG cells cultured in serum-free medium (SF) with FI. (b) SVG cells cultured in SF medium containing FI for 5 days and then fed with Eagles minimum essential medium (EMEM) containing 10% fetal bovine serum (FBS). (c) Untreated control

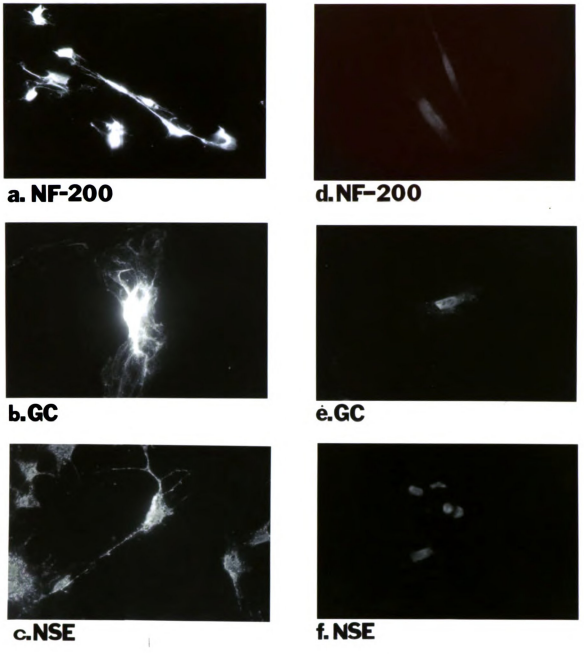


Figure 4.6. The biochemical changes are not reversible after initial SVG cell differentiation in serum-free medium with 5 μM forskolin + 200 μM IBMX. SVG cells cultured in serum-free medium with 5 μM forskolin + 200 μM IBMX for 5 days and then later re-fed Eagles minimum essential medium with 10% fetal bovine serum (FBS) retain expression of (a) neural filament 200 kDa (NF-200), (b) galactocerebroside (GC), and (c) neuron specific enolase (NSE). (d-e) Cells cultured in serum-free medium for 5 days and then re-fed EMEM, 10% FBS do not express any of the cell specific antigens.

4.4– Analysis of Cell Proliferation [Criterion iv]

4.4.a. – Uptake of [^{3}H]thymidine

In order to determine if the cAMP-inducing compounds forskolin and IBMX were inhibiting cell growth, one criterion for differentiation, under the conditions used to induce the morphological change and expression of neuronal markers incorporation of [^{3}H]thymidine was assayed. Total DNA synthesis in SVG cells was measured after treatment with 5 μM forskolin or 5 μM forskolin + 200 μM IBMX by determining the incorporation of [^{3}H]thymidine into trichloracetic-acid-precipitable material. The data in Table 4.1 show that 5 μM forskolin or 5 μM forskolin + 200 μM IBMX treated cells have a significantly reduced incorporation of [^{3}H]thymidine as compared to cells cultured without the treatments (EMEM 10% FBS, EMEM 5% FBS, serum-free). The type of medium and serum content also had a significant effect on the proliferation of SVG cultures, whether the treatments were present or not. Culturing cells in serum-free medium alone also significantly reduced the uptake of [^{3}H]thymidine compared to cultures where serum was present. There was no significant difference between cells cultured in EMEM containing 10% or 5% fetal bovine serum with or without treatment.

Table 4.2. Incorporation of [^{3}H]thymidine by SVG cells in response to treatment with 5 μM forskolin or 5 μM forskolin + 200μM IBMX dissolved in medium containing 10%FBS, 5% FBS, or medium without serum.

Treatment	CPM ± SD
EMEM 10% FBS	$1.140 \times 10^4 \pm 0\ 551.23$
EMEM 10% FBS + forskolin	$0.789 \times 10^4 \pm 0.186 \times 10^4$ *
EMEM 10% FBS + forskolin and IBMX	$0.132 \times 10^4 \pm 115.3 \times 10^4$ *
EMEM 5% FBS	$1.670 \times 10^4 \pm 978.6$
EMEM 5% FBS + forskolin	$1.270 \times 10^4 \pm 509.88$
EMEM 5% FBS + forskolin and IBMX	$0.128 \times 10^4 \pm 226.6$ *
Serum-free	$0.32 \times 10^4 \pm 131.7$ †
Serum-free + forskolin	$0.23 \times 10^4 \pm 860.46$
Serum-free + forskolin and IBMX	545.03 ± 89.51 *

- *Indicates a significant difference from untreated controls in each group (EMEM 10% FBS, EMEM 5% FBS, serum-free) at $p<0.05$.
- † Note: culturing cells in serum-free medium, without treatment, also significantly reduces incorporation of [^{3}H]thymidine compared to cultures where serum is present (EMEM 10% FBS, EMEM 5% FBS) at $p<0.05$.

Data represent the mean ± SD of three determinations.
EMEM = Eagles minimum essential medium.
FBS = fetal bovine serum

4.4.b. – Cell Counting

In addition, the proliferation of the SVG cells after treatment with 5 μM forskolin or 5 μM forskolin + 200μM IBMX was determined by cell counting. SVG cells were plated (in 12 well plates) and cultured for 1-7 days in the presence of EMEM 5% FBS, EMEM 10% FBS, or serum-free medium with and without the treatments. Table 4.2 shows that the number of cells present after two days of culture in the presence of 5 μM forskolin or 5 μM forskolin + 200μM IBMX was significantly decreased compared to cultures where there was no treatment. Statistical signifigance was determined using a one-way ANOVA followed by Tukey's post hoc test to compare treatment groups to the untreated control at a probability of $p<0.05$. Furthermore, cells grown in the serum-free environment with treatment had significantly decreased cell numbers compared to cells cultured with EMEM 10% FBS or EMEM 5% FBS with or without the treatments at $p<0.05$ using a one-way ANOVA followed by Tukey's post hoc test. The significant decrease in cell numbers was not due to cell death as evidenced by a very low percentage (23%) of cells that took up the trypan blue dye regardless of the treatment or presence of serum (Table 4.4).

Table 4.3. Proliferative response of SVG cells to treatment with cAMP-inducers after 2 -- 7 days in culture.

	Control	Forskolin	Forskolin + IBMX
DAY2			
NB	$20.3 \times 10^3 \pm 1.8 \times 10^3$†	$10.6 \times 10^3 \pm 8.9 \times 10^3$	$7.8 \times 10^3 \pm 2.1 \times 10^3$
EMEM 5% FBS	$44.7 \times 10^3 \pm 18.6 \times 10^3$	$15.1 \times 10^3 \pm 4.6 \times 10^3$	$17.5 \times 10^3 \pm 17.3 \times 10^3$
EMEM 10% FBS	$57.4 \times 10^3 \pm 7.1 \times 10^3$	$21.1 \times 10^3 \pm 10.0 \times 10^3$*	$21.8 \times 10^3 \pm 4.3 \times 10^3$*
DAY3			
NB	$26.5 \times 10^3 \pm 4.8 \times 10^3$†‡	$13.8 \times 10^3 \pm 4.1 \times 10^3$*†‡	$12.3 \times 10^3 \pm 1.6 \times 10^3$*‡
EMEM 5% FBS	$127.7 \times 10^3 \pm 19.2 \times 10^3$	$23.3 \times 10^3 \pm 17.2 \times 10^3$*	$41.0 \times 10^3 \pm 11.6 \times 10^3$*
EMEM 10% FBS	$118.4 \times 10^3 \pm 11.4 \times 10^3$	$86.3 \times 10^3 \pm 18.2 \times 10^3$	$32.1 \times 10^3 \pm 8.2 \times 10^3$*
DAY5			
NB	$15.8 \times 10^3 \pm 4.3 \times 10^3$†‡	$14.6 \times 10^3 \pm 2.4 \times 10^3$†‡	$5.7 \times 10^3 \pm 1.9 \times 10^3$*†‡
EMEM 5% FBS	$268.3 \times 10^3 \pm 29.1 \times 10^3$	$152.3 \times 10^3 \pm 50.0 \times 10^3$*	$35.5 \times 10^3 \pm 9.4 \times 10^3$*
EMEM 10% FBS	$267.2 \times 10^3 \pm 23.0 \times 10^3$	$86.3 \times 10^3 \pm 18.2 \times 10^3$*	$28.2 \times 10^3 \pm 12.7 \times 10^3$*
DAY7			
Serum-free	$17.8 \times 10^3 \pm 4.2 \times 10^3$†‡	$4.6 \times 10^3 \pm 0.4 \times 10^3$*†‡	$2.4 \times 10^3 \pm 4.3 \times 10^3$*†‡
EMEM 5% FBS	$357.3 \times 10^3 \pm 40.1 \times 10^3$	$126.9 \times 10^3 \pm 6.2 \times 10^3$*	$23.6 \times 10^3 \pm 4.3 \times 10^3$*
EMEM 10% FBS	$362.5 \times 10^3 \pm 44.8 \times 10^3$	$224.6 \times 10^3 \pm 10.3 \times 10^3$*	$17.3 \times 10^3 \pm 4.3 \times 10^3$*

SVG cells were treated with forskolin or forskolin + IBMX or left untreated for 2-7 days. After the specified treatment time-point the cells were trypsinized and the number of cells in each treatment group was counted using a hemocytometer. Results are expressed as mean number of cells ± SD of three replicates/treatment. * Indicates a significant difference from the untreated control group (serum-free, EMEM 5% FBS, EMEM 10% FBS) at $p<0.05$. † Indicates a significant difference between serum-free and EMEM 10% FBS at $p<0.05$. ‡ Indicates significant difference from serum-free and EMEM 5% FBS.

Table 4.4. Measurement of SVG cell cytotoxicity after treatment with cAMP-inducers.

	Control	Forskolin	Forskolin + IBMX
DAY2			
NB	13%	28%	20%
EMEM 5% FBS	14%	16%	23%
EMEM 10% FBS	13%	17%	22%
DAY3			
NB	10%	3%	14%
EMEM 5% FBS	4%	6%	5%
EMEM 10% FBS	10%	7%	9%
DAY5			
NB	7%	3%	8%
EMEM 5% FBS	4%	6%	7%
EMEM 10% FBS	3%	4%	4%
DAY7			
NB	11%	5%	15%
EMEM 5% FBS	5%	12%	5%
EMEM 10% FBS	5%	7%	10%

SVG cells were treated with forskolin or forskolin + IBMX or left untreated for 2-7 days. After the specified treatment time-point trypan blue was added to cultures and allowed to incubate for 5 min at 37 ^{0}C. The cells were trypsinized and the number of cells containing the trypan blue, in each treatment group, were counted using a hemocytometer. Data represent the mean from three replicates/treatment. NB = Neurobasal (serum-free) medium, EMEM = Eagles minimum essential medium, FBS = fetal bovine serum

4.5. – Conclusions

Along with the increase in gap junctional intercellular communication, there was also a noticeable change in cell morphology after treatment of SVG cell cultures for 24 hr with forskolin + IBMX. The forskolin/IBMX combination dissolved in neurobasal medium appeared to stimulate differentiation of a greater number of process-bearing cells than addition of forskolin or IBMX alone and compared to cultures maintained in EMEM with or with out treatment. The morphological change was also associated with a change

in protein expression from astrocyte type filaments to neuronal and at least two oligodendroglial type protein markers.

Furthermore, the differentiation is not reversible, suggesting that it is terminal, at least under the conditions investigated here. The changes that occur in the cells after treatment with forskolin and IBMX in the serum-free environment can not be eliminated after removing the signal for differentiation (forskolin + IBMX) and placing in medium with serum. Placing cells in the serum-free environment without treatment after they have been exposed to forskolin + IBMX causes detachment from the substrate after two days. Furthermore, growing cells in serum-free medium without forskolin + IBMX and then placing them in eagles minimum essential medium with 10% FBS without forskolin + IBMX does not induce morphological or biochemical differentiation.

The cAMP-inducers, forskolin and IBMX, also cause a significant decrease in DNA synthesis and cell number after three days of treatment that is due to a decrease in cell proliferation and not cell death. Together the results from Chapter 4 suggest that the SVG cells have the ability to differentiate and that the differentiation involves experssion of neuronal and oligodendroglial antigens, a decreased growth rate, and commitment to a specific cell lineage.

CHAPTER 5 – RESULTS
RELATIONSHIP BETWEEN OBSERVED DIFFERENTIATION AND CELL – CELL COMMUNICATION (CORRELATION OR CAUSATION)

5.1 – Effect of Serum on Cell Morphology

Cells cultured in EMEM/10% FBS, containing forskolin + IBMX, exhibit outgrowth of processes in approximately 30% of the cell populations after two days of treatment (Figure 5.1a). The remaining 70% of cell populations maintain an epithelial phenotype. When the serum concentration is reduced to 5% FBS in EMEM, outgrowth of processes occurs within 24 hr (Figure 5.1 b), however, only 30% of the population possesses the process bearing phenotype. Longer incubations in the EMEM containing both the treatments (forskolin or forskolin + IBMX) and serum do not increase the number of process bearing cells even after 5 days of culture in the presence of forskolin + IBMX (Figure 5.1 c, d). Cells that are grown in EMEM 10% FBS or serum-free medium (neurobasal medium) without treatment do not exhibit morphological changes at any time point examined (24 hrs – 7 days). The only instance where dramatic morphological changes, exemplified by extension of neurite-like processes from the cell body, are observed are when SVG cells are cultured in serum-free medium (neurobasal medium) containing forskolin + IBMX.

5.2. – Effect of Serum on Expression of Connexin Protein

5.2.a. – Expression of Connexin 43 Protein

In order to determine whether different serum concentrations would have an effect on the connexin 43 protein, SVG cells were cultured in Eagles minimum essential medium containing 5% or 10% fetal bovine serum and 5 μM forskolin + 200 μM IBMX. Figure

5.2 is a Western blot of SVG cell total protein extracts from cells cultured in the above manner. Figure 5.2 shows that connexin 43 protein remains unaffected by changing the concentration of serum in SVG cell medium. In fact, there appears to be an enhancement in the amount of protein present when cells are cultured with the treatment plus serum versus those with the treatment dissolved in serum-free medium. The presence of Cx43 protein bands in the untreated SVG protein extracts, isolated from cells cultured in serum (Figure 5.2; 5% and 10% FBS, NT), suggests that serum in the media may provide factors to stabilize the protein, enhance its translation or inhibit its degradation. Furthermore, Figure 5.2 also shows that the treatments 5 μM forskolin + 200 μM IBMX are most likely inhibiting the degradation of the P2 form of Cx43 (the P2 form is phosphorylated twice and thought to form the functional channel) and stabilizing it in the membrane. The P2 band is enhanced in all treated extracts taken from cells grown in both the serum and serum-free medium. It is important to note that in the untreated cells grown in 5-10% serum (Figure5.2; NT), there is a weaker P2 Cx43 band compared to those cells treated with cAMP activators. The Cx43 band at ~ 44 kDa or P1 is also enhanced in untreated extracts suggesting that cAMP production is able to shift the Cx43 protein to the open state via indirect phosphorylation events as well as inhibit its degradation.

Figure 5.1. SVG cells cultured in medium containing serum along with forskolin and IBMX do not morphologically differentiate. After 2 days of treatment with 5μM forskolin + 200 μM IBMX dissolved in Eagles minimum essential medium (EMEM) with 10% or 5% fetal bovine serum (FBS) approximately 30% of the cells exhibit a morphological change (a-b). 5 days of treatment in EMEM with serum does not increase the number of cells that morphologically differentiate (c-d). Untreated cells grown in serum (e). Cells treated with 5μM forskolin + 200 μM IBMX in serum-free medium for 24 hrs (f). Data are representative of 3 independent experiments.

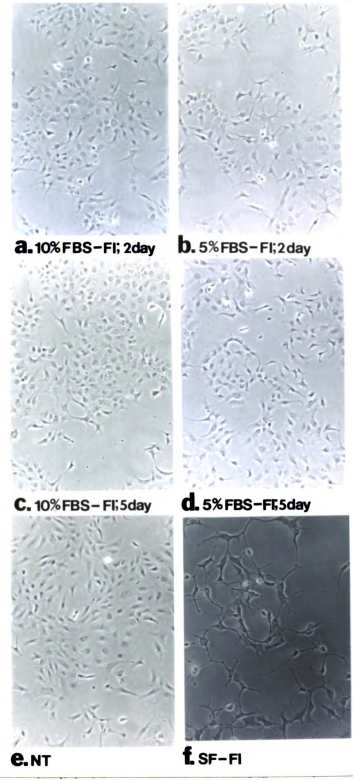

a. 10%FBS-FI; 2day
b. 5%FBS-FI; 2day
c. 10%FBS-FI; 5day
d. 5%FBS-FI; 5day
e. NT
f. SF-FI

Figure 5.1

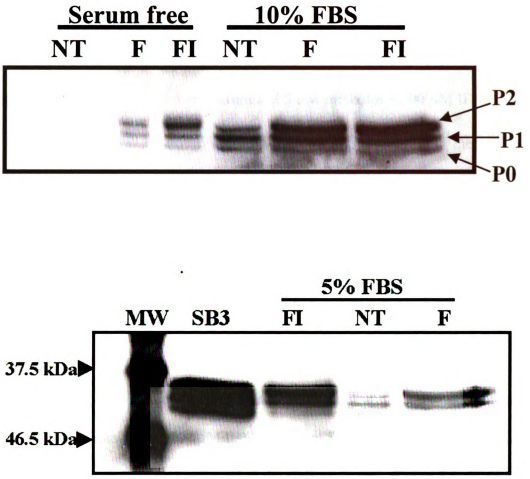


Figure 5.2. Effect of serum on forskolin + IBMX treated SVG connexin 43 protein. Western analysis using Cx43-specific antibodies reveals that Cx43 protein is present in total protein extracts regardless of serum, the exception being the untreated cell protein harvested from cells grown in serum-free medium only. NT = untreated; F = 5 μM forskolin; FI = 5 μM forskolin + 200 μM IBMX; SB3 = human epithelial kidney cells (positive control); MW = molecular weight markers; FBS = fetal bovine serum Data are representative of at least 4 independent experiments.

5.2.b. – Expression of Connexin 32 Protein

In order to determine whether different serum concentrations would have an effect on the connexin 32 protein SVG cells were cultured in eagles minimum essential medium containing 5% or 10% fetal bovine serum and 5 μM forskolin + 200 μM IBMX. Figure 5.3 shows that connexin 32 protein is enhanced in cells that are cultured with the treatment plus serum versus those with the treatment dissolved in serum-free medium. The results in Figure 5.3 are similar to what is observed when protein extracts are taken from cells cultured in serum-free medium (Figure 3.3). Data are representative of 3 independent experiments.

5.2.c. – Expression of Connexin 26 Protein

In order to determine whether serum would have an effect on the connexin 26 protein, SVG cells were cultured in eagles minimum essential medium containing 5% fetal bovine serum and 5 μM forskolin + 200 μM IBMX. Figure 5.4 is a Western blot of SVG cell total protein extracts from cells cultured in the above manner. Figure 5.4 shows that connexin 26 protein remains unaffected by adding serum to SVG cell medium. The only noticeable difference between cells cultured in serum versus those cultured in serum-free medium is the presence of weak Cx26 protein bands in the untreated extracts versus those from serum-free extracts (Figure 3.4). Again, as with the Cx43 and Cx32 proteins, serum probably contains factors that contribute to connexin stability as well as enhancement of translation or transcription. Data are representative of 3 independent experiments.

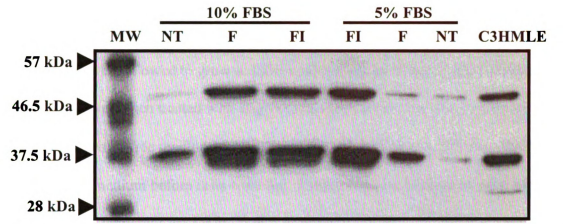


Figure 5.3. Effect of serum on forskolin + IBMX treated SVG cell connexin 32 protein. Western blot analysis using Cx32-specific antibodies reveals the presence of Cx32 protein in protein extracts isolated from cells grown in eagles minimum essential medium containing 5% fetal bovine serum (FBS) or 10% FBS. NT = untreated; F = 5 μM forskolin; FI = 5 μM forskolin + 200 μM IBMX; C3HMLE = Mouse liver hepatocytes (positive control); MW = molecular weight markers.

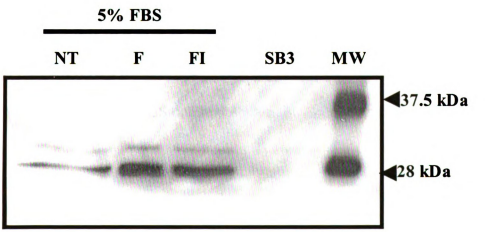


Figure 5.4. Effect of serum on forskolin + IBMX treated SVG Cx26 protein. Western blot analysis using Cx26-specific antibodies reveals the presence of Cx26 protein in protein extracts isolated from cells grown in Eagles minimum essential medium containing 5% fetal bovine serum (FBS). NT = untreated; F = 5 μM forskolin; FI = 5 μM forskolin + 200 μM IBMX; SB3 = Human epithelial kidney cells (negative control); MW = molecular weight markers.

5.3. – Affect of Serum on Dye Transfer

The technique of scrape load dye transfer (SL/DT) was used to determine whether the connexin channels could functionally transfer dye from cell to cell in the presence of serum. Cells were allowed to grow to form a monolayer in 35 mm tissue culture grade dishes. Cells were then treated with Eagles minimum essential medium containing 5% or 10% FBS or serum-free medium. The treatment 5 μM forskolin + 200 μM IBMX was added to each medium before cells were fed. Cells were then allowed to grow in the presence of the treatment for 3 days before performing SL/DT. Three dishes per treatment were prepared to assure consistency in the result. Data are representative of 2 independent experiments. Figure 5.5 is representative SL/DT data from one experiment. In all cases, regardless of the presence of serum, cells were able to transfer dye as long as treatment was present in the medium (Figure 5.5a, b, and c). Figure 5.5d is a photomicrograph of untreated cells grown in serum-free medium without treatment; note that the cells do not transfer dye. The SVG cells did not transfer dye when they were grown in EMEM/10% FBS or EMEM/5% FBS minus the forskolin + IBMX treatment.

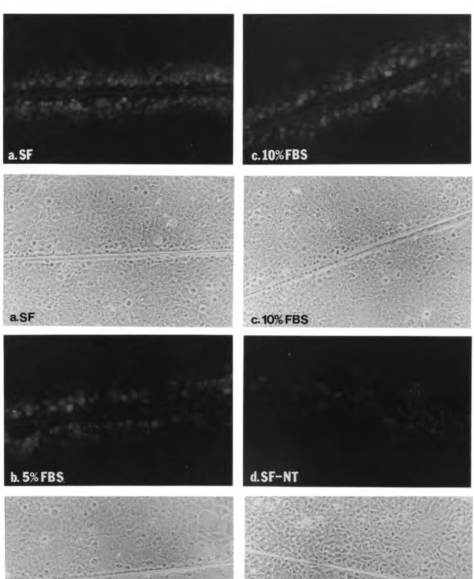


Figure 5.5. Scrape Load/Dye Transfer of SVG cells treated for 48 hr in different media containing 5μM forskolin + 200 μM IBMX . (a. SF) 5μM forskolin + 200 μM IBMX in serum-free medium, (b. 5% FBS) 5μM forskolin + 200 μM IBMX in Eagles minimum essential medium (EMEM) + 5% fetal bovine serum (FBS). (c. 10% FBS) 5μM forskolin + 200 μM IBMX in EMEM + 10% FBS. (d) Serum-free medium without treatment (SF-NT). Phase/contrast images appear directly under the corresponding fluorescent image.

5.4 – Inhibition of Process Outgrowth by Cell Plating and the Effect on Connexin 43

5.4.a. – Cell Culture Conditions that can Inhibit Process Outgrowth with Treatment

Cells that are plated extremely close together or become confluent within 24 hr (> 20,000 cells/well in a four-well chamberslide) will not exhibit extensive outgrowth of processes in the serum-free environment with forskolin + IBMX treatment (Figure 5.6a). Furthermore, cells that are plated sparsely so that there are approximately 500-1000 cells/well in a four-well chamber slide will not exhibit morphological changes even in serum-free medium with forskolin + IBMX (Figure 5.6b). Both of the above cell culture conditions produce morphologies similar to the untreated SVG cells (Figure 5.6c). Figure 5.6d is a phase/contrast picture of SVG cells plated at 10,000-20,000 cells/well in serum-free medium containing forskolin + IBMX as a comparison; notice extensive outgrowth of neurite-like processes. Data are representative of 3 independent experiments.

5.4.b – Expression of Connexin 43 at Different Plating Densities

To determine whether the different plating densities have an effect on connexin 43, plaque formation on the membrane of SVG cells immunofluorescent staining was performed. Cells were plated in the same manner as described above; briefly, SVG cells were plated very dense (> 20,000 cells/well), very sparse (500-1000 cells/well), or at a confluency to promote process outgrowth (between 10,000 – 20, 000 cells/well). Figure 5.7a shows that although no morphological change occurs when cells are densely plated there are numerous punctate connexin 43 plaques at most cell membrane contacts. Figure 5.7b shows that when cells are not touching and are plated very sparse, there is no Cx43 immunofluorescence at the cell membrane. Figure 5.7c is immunofluorescent staining of untreated SVG cell cultures which exhibit very little connexin 43 plaque formation and

figure 5.7d is a photomicrograph of SVG cells plated at 20,000 cells/well and show numerous Cx43 immunofluorescent plaques at cell–cell and cell–neurite contacts. Data are representative of 3 independent experiments.

Figure 5.6. The density of cell plating can effect cell morphology regardless of treatment. (a) SVG cells plated at > 20,000 cells/well (b) SVG cells plated at 500-1000 cells/well. (c) SVG cells plated at ~ 10,000-20,000 cells/well. (d) SVG cells plated at ~ 10,000-20,000 cells/well with treatment as a comparison. SF-FI = serum-free medium with 5 μM forskolin + 200 μM IBMX; SF-NT= serum-free medium without treatment.

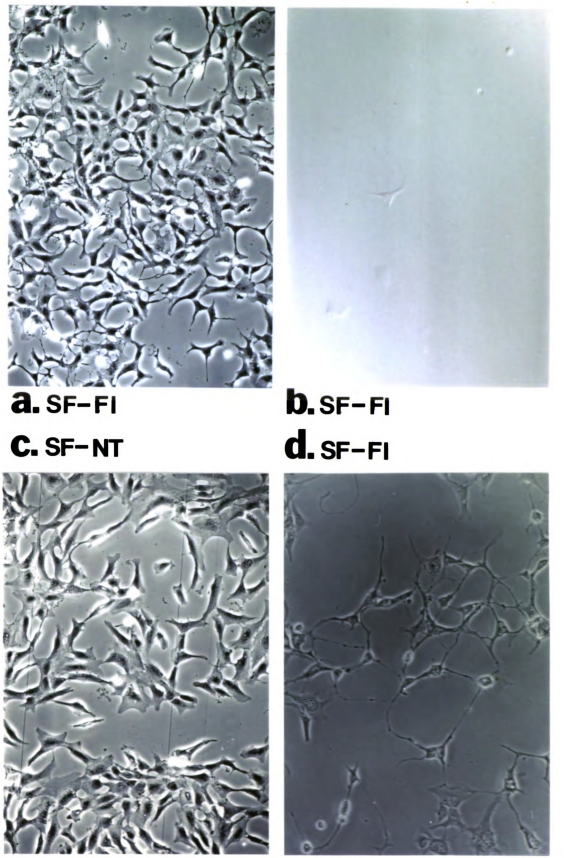


Figure 5.6

Figure 5.7. Plating density and the effect on connexin 43 plaque formation. (a) SVG cells plated in a confluent monolayer with serum-free medium containing 5 μM forskolin + 200 μM IBMX have Cx43-specific immunoreactivity. (b) Isolated cells plated at 500-1000 cells/well show no Cx43-specific immunoreactivity. (c) Untreated control plated at 10,000-20,000 cells/well. (d) Cells plated at 10,000-20,000 cells/well and treated with 5 μM forskolin + 200 μM IBMX. SF-FI = serum-free medium with 5 μM forskolin + 200 μM IBMX.

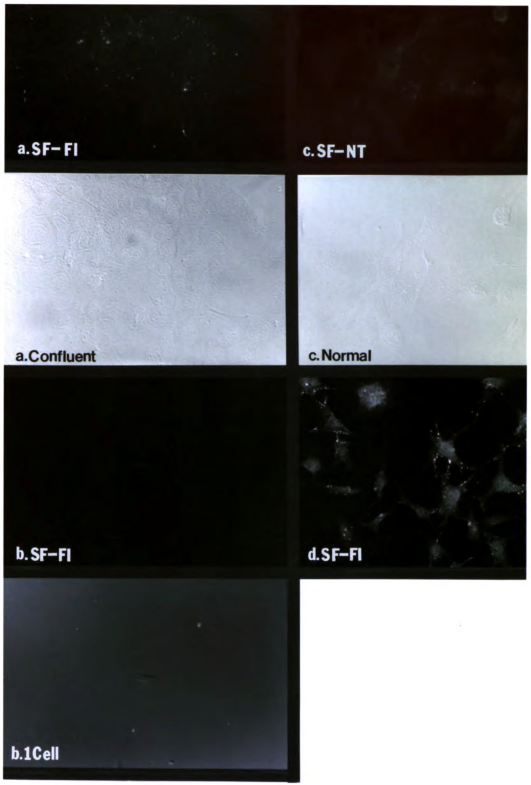


Figure 5.7

5.5. – Cell Culture Conditions that Allow Processes Outgrowth without Cell-Cell Contact

5.5.a – Processes Outgrowth without Cell Cell Contact

Figure 5.8a shows SVG cells plated lightly so that cell membranes are not allowed to touch. To ensure that cells do not contact other cells, approximately 15,000-20,000 cells were plated per well in a four well chamber slide. Cells were then allowed to attach for approximately 4 hours in Eagles minimum essential medium containing 10% fetal bovine serum. The EMEM was then removed, cells were washed in PBS to remove any unattached cells, and serum-free medium was added containing the forskolin + IBMX treatment. The cells were allowed to grow for 10 hours and then fixed with 1% Methanol/95% acetic acid for immunofluorescence and phase/contrast microscopy. The cells in Figure 5.8a continue to exhibit process outgrowth although their cell membranes were not touching. Furthermore, it appears that cells were extending their processes towards other cells or growing towards one another. Figure 5.8b is a phase/contrast image of cells plated lightly and allowed to attach for 10 hours before treatment so that membranes are touching; notice more extensive process outgrowth. Figure 5.8c is a phase/contrast image of cells plated similarly to the cells in Figure 5.8b, but without treatment; notice no outgrowth of processes. Data are representative of 4 independent experiments.

5.5.b – Biochemical Differentiation without Cell-Cell Contact

Cells were cultured in the same manner as described in for Figure 5.8a. To ensure that cells do not contact other cells approximately 15,000-20,000 cells were plated per well in a four well chamber slide. Cells were then allowed to attach for approximately 4 hours in Eagles minimum essential medium containing 10% fetal bovine serum. The EMEM was

removed, cells were washed in PBS to remove any unattached cells and serum, and serum-free medium was added containing the forskolin + IBMX treatment. The cells were allowed to grow for 10 hours and then fixed with 95% Methanol/1% acetic acid or 1% formalin for immunofluorescence.

The cells in Figure 5.9 express protein markers for specific neural cell types although their cell membranes were not touching. Notice that cells that are completely isolated from other cells do not exhibit a morphological change and do not express biochemical markers for differentiation (Figure 5.10). Data are representative of 4 independent experiments.

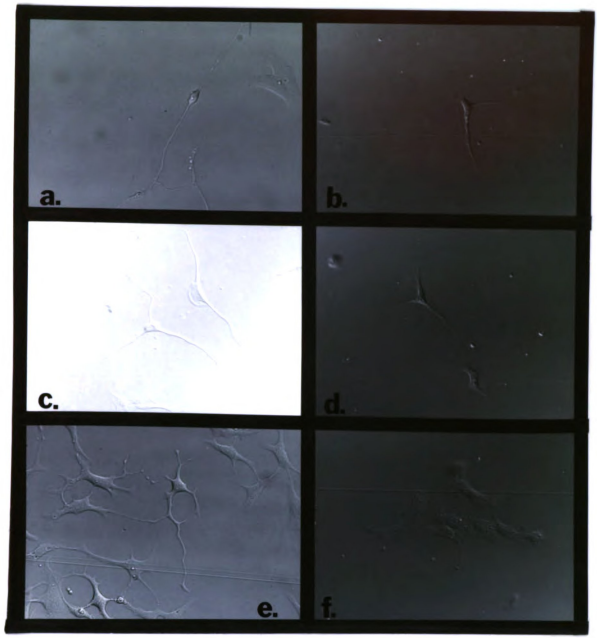


Figure 5.8. SVG cells will morphologically differentiate as long as they are in close proximity to other cells. (a-d) Cells plated for 4 hr serum before treatment with 5 μM forskolin + 200 μM IBMX in serum-free medium will not divide and will not form membrane contacts with other cells. (e) Cells plated for 10 hr in medium with serum will divide before treatment with 5 μM forskolin + 200 μM IBMX in serum-free medium and will make membrane contacts. (d) untreated control.

Figure 5.9. SVG cells will biochemically differentiate at low density even though they are not touching other cells. (a) Neural filament 200 kDa (NF-200), (b) Neuron specific enolase (NSE), (c) Nerve growth factor (NGF), (d) Glial fibrillary acidic protein (GFAP). DIC images appear directly bellow the corresponding immunofluorescent image.

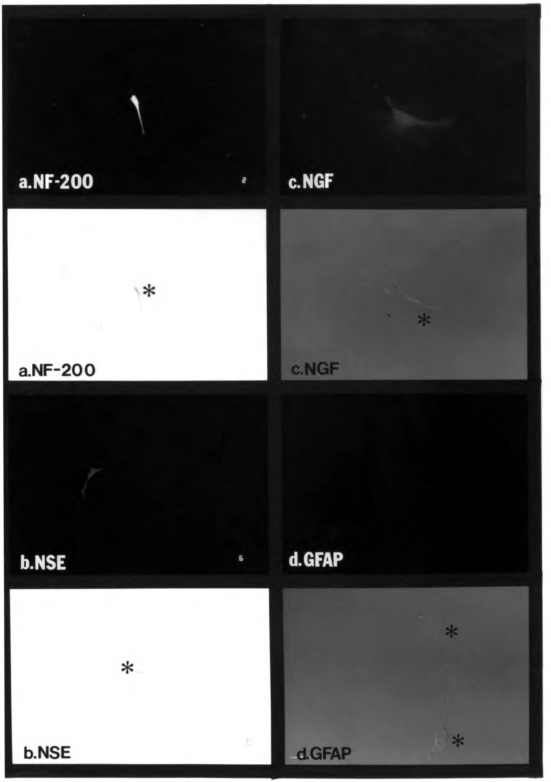


Figure 5.9

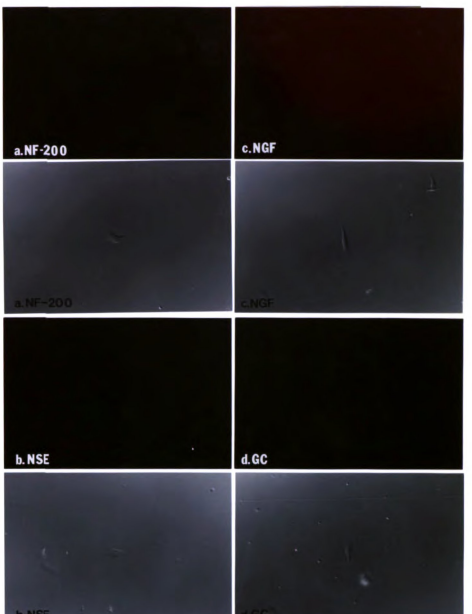
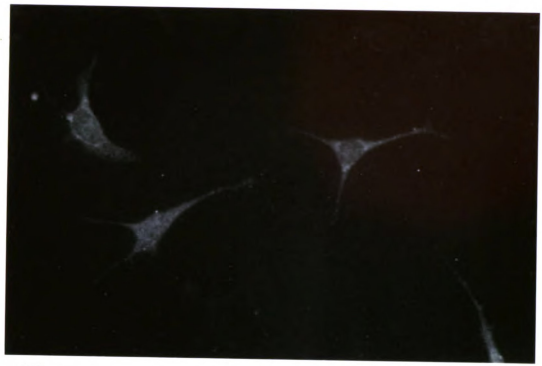


Figure 5.10. Individual SVG cells cultured so that they are isolated from other cells will not exhibit outgrowth of neurite-like processes or biochemically differentiate. (a) Immunofluorescent staining for neural filament 200 kDa (NF-200), (b) neuron specific enolase (NSE), (c) nerve growth factor (NGF), and (d) galactocerebroside (GC).

5.5.c – Connexin 43 Staining without Cell-to-Cell Contact

The cell cultures used in Figure 5.8a were also used in immunolabeling experiments with Cx43-specific antibodies as described in Experimental Procedures. Results presented in Figure 5.11a show that cells that do not contact other cells do not exhibit intense punctate labeling of connexin 43 gap junction channels as do cells that have extensive membrane contacts (Figure 5.11b). Although cells in Figure 5.11a have neurite-like processes the expression of connexins is similar to that of untreated SVG cells that do appear to have membrane contacts. Data are representative of 2 independent experiments.

Figure 5.11. SVG cells that do not contact neighboring cell processes or cell bodies do not exhibit punctate Cx43-specific immunofluorescence. (a) SVG cells immunofluorescently stained with Cx43-specific antibodies that do not have membrane contact with other cells will exhibit cytoplasmic staining only. (b) SVG cells that have membrane contacts with other cells exhibit punctate Cx43 immunoreactivity at areas of membrane contact.



a.

b.

Figure 5.11

5.6. – Conclusions

The morphological results from cells grown in different serum concentrations (Figure 5.1) show that depending on the type of media, treatment, and percentage of serum that is added the number of cells maintaining the processes bearing phenotype can be altered. Although the changes in morphology can be inhibited by treating cells with forskolin + IBMX, dissolved in medium containing serum, the connexin proteins (Cx43, 32, 26) remain uneffected on western blots (Figures 5.2, 5.3, 5.4) and cells have functional dye coupling even though serum is present (Figure 5.5).

Furthermore, outgrowth of neurite-like processes can be inhibited by densely plating cells so they become confluent within 24 hours even with treatment dissolved in serum-free medium (Figure 5.6). The highly confluent cells maintain connexin 43 expression on the outer membrane where they make contacts with neighboring cells as long as the forskolin + IBMX treatment is present (Figure 5.7). These cells can also functionally transfer dye (Figure 5.5c).

Conversely cells can be plated so that they have no membrane contact but exhibit neurite-like outgrowth (Figure 5.8). Cells plated in this manner do not form gap junctions (Channels that allow cytoplasmic continuity between two or more cells) but if they lie in close proximity, they extend processes. These cells are also able to biochemically differentiate without making cell-to-cell membrane contact as long as they are close to other cells (Figure 5.9). Formation of punctate connexin 43 plaques is not seen in cells that do not make cellular contacts (Figure 5.10).

From these results, it is evident that the morphological and biochemical differentiation is not dependent on cellular contacts and communication through gap junctions.

It does appear, however, that extracellular signals are important for the differentiation due to the observation that cells that are completely isolated from other cells will not extend processes or biochemically differentiate. This suggests the need for a signal that is secreted by neighboring cells which directs the extension of processes and biochemical differentiation.

CHAPTER 6 – RESULTS
TIME LINE OF EVENTS LEADING TO INDUCTION OF CELL – CELL COMMUNICATION AND DIFFERENTIATION

6.1. – Analysis of the Cyclic AMP Dependent Pathway

6.1.a – Measurement and Timing of cAMP

Cyclic AMP was measured at various time points to ensure that the treatment, 5 μM forskolin + 200 μM IBMX, was truly inducing an increase in cAMP and that this increase was occurring at the appropriate time prior to differentiation and induction of cell-cell communication. Figure 6.1 shows that by 5 sec – 10 sec measurable levels of cAMP were detected at 0.5 pmol/ml – 1.5 pmol/ml respectively and increases logarithmically with time. After about 30 min – 1 hr there appears to be a threshold level of cAMP. Figure 6.2 is the standard curve used to calculate the amount of cAMP in sample supernates.

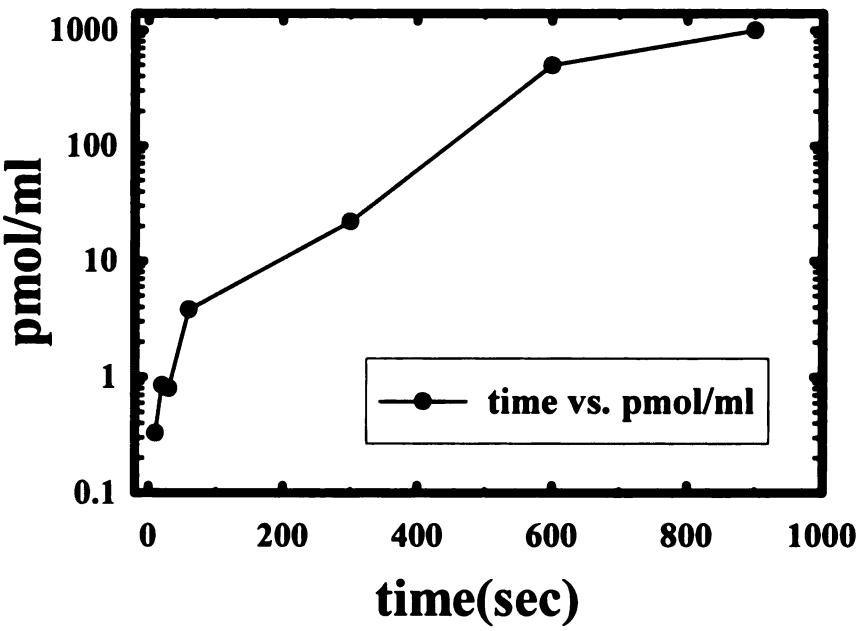


Figure 6.1. Increase in amount of cAMP (pmol/ml) over a time period of 5 seconds to 1 hour. Representative of 2 independent experiments.

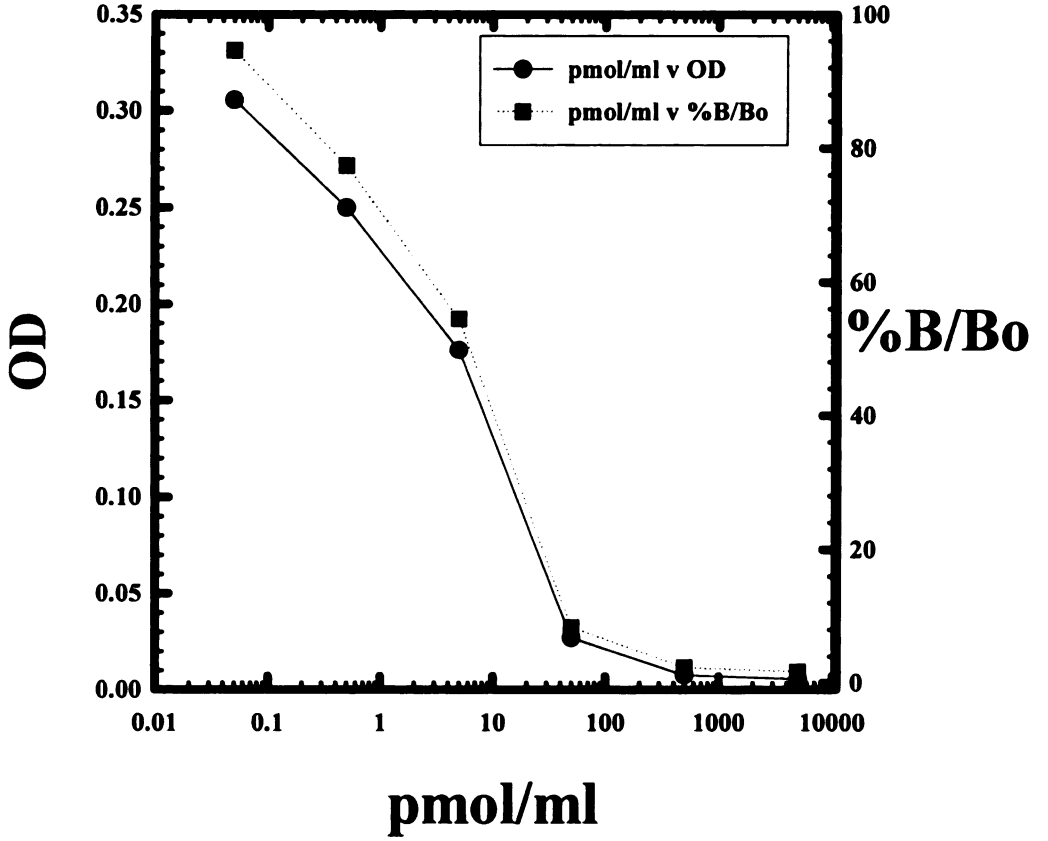


Figure 6.2. Standard curve used to obtain values for the concentration of cAMP in the SVG sample supernates. OD (absorbance at 405 nm λ) vs. pmol/ml of known cAMP standard. B/B_0 = bound / unbound

6.1.b. – Measurement and Timing of PKA Activity

Table 6.1 provides data on the activity of protein kinase A in the membrane (particulate) fractions of SVG cells treated with 5 μM forskolin + 200 μM IBMX. PKA activity was measured at the time points 10 sec, 20 sec, 30 sec, and 5 min after addition of the cAMP-inducers. The results indicate that measurable PKA activity in SVG cells treated with 5 μM forskolin + 200 μM IBMX occurred by 10 sec, after treatment, and was significantly enhanced above that of the untreated SVG cell extract. Two independent experiments were carried out with two replicates per treatment performed in each experiment.

Table 6.1. Protein kinase A activity in the particulate fraction of SVG cell extract treated with 5 μM forskolin + 200 μM IBMX.

Treatment	PKA activity (pmol of [^{32}P]phosphate/min	% Activated PKA (pmol/min activated PKA/ pmol/min total PKA)
Untreated	86.18	6%
10 sec	221.01*	44%
20sec	669.33*	83%
30 sec	679.04*	86%
5 min	721.27*	90%

PKA activity was measured in 2 experiments with two replicates / treatment (n=2). Data represent the mean of two replicates / treatment from 2 independent experiments. All time points measured were treated with 5 μM forskolin + 200 μM IBMX. * Indicates a significant difference from the untreated at $p < 0.05$

6.1.c. – Timing of CREB Activation Based on Phosphorylation State

CREB or cyclic AMP response element binding protein is a transcription factor that is directly phosphorylated by PKA leading to its activation and ability to bind response elements on various genes including the connexin genes for Cx43 and Cx32 (Saez et al., 1990). In this instance CREB phosphorylation was used as a marker for functionality of the adenyly cyclase/PKA dependent pathway to ensure that this pathway was functioning appropriately. Figure 6.3A shows that after 1 min there is detectable activation of CREB as evidenced by the appearance of the phosphorylated form after treatment of SVG cells with 5 μM forskolin + 200 μM IBMX. Untreated SVG cells do not exhibit the phosphorylated form of CREB (Figure 6.3A; NT) but do contain the inactive protein (Figure 6.3B; NT). After 24 hours of treatment with the forskolin / IBMX combination, CREB becomes inactive as evidenced by the inability to detect phosphorylated CREB protein (Figure 6.3A; 24 hr). Although there is no active CREB after 24 hr (Figure 6.3A; 24 hr), there is a sustained increase in the inactive form of CREB at the 24 hr time point (Figure 6.3B; 24 hr).

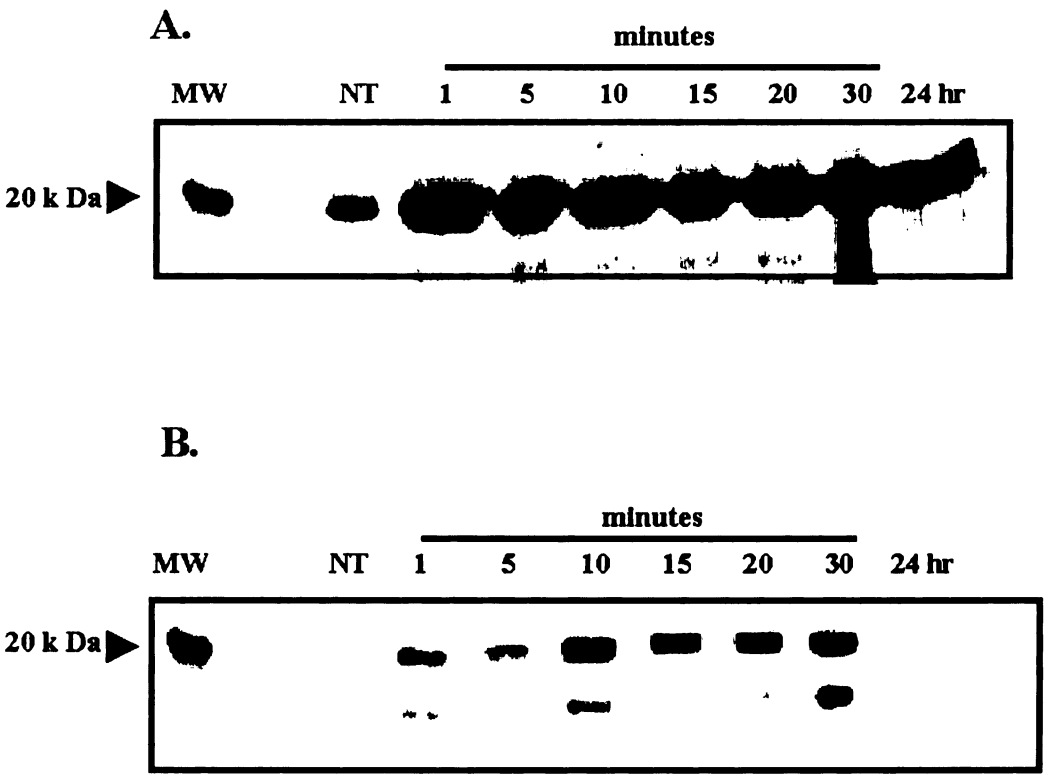


Figure 6.3. Effect of forskolin + IBMX on CREB phosphorylation in total protein extracts isolated from SVG cells at various time points. A. Western blot analysis using antibodies specific for the non-phosphorylated form of CREB. B. Western blot analysis using antibodies specific for the phosphorylated form of CREB. CREB appears at approximately 20 kDa. The band below 20 kDa is ATF-1, a viral transcription factor similar to CREB. Data are representative of 4 independent experiments.

6.2. – Analysis of Connexin 43 Phosphorylation and Transcription

6.2.a. – Timing of Connexin 43 Phosphorylation

Connexin 43 is indirectly phosphorylated via a cAMP dependent mechanism (for review see Introduction). Because phosphorylation of individual Cx43 protein subunits is extremely important for functionality of gap junction channels formed by Cx43, I wanted to know at approximately what time point the Cx43 in the SVG cells was phosphorylated. Knowing when Cx43 is phosphorylated indicates the time point when the SVG cells might acquire communication competence; it is also a good marker of the functionality of the cyclic AMP pathway in the SVG cells.

Figure 6.4 shows that connexin 43 is phosphorylated at approximately 1 minute after addition of forskolin + IBMX to SVG cell cultures. The steady state level of the connexin 43 P2 band (the phosphorylated form responsible for channel forming properties) remains present after 6 hr treatment. Data are representative of 3 independent experiments.

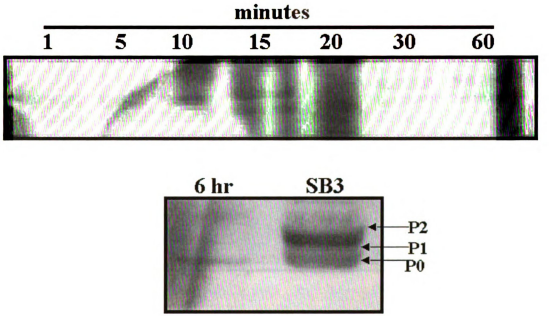


Figure 6.4. Timing of connexin 43 phosphorylation after treatment of SVG cell cultures with forskolin + IBMX. Connexin 43 protein is phosphorylated approximately 1 minute after addition of 5 μM forskolin + 200 μM IBMX dissolved in serum-free medium. SB3 = Human kidney epithelial cells (positive control).

6.2.b – RT-PCR for Connexin 43 mRNA

Figure 6.5 is an RT-PCR reaction for connexin 43 mRNA after treatment with 5 μM forskolin + 200 μM IBMX for 2 hr, 4 hr, 6 hr and 24 hours. The steady-state level of connexin 43 is increased after 4 hr treatment as compared to the untreated control (Figure 6.4; NT) and the culture treated for 2 hr (Figure 6.5; 2 hr).

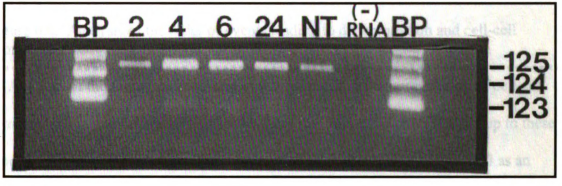


Figure 6.5. RT-PCR for connexin 43 mRNA in SVG cells treated with forskolin + IBMX. Analysis of the steady-state level of Cx43 mRNA shows that after 4 hr treatment with 5μM forskolin + 200 μM IBMX there is an increase in the amount of Cx43 mRNA compared to the 2 hr treatment or the untreated control (NT). Data are representative of 4 independent experiments.

6.3 - Conclusions

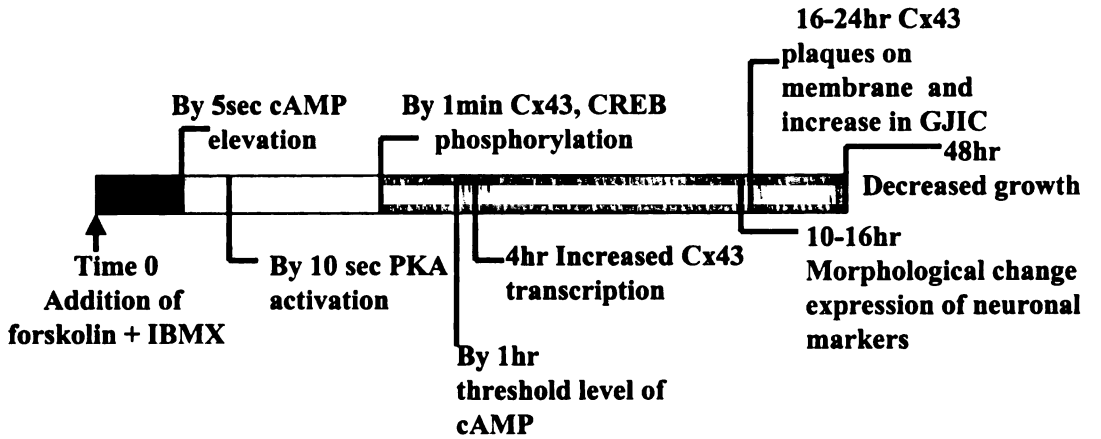


Figure 6.6. Analysis of the order of events leading to differentiation and cell-cell communication.

Analysis of the timing of important events involved in the upregulation of cell-cell communication and differentiation shows there is a sequential order leading up to these processes. Figure 6.6 is a time line showing that an increase in cAMP as well as an increase in PKA activity occurs within seconds. Phosphorylation of CREB and Cx43 occurs within a minute of adding forskolin and IBMX, and within 50 sec of PKA activation (Figure 6.6). After 4 hours treatment with forskolin and IBMX there is an increase in the amount of Cx43 mRNA. Approximately 16-24 hr after treatment or within 12-20 hours after an increase in Cx43 mRNA, there is formation of connexin 43 plaques on the membrane of the SVG cells and functional cell-cell communication (Figures 3.1 and 3.5). Although there is a measureable increase in cAMP and PKA activity within seconds the differentiaiton does not occur until 24 hrs after addition of the cAMP elevating compounds. The initial small elevation in cAMP is enough to induce phosphorylation of various proteins like Cx43 and CREB and may even initiate cell-cell communication, if cells form a monolayer, but it is probably not enough to induce differentiation. The extension of neurite-like process most likely involves sustained

increases in cAMP and a threshold-level that occurs at one hour or more after treatment. Furthermore, results examining dye-transfer in cells treated with forskolin alone are well coupled (66%, Table 3.1) but there is little neurite-like extension suggesting that increases in cAMP induced by forskolin are not sustained, due to cAMP breakdown by phosphodiesterases, and allow for cell-cell communication but not differentiation. Large, sustained increases in cAMP are required for differentiation, which are only obtained when the phosphodiesterase inhibitor IBMX is added simultaniously with the forskolin.

Results presented in Figure 5.8a show that if cells are plated sparsely, so that membranes are not touching, outgrowth of processes occurs within 10 hours and can precede the increase in cell-cell communication. Figure 6.6 provides further evidence that dye-coupling and differentiation are highly correlative events but are not causally dependent on one another under these conditions.

CHAPTER 7 – RESULTS
NEUROTROPHIC FACTORS AND MITOGENS (INITIATORS OR INHIBITORS OF DIFFERENTIATION)

7.1 – Neurotrophic Factors an Overview

The results obtained in Chapters 5 and 6 demonstrate that the initiation of the morphological or biochemical change observed in the SVG cells after treatment with forskolin and IBMX are not causally related to the increase in cell-cell communication. The question still stands as to what is really behind the observed differentiation. The SVG cells are known to secrete at least one neurotrophic factor, NGF (Tornatore et al., 1996), and have neurotrophic potential and induce PC12 cell differentiation in co-cultures (Tornatore et al., 1996). Neurotrophins are known to promote survival, differentiation, and neurite outgrowth of developing neurons (Wang et al., 1998). Application of BDNF to cortical stem cells yields pyramidal-like neurons, supports survival of hippocampal stem cell-derived neurons, and can also induce differentiation of hippocampal stem cell-derived neurons into pyramidal-like neurons (Shetty and Turner, 1998). BDNF has also been shown to induce differentiation of GABAergic neurons (Arsenijevic and Weiss, 1998). Hanson et al. 1998 have shown that a combination of trophic factors (CNTF, BDNF, GDNF, FGF, HGF), together with cAMP elevation, promotes long-term survival of spinal motor neurons in serum-free culture. The trophic factors alone were not capable of maintaining differentiation or supporting spinal motor neuron survival in a serum-free environment (Hanson et al., 1998).

Furthermore, elevations in cAMP are known to induce changes in secretion, translation, and transcription of neurotrophic factor protein and mRNA. Glial derived neurotrophic factor release can be increased 2-3 fold with forskolin or db-cAMP in

neuroblastoma cells and U-87MG glioblastoma cells (Verity et al., 1999). Modulators of the cAMP pathway will induce an increase in nerve growth factor mRNA expression and NGF protein release in microglia (Heese et al., 1997). It therefore seemed logical that if SVG cells can secrete one neurotrophic factor (NGF) then the serum-free environment along with the elevation in cAMP might also increase the secretion of NGF which in turn might stimulate outgrowth of neurite processes. It was already established that removing the cells from serum and placing them in a serum-free environment with forskolin + IBMX increased the observed fluorescence intensity of NGF versus cells cultured in serum with no treatment (Figure 3.4). Application of mouse 7S NGF to SVG cultures did not initiate neurite-like outgrowth independent of cAMP elevation, but did seem to promote survival of cells in the serum-free environment past 7 days (based on 4 independent observations) Therefore, NGF, as well as ciliary neurotrophic factor (CNTF) and brain derived neurotrophic factor (BDNF) mRNA and protein was examined in the SVG cells to determine whether increases in cAMP would alter expression of these three factors after treatment with 5 μM forskolin + 200 μM IBMX.

7.2. – RT-PCR of neurotrophic Factor mRNA

7.2.a. – Neural Growth Factor or NGF

RT-PCR of NGF mRNA revealed that at least the steady–state level of this factor was not effected by elevation of cAMP with forskolin + IBMX (Figure 7.1). However, from results presented in Figure 4.3 cAMP elevation does appear to enhance NGF mRNA expression.

7.2.b. – Ciliary Neurotrophic Factor or CNTF

RT-PCR of CNTF mRNA revealed that the steady–state level of this factor was increased after 24 hr of treatment with forskolin + IBMX (Figure 7.2).

7.2.c. – Brain Derived Neurotrophic Factor or BDNF

RT-PCR of BDNF mRNA revealed that the steady–state level of this factor was increased after 2 hours of treatment and continued to increase until 6 hours treatment with forskolin + IBMX (Figure 7.3).

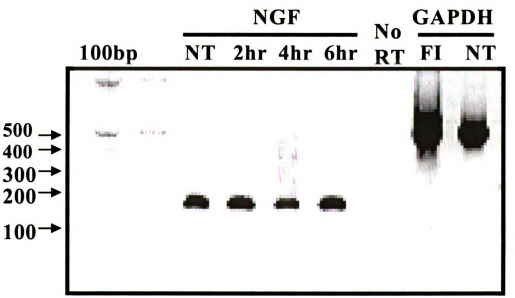


Figure 7.1. RT-PCR of nerve growth factor mRNA at 2, 4, and 6 hours after treatment with 5 μM forskolin + 200 μM IBMX. A 100 base pair DNA ladder was used as a base pair standard. NGF is approximately 200 bp. NT = no treatment; FI = 5 μM forskolin + 200 μM IBMX; GAPDH = glyceraldehyde phosphate dehydrogenase (positive control). No RT = elimination of reverse transcriptase from the reaction to ensure no DNA contamination. Data represent 4 independent experiments.

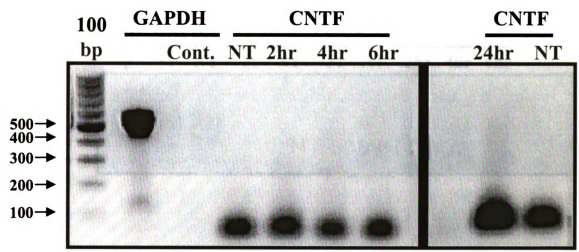


Figure 7.2. RT-PCR of ciliary neurotrophic factor (CNTF) mRNA at 2, 4, 6, and 24 hours after treatment with 5 μM forskolin + 200 μM IBMX. A 100 base pair DNA ladder was used as a base pair standard. CNTF is approximately 100 bp. NT = no treatment; FI = 5 μM forskolin + 200 μM IBMX; GAPDH = glyceraldehyde phosphate dehydrogenase (positive control). GAPDH cont. = GAPDH primer sequence is reversed, it should not work in the PCR reaction (negative control). Data represent 4 independent experiments

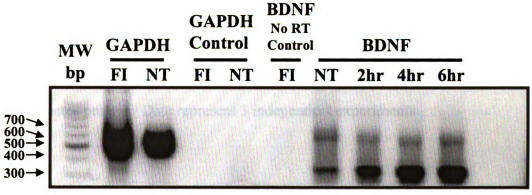


Figure 7.3. RT-PCR of brain derived neurotrophic factor (BDNF) mRNA at 2, 4, and 6 hours after treatment with 5 μM forskolin + 200 μM IBMX. A 100 base pair DNA ladder was used as a base pair standard. BDNF is approximately 365 bp. NT = no treatment; FI = 5 μM forskolin + 200 μM IBMX; GAPDH = glyceraldehyde phosphate dehydrogenase (positive control). No RT = elimination of reverse transcriptase from the reaction to ensure no DNA contamination. GAPDH cont. = GAPDH primer sequence is reversed, it should not work in the PCR reaction (negative control). Data represent 4 independent experiments

7.3. – Western Analysis of NGF and BDNF

Western analysis of total protein extracts taken from SVG cells after treatment for 24 hr (Figure 7.4; FI) revealed an increase in NGF and a minor increase in BDNF protein compared to untreated SVG cells (Figure 7.4 A and B; NT) cultured in serum-free medium. The increase in NGF on western blots and that observed with immunofluorescent staining is most likely due to cAMP elevation and might be one signal for differentiation. However, the qualitative increase measured by western analysis and immunofluorescence was not quantitated and a more quantitative analysis (such as an immunoassay) is needed to assess whether cAMP is upregulating neurotrophic factor protein. Data represent 3 independent experiments

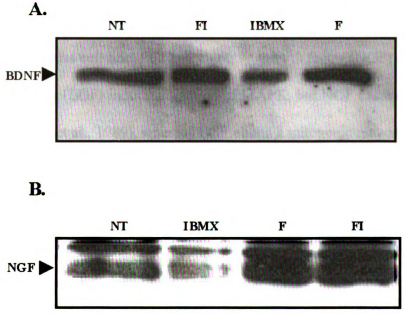


Figure 7.4. Western analysis of neural growth factor (NGF) (A) and brain derived neurotrophic factor (BDNF) (B) taken from SVG cell extracts after 24 hr treatment with cAMP-inducers. MW = molecular weight markers; NT = no treatment; IBMX = 200 μM IBMX; F = 5 μM forskolin; FI = 5 μM forskolin + 200 μM IBMX.

7.4. – Mitogen Activated Protein Kinase Pathway an Overview

The two most thoroughly characterized MAP kinases are the extracellular signal-regulated kinases ERK2 (42-kDA) and ERK1 (44-kDa). Both kinases are expressed in a wide variety of tissues and cells lines (Boulton et al., 1990) and are activated in response to a diverse stimuli, including growth factor stimulation of DNA synthesis, differentiation, and secretion. Both kinases require phosphorylation of tyrosine and threonine residues to be fully active (Anderson et al., 1990; Gomez et al., 1990). In vertebrates, MAPK activity has been shown to fluctuate during the cell cycle with peaks of activity near G0/S and G2/M borders, suggesting that MAPK functions to regulate both entry into the cell cycle and progression through the cell cycle (Tammoto et al., 1992).

Growth factors activate MAPK and can promote either growth or differentiation. In the rat pheochromocytoma cell line, PC12, both nerve growth factor (NGF) and epidermal growth factor (EGF) induce activation of MAPK, but NGF causes differentiation (Rudkin et al., 1989), whereas EGF elicits a mitogenic response (Graves et al., 1995). An explanation for the different responses elicited by NGF and EGF involve quantitative differences in MAPK activation induced by the two growth factors. NGF induces a sustained activation of MAPK while the EGF activation is transient (Traverse et al., 1992; Nguyen et al., 1993). In PC12 cells, MAPK has been shown to synergize with the cAMP pathway to produce the differentiated phenotype. Cyclic-AMP agonists or analogues will synergize with phorbol esters or growth factors (TPA or NGF) for induction of MAPK activation in PC12 cells (Gunning et al., 1981; Heidemann et al., 1985; Frödin et al., 1994). Frödin et al. (1994) have shown that elevation of cAMP in

PC12 cells with IBMX, forskolin, or cAMP-analogous will increase activity of MAP kinase kinase an immediate upstream activator of ERK1. The cAMP elevation causes a more than additive or synergistic activation of ERK1 in combination with growth factor or phorbal ester treatment. Mark et al. (1995) have also demonstrated that growth factor stimulation of MAPK is enhanced by KCl depolarization, which causes an increase in cAMP. PC12 cells were treated with EGF and KCl, NGF (+ control) or EGF alone. EGF is mitogenic but when coupled to KCl-depolarization an increase in cAMP was observed along with an increase in neurite outgrowth over that observed with NGF.

The SVG cells express the two MAPK subtypes ERK1 and ERK2 when grown in medium with serum. From results presented in Chapter 4 and 5, it is clear that there are differences in proliferation and DNA synthesis when the SVG cells are grown in serum versus serum-free medium. Furthermore, cells that have been exposed to the serum-free medium with cAMP-inducers do not reverse their differentiation even after they have been placed back under mitogenic stimulation (Chapter 4 Figures 4.5 and 4.6). It is, therefore, appropriate to state that SVG cells like the PC12 cells depend on MAPK for proliferation and differentiation.

7.5. – Morphological Changes After Treatment with Growth Factors

Results in Figure 7.5 show that treatment of SVG cells with mitogenic stimuli will not allow for extensive process outgrowth as is observed when cells are treated strictly with cAMP-inducers. Cells treated with epidermal growth factor (EGF) + forskolin and IBMX dissolved in serum-free medium (Figure 7.5a) do not morphologically differentiate to the same extent, assayed in four independent experiments, as cells that are treated with forskolin and IBMX alone in serum-free medium (Figure 7.5c). On the other

hand, cells that are treated with insulin + forskolin and IBMX dissolved in serum-free medium (Figure 7.5b) exhibit a morphological change similar to those treated with just forskolin and IBMX (Figure 7.5c). However, cells that have insulin dissolved in the medium quickly become overgrown and do not maintain the differentiation, similar to what is observed when cells are treated with forskolin + IBMX in medium with serum (EMEM 10% or 5% FBS). SVG cells cultured in serum-free medium (neurobasal medium) with insulin morphologically change after 24 – 48 hrs but after 5 days in culture most cells do not have processes and appear much like the untreated cultures or cultures where cells are given the forskolin + IBMX treatment in medium containing serum. Cells that are grown in serum-free medium (neurobasal medium) without the forskolin + IBMX treatment do not morphologically change (Figure 7.5d). Data represent 4 independent experiments.

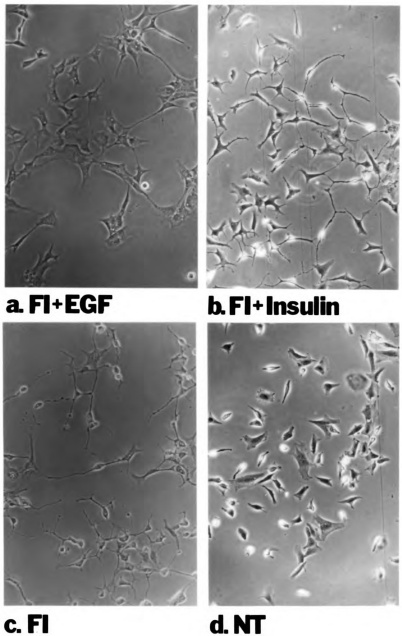


Figure 7.5. Phase/contrast images of SVG cells treated with growth factors or cAMP-inducers dissolved in serum-free medium. (a) 5 μM forskolin + 200 μM IBMX + 100 ng/ml epidermal growth factor (EGF), (b) 5 μM forskolin + 200 μM IBMX + 500 μg/ml insulin, (c) 5 μM forskolin + 200 μM IBMX or (d) serum-free medium alone.

7.6. – Effect of Forskolin + IBMX and Growth Factors on MAPK

Results from Figure 7.6 show that when serum-free medium is used to culture SVG cells, MAPK activity can be altered depending on what is added to the medium. To become active, the MAPK kinase subtypes ERK1 and ERK2 must be dually phosphorylated on threonine and thyrosine. Figure 7.6A is a western blot using anti-MAPK antibodies specific for the phosphorylated forms of the MAPK subtypes ERK1 and ERK2. When EGF is added to serum-free medium, in the SVG cells, the ERK2 subtype is most active (Figure 7.6 EGF). When cAMP-inducers are added to the medium, there is very little ERK2 activity (Figure 7.6; I, F, FI). When forskolin is added in addition to growth factors (EGF, NGF), there is a slight reduction in the active form compared to addition of the growth factor alone (Figure 7.6; F-EGF, F-NGF). TPA (12-O-tetradecanoylphorbol-13-acetate) will activate protein kinase C which will then phosphorylate and activate MAPK (Figure 7.6 TPA). Simultaneous addition of forskolin with TPA inhibit the activation of ERK2 (Figure 7.6 F-TPA). Figure 7.6B is a western blot using anti-MAPK antibodies specific for the inactive or non-phosphorylated form of the subtypes ERK1 and ERK2. Notice that the SVG cells express both ERK1 and ERK2 subtypes (Figure 7.6B), however, only the ERK2 is activated when cells are treated with growth factors such as EGF.

Figure 7.7 shows that when insulin is added to the serum-free medium there is a sustained activation of both MAPK subtypes ERK1 and ERK2 whether there is addition of growth factors, cAMP-inducers or no treatment at all. Figure 7.7A is a western blot using anti-MAPK antibodies specific for the phosphorylated forms of the MAPK subtypes ERK1 and ERK2. Unlike the growth factors EGF and NGF, which only

stimulate ERK2 phosphorylation, (Figure 7.6; EGF, NGF) insulin can activate both forms of MAPK (Figure 7.7). Figure 7.7B is a western blot using anti-MAPK antibodies specific for the inactive or non-phosphorylated form of MAPK.

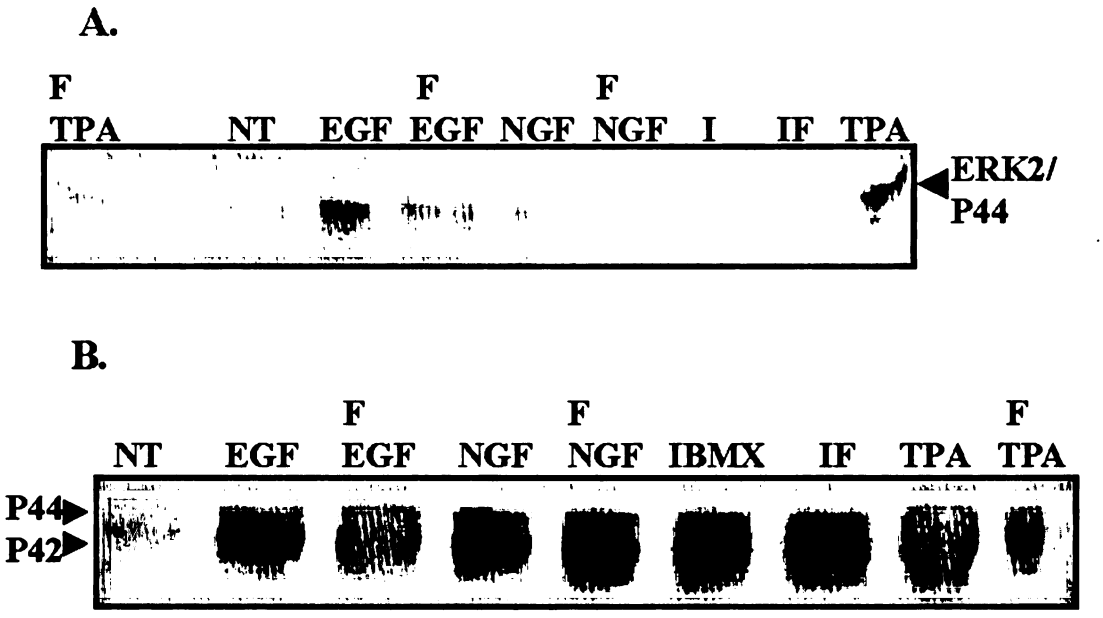


Figure 7.6. Western analysis of the mitogen activated protein kinase (MAPK) subtypes ERK1 and ERK2 after treatment with cAMP-inducers and growth factors in serum-free medium without insulin. (A) The activated or phosphorylated form of MAPK subtypes ERK1 and ERK2. (B) The inactive or unphosphorylated MAPK subtypes ERK1 and ERK2. NT = untreated; EGF = 100 ng/ml epidermal growth factor; NGF = nerve growth factor 100 ng/ml; F = 5 μM forskolin; I = 200 μM IBMX; FI = 5 μM forskolin + 200 μM IBMX; TPA = 1 μg/ml, 12-O-tetradecanoylphorbol-13-acetate.

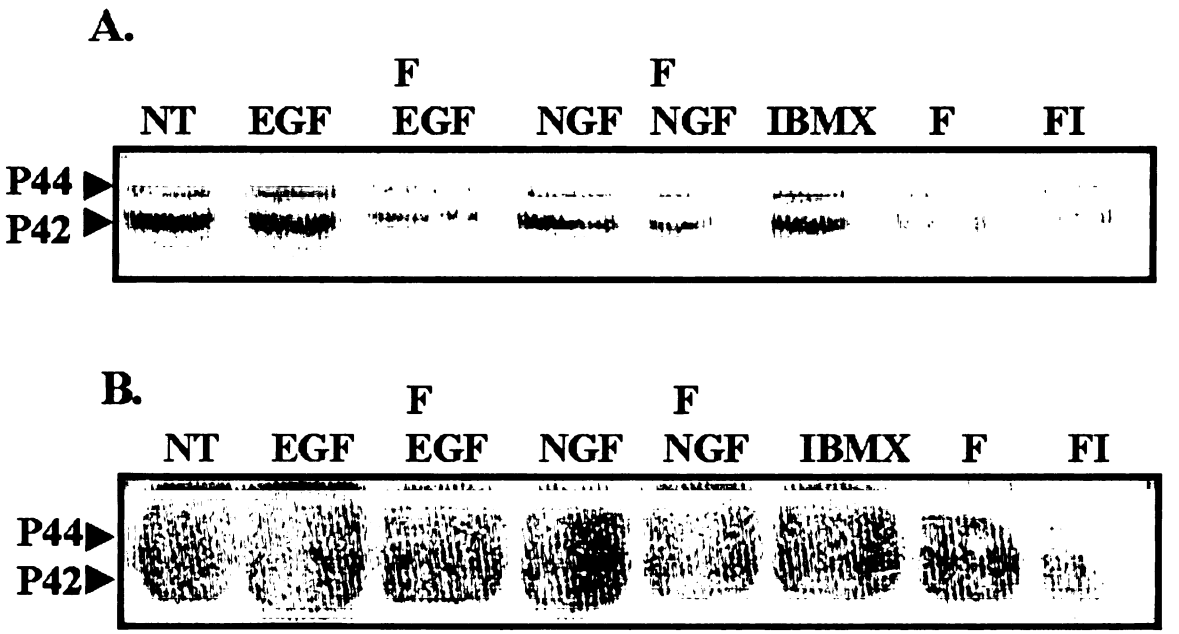


Figure 7.7. Western analysis of the mitogen activated protein kinase (MAPK) subtypes ERK1 and ERK2 after treatment with cAMP-inducers and growth factors in serum-free medium with insulin. (A) The activated or phosphorylated form of MAPK subtypes ERK1 and ERK2. (B) The inactive or unphosphorylated MAPK subtypes ERK1 and ERK2. NT = untreated; EGF = 100 ng/ml epidermal growth factor; NGF = nerve growth factor 100 ng/ml; F = 5 μM forskolin; I = 200 μM IBMX; FI = 5 μM forskolin + 200 μM IBMX.

7.7. – Conclusions

Results from RT-PCR reveal that there are changes in BDNF and CNTF mRNA upon treatment with forskolin + IBMX while the steady-state level of NGF mRNA remains constant. Results from western analysis reveal that the steady-state level of NGF and BDNF protein appears to increase after 24 hrs treatment with the forskolin + IBMX. Both results provide some preliminary evidence that neurotrophic factors may be regulated by cAMP in the SVG cells. Furthermore, the upregulation in the mRNA of BDNF appears at a time prior to neurite outgrowth suggesting that it could play a role in initiation of a morphological change if these cells do in fact have BDNF receptors.

Western analysis and even RT-PCR are not the best ways to examine these factors although they do demonstrate that the SVG cells express these factors, an observation that has not been documented for BDNF or CNTF in this cell line. The next step in investigating a possible role for neurotrophic factors in the differentiation of the SVG cells is to quantitatively measure the timing of secretion of these factors in the cell supernates or protein extracts to determine whether there is an increase in their secretion upon stimulation of the cAMP pathway. Furthermore, it remains to be demonstrated whether the SVG cells have receptors for the neurotrophic factors described here.

Analysis of the effect of growth factors on the morphology of the SVG cells reveals that individual growth factors can inhibit the morphology change. Although insulin (24 hr treatment) appears to stimulate a similar change as seen in cultures treated with forskolin and IBMX alone incubations beyond 24 hr reveal an inability to maintain the morphological process outgrowth. Analysis of MAPK subtypes and their activity when

cells are treated with growth factors shows the ERK2 subtype is active particularly with EGF treatment.

A very different result is observed when insulin is added to the medium. Both MAPK subtypes ERK1 and ERK2 are active and concomitantly there is a more substantial morphological change with the insulin / forskolin + IBMX treatment than with the EGF / forskolin + IBMX treatment. The differential activity of the two MAPK subtypes may underlie the ability of the SVG cells to differentiate or proliferate, as well as, the duration of the MAPK activation.

In PC12 cells, mitogens like EGF stimulate proliferation while NGF induces differentiation due, in part, to differences in MAPK activity (Graves et al., 1995). Furthermore, ERK1 is the subtype that is most active in PC12 cells when they differentiate (Frödin et al., 1994). However, in the SVG cells ERK2 is the subtype that is activated by EGF, NGF, and TPA. It is possible that in the SVG cells, ERK2 is responsible for signaling events involved in proliferation. When insulin or serum is added, there is activation of both ERK1 and ERK2 in the SVG cells. The SVG cells may require ERK1 activation for signaling events involved in differentiation. However, because ERK2 is still active when insulin or serum is added to the medium, differentiation signals may be overridden and the cells eventually lose the differentiated phenotype.

The active MAPK may be the reason that the SVG cells will not fully differentiate when placed in medium containing EGF, insulin, or serum. These results show that unlike PC12 cells, SVG cells do not respond to growth factor treatment by extending processes neither do they show a synergistic differentiation when treated with growth

factors and cAMP-inducers. In fact, SVG cells are just the opposite; growth factors appear to inhibit the differentiation. However, it may be the environment that determines whether the mitogen activated protein kinase pathway will override the cAMP pathway to allow or inhibit differentiation. From these results, it appears that in the SVG cells, the initial differentiation is MAPK-independent and that active MAPK subtype ERK2 may be inhibiting the differentiation.

CHAPTER 8 – DISCUSSION

8.1. - Overview

In this dissertation, the novel cell line, SVG, has been characterized in the context of its ability to express gap junction channels, exhibit dye coupling, and differentiate, to answer the hypothesis that gap junctional intercellular communication is a causative element in controlling and maintaining cellular differentiation. At least three significant findings have come out of this dissertation. The first finding is that the SVG cells express the three main connexin subtypes found in the central nervous system and significant increases in dye coupling and connexin protein expression can be upregulated by treatment of the cells with the compounds forskolin and IBMX. The second major finding demonstrates that the cells can differentiate when given the appropriate cellular environment, into at least two cell types, suggesting that the SVG cell line is a multipotent progenitor cell line. The differentiation is not reversible following removal of the differentiation inducing stimulus, suggesting that cAMP elevation, in the SVG cell line, not only induces differentiation but might also be a signal causing commitment to a particular cell lineage. Thirdly, it appears that the relationship between the upregulation in dye coupling and differentiation is highly correlative but not causative as these two events can be disassociated. Lastly, results show that a number of different factors are controlling the ability of the SVG cells to differentiate including cellular environment (serum vs. serum-free) and the control this has over proliferation and differentiation, extracellular signals such as possible secretion of neurotrophic factors, and density of cell plating.

8.2 – Significance of Connexin Protein Expression and Dye Coupling

The finding that the SVG cells express connexins 43, 32 and 26 simultaneously after treatment with forskolin + IBMX in serum-free medium makes this cell line a good model of the central nervous system as far as connexins are concerned. As discussed earlier, the mature central nervous system expresses all three connexins with a great number of cell types expressing Cx43, including astrocytes and neurons and a smaller subset of neurons and oligodendroyctes expressing Cx32 while Cx26 is found in populations of neurons as well. The SVG cells, like the CNS, express Cx43 ubiquitously while Cx32 is localized to smaller cell populations. Connexin 26 appears to be expressed in more cells than connexin 32 and is similar, in expression, to Cx43.

The need for stimulation of the cAMP-pathway to upregulate dye-coupling in the SVG cells suggests that regulation of the connexin subtypes in these cells is perturbed and that elevation of cAMP levels is one way to restore connexin functionality. Upregulation of gap junctional coupling by agents that increase intracellular concentrations of cAMP has been demonstrated in a number of cell types including hepatocytes (Sáez et al., 1989; Traub et al., 1987), fibroblasts (Flagg-Newton et al., 1981), lung epithelial cells (Banoub et al., 1996), sympathetic neurons (Kessler et al., 1984), and gonadotropin-releasing hormone neurons (Matesic et al., 1996).

In this study, an increase in intracellular coupling was observed after a 24-48 hr incubation with the cAMP-inducing agents. The earliest time point where dye coupling was observed, after forskolin + IBMX treatment, was 16 hr and the latest time point was 72 hours. There might have been further increases in dye coupling after a treatment period of 72 hr that were not measured, however, preliminary results from scrape load /

dye transfer showed that after 48 hr there was little change in dye coupling between the 48 and 72 hour time points. After about 48 hr – 72 hours dye did not transfer past the 4th row of cells from the scrape line suggesting a threshold level of cell-cell coupling after 48 hours. Further analysis of connexin protein showed that this slower increase in communication was consistent with an increased number of connexin 43, 32, and 26 plaques formed at the ends of cell processes. However, connexin 43 is post-translationally phosphorylated in brain, heart, and kidney (Hossian et al., 1994; Laird et al., 1991; Musil et al., 1990a, 1990b, 1991). It has also been documented that activation of the adenylyl cyclase/cAMP pathway can induce phosphorylation of Cx43 and Cx32 (Sáez et al., 1989, 1986). Phosphorylation of Cx43 is associated with a more rapid increase in cell-cell communication, taking only minutes instead of hours while Cx32 does not require phosphorylation to become functional (Sáez et al., 1986; ; Traub et al., 1987). The rapid increase in cell-cell communication was not observed in the SVG cells and the earliest time point at which cell-cell coupling was seen was 16hr, although, in Chapter 6, it was demonstrated that the Cx43 protein exists in a higher phosphorylated form after 1 min of treatment with cAMP-inducing compounds. One explanation for the slower onset of functional dye-transfer might be the need for increased protein synthesis, decreased breakdown of the connexin mRNA and protein, and trafficking of connexon hemichannels to the cell membrane. Evidence for this notion comes from the observation that very little Cx43, Cx32 or Cx26 protein is seen at membrane appositions between untreated SVG cells, after examination using immunofluorescent techniques. We have not investigated connexin transcription, degradation, or channel formation and trafficking in the SVG cells. However, defective connexin trafficking and packaging at the cell

membrane has been observed in other cell types where it was found that upregulation of the adenylyl cyclase/cAMP pathway would enhance connexin aggregation and channel formation (Sáez et al., 1990). Another explanation for the slower onset to functional dye-transfer is the cell plating density. In most cases the SVG cells were plated at a 50% cell density so that outgrowth of processes could take place. It is most likely that plating at a lower density did not allow for cell-to-cell membrane contact and hence no gap junctional communication until the SVG cells were able to extend processes and make membrane contact with each other.

Investigation of the effect of serum on Cx43, Cx32, and Cx26 in the SVG cells further supports the concept that an increase in the connexin half-life and/or a decrease in degradation at the membrane may be the mechanism by which cAMP is altering dye coupling and connexin protein expression. The serum-free environment drastically reduces DNA synthesis and the proliferative potential of the SVG cells (Table 4.1) which can lead to arrest of the cell cycle. It could be that in the serum-free environment connexins that are degraded (remember half-life is only 1 hr) are never replaced. In the serum-free environment, there is most likely a decrease in the amount of connexin DNA that is available to be transcribed and a subsequent decrease in connexin mRNA that is available for translation. Serum supports DNA synthesis and even though there is no significant dye coupling or connexin plaque formation, connexin DNA is still available for transcription and later translation so that connexin protein can be seen on western blots. When cAMP is elevated with forskolin and IBMX there is probably a stabilization of the connexin at the membrane (specifically the P2 form of Cx43), as well as a possible stabilization of the connexin mRNA so that more connexin can be translated and

trafficked to the membrane. The end result is an observed increase in total connexin protein seen on western blots in the serum-free environment.

8.3. – Significance of the Differentiation

Results show that after plating SVG cells in serum-free medium for 10-24 hours with the cAMP-inducers forskolin + IBMX, there was a change in cell morphology that could be maintained for 7 days in serum-free medium and a number of weeks after removal from the serum-free environment. The morphology is associated with an outgrowth of processes from cell bodies. The cells cultured in serum-free medium with forskolin + IBMX express many neuronal markers along with the morphological change but their morphology remains rather flat with shorter neurite-like processes than what is observed in true neurons. This may be due to lack of additional differentiation signals. Seigel et al. (1996) isolated and immortalized cerebellar cells from one-week-old rats. The immortalized cerebellar cells displayed many neuronal characteristics including glutamate toxicity, neurofilaments, neuron specific enolase, and did not show expression of any glial markers (GFAP or S100). Despite the neuronal characteristics these cells had very limited neurite process outgrowth and flattened cell bodies due to lack of further differentiation signals.

There could be at least three explanations for the observed morphological and biochemical change: (1) stimulation of the cAMP pathway induces astroglial cells to express neuronal type markers, (2) stimulation of the cAMP pathway induces structural and biochemical plasticity or (3) the SVG cell cultures represent a multipotent progenitor cell population with the ability to differentiate when placed in the proper environment.

It has been shown *in vivo* that astroglial cells, in cases of acute brain injury, will become reactive astrocytes that can express the neuron specific markers, neuron specific enolase and microtubule associated protein two (Lin et al., 1994). However, reactive astrocytes typically retain strong expression of the glial cell marker glial fibrillary acidic protein while gaining expression of neuron-specific markers. I found that SVG cells had weak expression of GFAP and lost expression of the GFAP when they were treated with the forskolin + IBMX. SVG cells then gained expression of the neuronal specific markers neural filament 200 and neuron specific enolase. Further, the treated SVG cells also gained expression of galactocerebroside and myelin basic protein; both proteins are components of myelin primarily found in oligodendrocytes and not typically found in reactive astrocytes or neurons. Other instances where cells have been shown to co-express glial markers and neuronal markers are in embryonic stem cell cultures that have been induced to differentiate. In some cases EGF-responsive precursor cells that have begun to differentiate for 2 hr-24 hr express GFAP and MAP 2abc (Rosser et al., 1997). This co-expression declines after 2 –7 days as the cells acquire neuronal or glial cell morphology (Rosser et al., 1997).

The SVG cells might display structural and biochemical plasticity upon elevation of cAMP in the serum-free environment. The CNS has a high degree of plasticity and changing the environment of neurons and astrocytes in specific CNS regions can elicit plastic changes involving morphology. One example of astrocyte plasticity is the hypothalamo-neurohypophysial system (HNS) within which lies the supraoptic nucleus (SON). Activation of the HNS by water deprivation or lactation in postpartum rats increases the percentage of neuronal somatic membrane in direct apposition (Hatton,

1997). This increase in neuronal interaction is due to retraction of glial processes from between the neurons which are then left with only small extracellular clefts to separate them (Hatton, 1997). The increase in axosomatic or dendritic synapses are postulated to facilitate an increase in excitability throughout the system and enhance release of neuronal peptides (Hatton, 1997). These structural changes are entirely reversible when the water deprivation is removed or the pups are weaned. In the SVG cells, however, the morphological and biochemical changes are not reversible after cells have been exposed to serum-free medium with treatment. Results in Chapter 4 show that cells that have differentiated do not reverse this differentiation upon removal of the differentiating stimulus.

The fact that the morphological and biochemical change was not reversible further suggests that the cells were differentiating rather than becoming reactive astrocytes, and that the differentiation is terminal. Reactive astrocytes that express neuronal antigens can reverse this phenotype where as the protein changes in the SVG cells are not reversible. The terminality of this differentiation is exemplified by the fact that placing the cells back into the medium with serum (without forskolin/IBMX treatment) does not reverse the processes extending phenotype as long as they have had prior exposure to the serum-free medium containing the forskolin/IBMX combination

However, removal of the forskolin/IBMX treatment by placing cells in serum-free medium causes detachment of all cells after 48 hours. Culturing SVG cells in the serum-free medium might cause the differenitated SVG cells to lose their ability to attach to the substrate or lose necessary cell adhesion molecules like NCAM. Factors in the serum might also be needed for cell survival, attachment to the substrate, and maintenance of

the differentiated phenotype. Culturing cells in medium containing serum along with the treatments does not induce this "terminal differentiation". Although some cells extend processes, the cultures are quickly overgrown with cells that resemble the untreated SVG controls possessing a very epithelial-like phenotype (Chapter 5). Similar results are observed when cells are given serum-free medium containing insulin with the forskolin + IBMX treatment (Chapter 7).

It is, therefore, concluded that treatment of the SVG cells with forskolin + IBMX is not generating reactive astrocytes or structural and biochemical plasticity but possibly inducing differentiation of a multipotent progenitor or stem cell population that can differentiate into oligodendrocytes or neurons, when placed under the appropriate growth conditions.

Evidence for the idea that these cells could represent a multipotent progenitor cell population comes from previous research showing that the three main cell types of the CNS (oligodendroyctes, neurons, and astrocytes) can develop from a multipotent progenitor cell isolated from the embryonic nervous system (Chalmers-Redman et al., 1997; McKay 1997; Reynolds et al., 1992, 1996; Weiss et al., 1996; Williams et al., 1991). Furthermore, changing the culture medium along with changes in the concentration and number of growth and neurotrophic factors have been shown to change the developmental pathway of progenitor cells *in vitro* (Raff et al., 1983; Chalmers-Redman et al., 1997; Gage 1998). Debate continues regarding the definition of a stem cell or progenitor cell, particularly in the CNS, and the criteria that have been established to distinguish between progenitor and stem cells are ill-defined. However, some common features that stem cells should exhibit are (1) the ability to proliferate, (2) exhibit self-

maintenance, (3) generate a large number of progeny including phenotypes of the tissue of origin, and (4) maintain multilineage potential (Reynolds and Weiss, 1996). Once a cell becomes restricted or committed to a lineage it is generally called a progenitor (Gage, 1998).

The SVG cells possess stem cell-like qualities in that they can self-renew when placed in medium containing serum or growth factors. However, due to the Simian virus 40 transfection/immortalization it is misleading to label the SVG cells as true "stem cells" because, at least in the nervous system, cells that are given the designation of "stem cell" are able to proliferate, self-renew, and differentiate without viral transfection. Due to their SV40 transfection/immortalization, it is difficult to determine whether these cells were ever true stem cells, because their ability to proliferate and self-renew might be solely due to SV40. However, two ways to test whether the SVG cells have the ability to proliferate and self-renew without the SV40 large T antigen are (1) to block production of the viral protein using antisense oligonucleotides or (2) inhibit the ability of SV40 large T antigen to bind RB and P53 by upregulation of a protein "sink" that would allow large T antigen binding without playing a role in the cell cycle.

The fact that the SVG cells can not reverse their differentiation, at least under the conditions described here, suggests that the SVG cells may represent a population of CNS progenitors that become committed with cAMP elevation. Commitment or lineage restriction in the CNS as defined by Gage, (1998) is a structural change in a DNA recombination event or a change in transcription factor expression that is passed on to each subsequent symmetric division. Commitment may also mean a change in DNA methylation or chromatin packing. The results showing that the SVG cells can not

reverse their phenotype or expression of protein markers suggests that whatever genetic changes have occurred they are irreversibly committed. However, although the SVG cells can not reverse their differentiated phenotype they still maintain the ability to proliferate, for a short time, as long as they are exposed to serum (possibly due to SV40 large –T transfection). This does not occur in true stem or progenitor cells isolated from the mammalian brain; once they are differentiated they can not proliferate. The finding that the SVG cells can still self-renew even after differentiation makes the SVG cell line a valuable tool for studying the underlying genetic changes necessary for commitment of CNS progenitor cells.

Analysis of the growth rate, or proliferation rate, of the SVG cells using cell counts and uptake of [^{3}H]thymidine clearly show that the serum-free environment coupled with the forskolin/IBMX treatment results in a significant decrease in the number of cells present in the cultures after three days as well as a significant decrease in DNA synthesis. It is also important to note that the serum-free environment alone can reduce the number of cells as well as DNA synthesis compared to untreated cultures grown in 5% or 10% serum. Cells grown in serum with treatment will not terminally differentiate. Although it appears that some cells will differentiate when exposed to forskolin/IBMX in medium with serum or serum-free medium with insulin, this differentiation is not maintained. The differentiation is either reversible or the morphologically differentiated cell dies leaving only the epithelial-like cells to grow.

Cells exposed to the serum-free environment terminally differentiate and it appears that the serum-free exposure is a crucial step in the ability of the SVG cells to differentiate regardless of treatment. The serum-free exposure may be the switch where

the SVG cells turn off genes critical for driving the cell cycle and inducing proliferation, and become committed.

The commitment in this case may be explained by changes in the cells' ability to respond to the same stimuli in different ways before and after the serum-free exposure with treatment. The mitogen activated protein kinase pathway (MAPK) could be a key signaling factor to determine whether the cells become committed and terminally differentiate or proliferate. As discussed in Chapter 7, SVG cells do express MAPK subtypes ERK1 and ERK2 (Figure 6.4). Depending on the culture medium and factors in it, both subtypes can be activated (as is the case with insulin or serum in the medium), inactive (serum-free medium), or only one subtype may be active (EGF, NGF). The differences in morphology observed with the different treatments can be associated with the different MAPK activities.

To obtain the terminal differentiation, SVG cells need to be exposed to elevated cAMP in a serum-free environment where MAPK is inactive. After cells have been exposed to serum-free medium with treatment for a prolonged period (> 7 days), they begin to detach from the substrate, suggesting that this condition can promote differentiation but not maintain the cells in the differentiated state. Results presented in Chapter 5 showed that once the cells had differentiated, placement in medium with serum could prolong the life of the differentiated cells for a number of days. In fact, treatment of cells with serum-free medium for 5 days, then removal of the serum-free treatment and re-feeding in Eagles minimum essential medium with 10% FBS can maintain the differentiated phenotype, even after subculturing.

These observations suggest that a signal derived from the MAPK cascade is insufficient to determine whether the SVG cells will grow or differentiate and that MAPK activation is interpreted in the context of growth, differentiation or cell-specific signals. Another notion might be that after serum-free exposure different MAPK subtypes are expressed in the SVG cells that provide signals for differentiation rather than proliferation. These ideas were not examined in this dissertation but it would be interesting to investigate the genetic changes that occur in the MAPK pathway after SVG cells are terminally differentiated.

It is also clear from results presented in Figure 5.7 that cell confluency is another factor in determining whether cells differentiate or continue to grow. When cells are grown in serum with the treatment they divide faster than if they are in the serum-free medium with the treatment as evidenced by Tables 4.1 and 4.2 in Chapter 4, and therefore, become more confluent than their counterparts grown without serum. In Figure 5.6 photographs of cells grown very close together show that cells clustered closely together tend not to extend processes while some of the cells on the outside of the clusters extend processes and morphologically differentiate. It may be that the physical barrier of having cells clustered together inhibits process extension or alternatively other cellular interactions including cell-cell communication may inhibit the morphological change. Cells that are tightly packed together may secrete factors of their own and have stronger cell – cell interactions that might be inhibitory to extension of processes while those cells on the outside of the clusters will have fewer cellular interactions and may respond better to the environmental stimuli for differentiation.

8.4 – Relationship between Differentiation and Cell-Cell Communication

Results from Western blot analysis of Cx43, Cx32, and Cx26 (Figure 5.2, Figure 5.3 and Figure 5.4) show that serum does not inhibit connexin expression as long as the cAMP-inducers are present in the medium. And, as already discussed, in every case serum seems to increase the amount of connexin protein observed in untreated cultures, although it is not to the level seen when treatment is added to the medium.

The results presented in Figure 5.7 show that even though there is no morphological change (Figure 5.7A) occurring in cultures plated very densely, there are still numerous Cx43 plaques at membrane oppositions between the cells as long as the cells are exposed to cAMP-inducing compounds. Conversely results presented in Figure 5.8 show that a morphological change can occur when cells have made no membrane contacts with other cells and that there is a definite lack of connexin 43 gap junctions between cells that are not touching (Figure 5.8A).

Results from the SL/DT analysis show that serum has no inhibitory effects on dye transfer in the SVG cells after plating in 5% or 10% fetal bovine serum (Figure 5.5) as long as treatment is added to the medium. A reduction or lack of dye transfer is only observed in cell cultures that are not treated with forskolin + IBMX. Furthermore, for the technique of SL/DT to work effectively, the cells need to be cultured to form a monolayer. As already discussed, when cells are closely packed to form a monolayer, they do not extend long processes possibly due to the physical barrier of other cells. However, as evidenced by Figure 5.5A, cells that are in the serum-free environment that are treated with forskolin + IBMX still transfer dye even though there is no morphological change.

The results presented in Chapter 5 clearly demonstrate that the upregulation in cell-cell communication and the observed morphological and biochemical differentiation are two independent events that have a highly correlative relationship in that when one occurs the other one is always present almost simultaneously. However, these events can be disassociated in a number of ways as follows:

1. Serum can inhibit the morphological change but does not inhibit dye-transfer or connexin protein expression.
2. Plating cells very densely can inhibit a morphological change but does not alter dye-transfer or connexin plaque formation on the membrane.
3. Plating cells so they do not touch other cells but are in close proximity to neighboring cells inhibits formation of connexin plaques but not the morphological change.
4. Plating cells so they do not touch other cells but are in close proximity to neighboring cells inhibits formation of connexin plaques but not the biochemical change.

One interesting observation that comes out of these results is that cells plated so there are no other cells around them, 500 cells/well (Figure 5.6b), will not morphologically or biochemically differentiate. This seems to suggest that cells need other cells around them, not necessarily to make physical cell-cell contact, but to receive factors secreted from other cells that may be important for cellular survival and differentiation. Furthermore, the importance of cells receiving extracellular signals is exemplified by the observation that cells that are close to other cells, but not touching, tend to extend processes towards those other cells rather than extending them in a direction where there is nothing. This suggests that secretion of some substance (possibly neurotrophic factors) from one cell may induce a neighboring cell to extend processes towards it.

As is evidenced by Figure 5.7d, cells that are plated so that they do make cell-cell contacts, but are not dense enough to inhibit process outgrowth, extend more processes per cell body than cells that do not make any cellular contacts at all. This observation suggests that perhaps intercellular communication is not necessary for initiating differentiation but may maintain the differentiation already established by passing a signal from cell-cell to induce formation of more cellular gap junction contacts by initiating outgrowth of processes.

8.5. - Conclusions

Figure 8.1 is a schematic bringing together the results showing the conditions required for differentiation. When SVG cells are cultured in the proliferation medium containing Eagles minimum essential medium (EMEM) with 5% or 10% fetal bovine serum (FBS) or serum-free medium with insulin MAPK (ERK1 and ERK2) is active and cells do not differentiate (Figure 8.1a). When 5 μM forskolin + 200 μM IBMX is added to the proliferation medium (PKA is now active at least for 24 hr) some cells morphologically differentiate (Figure 8.1 b) but most do not. The few cells that do differentiate either de-differentiate (Figure 8.1 c) or die (Figure 8.1 d) after prolonged culturing in the growth medium, while the non-differentiated cells grow (Figure 8.1 e). This suggests that in the growth medium cells are not able to respond to differentiation signals such as neurotrophic factor secretion or cAMP.

Removal of cells from the proliferation medium and culturing in serum-free medium with forskolin + IBMX enhances the number of cells that morphologically and biochemically differentiate (Figure 8.1 f). The outgrowth of processes and biochemical changes may be due to enhanced cellular response to secreted neurotrophic factors

(Figure 8.1 g). If cells are isolated from other cells, even in the serum-free environment with treatment, they do not differentiate possibly due to lack of extracellular signals from neighboring cells i.e. neurotrophic factor stimulation (Figure 8.1 h). If cells are plated too close to other cells there is no morphological differentiation most likely a result of the physical barrier of other cells or cellular interactions that inhibit the shape change (Figure 8.1 i).

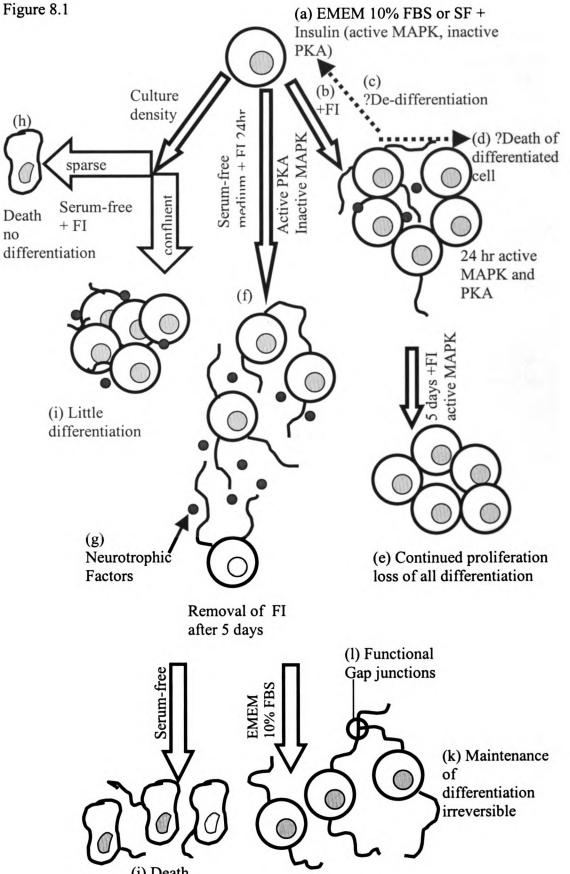
Cells that are cultured in the serum-free medium with forskolin + IBMX or serum-free medium alone for extended periods (7+ days) will eventually die (Figure 8.1 j). However, if cells growing in the serum-free medium with treatment are re-fed growth medium after 5 or more days they maintain their differentiated state (both biochemical and morphological) and survive (Figure 8.1 k). Cellular interactions may play a role in passing messages between cells that aid in maintenance of cellular differentiation. (Figure 8.1 l)

In the nervous system the relationship between gap junctional intercellular communication and differentiation is highly correlative and still not well defined. In some cases, particullary with *in vitro* experiments, there appears to be a causal relationship and blockage of gap junctional intercellular communication disrupts differentiation into neurons and glia (Bani-Yaghoub et al., 1999a, b). However in many *in vivo* systems, such as connexin 43 knockout mice (Perez-Velazquez et al., 1996) and the lens (Le and Musil, 1998), blockage of gap junctional communication has no effect on differentiation of any neuronal cell types or lens fiber cells. Furthermore, PC12 cells which have no functional gap junctional intercellular communication (Dowling-Warriner unpublished results, personal communication D.C. Spray Albert Einstein College of

Medicine Bronx, NY 10461) can differentiate into sympathetic neurons upon stimulation with nerve growth factor (Greene et al., 1976). Induction of intercellular communication in PC12 cells by transfection with various connexins is inhibitory to the differentiation (D.C. Spray Albert Einstein College of Medicine Bronx, NY 10461). The results presented in this dissertation show that the induced cell-cell communication does not appear to affect the initial morphological and biochemical differentiation. In conclusion the results presented in this dissertation do not support the hypothesis that gap junctional intercellular communication is a causative element in controlling cellular differentiation in the SVG model system but most likely is a consequence of the differentiated phenotype.

Figure 8.1. General schematic of cell behavior based on culturing conditions and activation of second messenger pathways.

Figure 8.1


(a) EMEM 10% FBS or SF + Insulin (active MAPK, inactive PKA)
(b) +FI
(c) ?De-differentiation
(d) ?Death of differentiated cell
24 hr active MAPK and PKA
Culture density
(h)
sparse
Death no differentiation
Serum-free + FI
confluent
Serum-free medium + FI ?4hr
Active PKA Inactive MAPK
(f)
5 days +FI active MAPK
(i) Little differentiation
(g) Neurotrophic Factors
(e) Continued proliferation loss of all differentiation
Removal of FI after 5 days
(l) Functional Gap junctions
Serum-free
EMEM 10% FBS
(k) Maintenance of differentiation irreversible
(j) Death

REFERENCES

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Abudata V., Eyzaguirre C (1994) Electrical coupling between cultured glomus cells of the rat carotid body: observations with current and voltage clamping. *Brain Res.* **664,** 257-265.

Allendoerfer K. L., Cabelli R. J., Escandon E., Kaplan D. R., Nikolics K., Shatz C. J. (1994) Regulation of neurotrophin receptors during the maturation of the mammalian visual system. *J. Neurosci.* **14,** 1795-1781.

Anderson N. G., Maller J. L., Tonks N. K., Sturgill T. W. (1990) Requirement for integration of signals from two distinct phosphorylation pathways for activation of MAP kinase. *Nature* **343,** 651-653.

Andrews P. W., Damjanov I., Simon D., Banting G. S., Carlin C., Dracopoli N. C., Fogh J. (1984) Pluripotent embryonal carcinoma clones derived from the human teratocarcinoma cell line Tera-2. *Lab. Invest.* **50,** 147-162.

Anzini P., Neuberg D. H.-H., Schachner M., Nelles E., Willecke K., Zielaseh J., Toyka K., Suter U., Martini R. (1997) Structural abnormalities and devicient maintenance of peripheral nerve myelin in mice lacking the gap junction protein connexin 32. *J. Neurosci.* **17,** 4545-4551.

Arellano R. O., Ramón F., et al. (1988) Calmodulin acts as intermediary for the effect of calcium on gap junction from crayfish lateral axons. *J. Membr. Biol.* **101,** 119—131.

Arellano R. O., Rivera A., Ramón F., (1990) Protein phosphorylation and hydrogen ions modulate calcium-induced closure of gap junction channels. *Biophys. J.* **57,** 363-367.

Arsenijevic Y., Weiss S. (1998) Insulin-like growth factor-I is a differentiation factor for postmitotic CNS stem cell-derived neuronal precursors: distinct actions from those of brain-derived neurotrophic factor. *J. Neurosci.* **18,** 2118-2128.

Atkinson M. M., Menko A. S., Johnson R. G., Sheppard J. R., Sheridan J. D. (1981) Rapid and reversible reduction of junctional permeability in cells infected with a temperature sensitive mutant of avian sarcoma virus. *J. Cell Biol.* **91,** 573-578.

Atkinson M. M., Lampe P. D., Lin H. H., Kollander R., Li X.-R., Kiang D. T. (1995) Cyclic AMP modifies the cellular distribution of connexin43 and induces a persistent increase in the junctional permeability of mouse mammary tumor cells. *J. Cell Sci.* **108,** 3079-3090.

Azarnia R., Dahl G., Loewenstein W. R., (1981) Cell junction and cyclic AMP: III. Promotion of membrane permeability and junctional membrane particles in a junction-deficient cell type. *J. Membr Biol* **63,** 133-146.

Azarnia R., Reddy S., Kmiecik T. E., et al. (1988) The cellular *src* gene product regulates junctional cell-to-cell communication. *Science* **239**, 398-401.

Balice-Gordan R. J., Bone L. J., Scherer S. S. (1998) Functional gap junctions in the schwann cell myelin sheath. *J. Cell Biol.* **142**, 1095-1104.

Banoub R. W., Fernstorm M., Malkinson A. M., Ruch R J. (1996) Enhancement of gap junctional intercellular communication by dibutyryl cyclic AMP in lung epithelial cells. *Anticancer Res.***16**, 3715-3720.

Bani-Yaghoub M., Bechberger J. F., Naus C. C. G. (1997) Reduction of connexin43 expression and dye-coupling during neuronal differentiation of human ntera2/clone d1 cells. *J. Neurosci. Res.* **49**,19-31.

Bani-Yaghoub M., bechberger J. F., Underhill M. T., Naus C. C. G. (1999a) The effects of gap junction blockage on neuronal differentiation of human ntera2/clone d1 cells. *Experimental Neurology* **156**, 16-32.

Bani-Yaghoub M., Underhill M. T., Naus C. C. G. (1999b) Gap junction blockage interferes with neuronal and astroglial differentiation of mouse p19 embryonal carcinoma cells. *Developmental Genetics* **24**, 69-81.

Batter D. K., Kessler J. A. (1991) Region-specific regulation of preproenkephalin RNA in cultured astrocytes. *Molec. Brain Res.* **11**, 65-69.

Batter D. K., Corpina R. A., Roy C., Spray D. C. (1992) Heterogeneity in gap junction expression in astrocytes cultured from different brain regions. *Glia* **6**, 213-221

Belliveau D. J., Kidder G. M., Naus C. G. (1991) Expression of gap junction genes during postnatal neural development. *Dev. Genetics* **12**, 308-317.

Belliveau D. J., Bechberger J. F., Rogers K. A., Naus C. G. (1997) Differential expression of gap junctions in neurons and astrocytes derived from p19 embryonal carcinoma cells. *Dev. Genetics* **21**, 187-200.

Belliveau D. J., Naus C. C. (1995) Cellular localization of gap junction mRNAs in developing rat brain. *Dev. Neurosci.* **17**, 81-96.

Bevilacqua A., Loch-Caruso R., Erickson R. P. (1989) Abnormal development and dye coupling produced by antisense RNA to gap junction protein in mouse preimplantation embryos. *Proc. Natl. Acad. Sci. U. S. A.* **86**, 5444-5448.

Bennett M. V. L., Spray D. C., Harris A. L. (1981) Gap junctions and development. *Trends Neurosci.* **4**, 159-163.

Bennett M. V. L. (1997) Gap junctions as electrical synapses. *J. Neurocytology* 26, 349-366.

Bergoffen J., Scherer S. S., Wang S., Scott M. O., Bone L. J., Paul D. L., Chen K., Lensch M. W., Chance P. F., Fischbeck K. H. (1993) Connexin Mutations in X-linked Charcot-Marie-Tooth disease. *Science* **262**, 2039-2042.

Berthound V. M., Iwanji V., Garcia A. M., Sáez J. C. (1992a) Connexins and glucagon receptors during development of rat hepatic acinus. *Am. J. Physiol.* **263**, G650-G658.

Berthound V. M., Leadbetter M. L. S., Hertzberg et al. (1992b) Connexin43 in MDCK cells: regulation by a tumor-promoting phorbol ester and Ca^{+2}. *Eur. J. Cell Biol.* **57**, 40-50.

Berthound V. M., Rook M. B., Traub O., Hertzberg E. L., Sáez J. C. (1993) On the mechanisms of cell uncoupling induced by a tumor promoter phorbol ester in clone 9 cells, a rat liver epithelial cell line. *Eur. J. Cell Biol.* **62**:384-396.

Blanc E. M., Bruce-Keller A. J., Mattson M. P. (1998) Astrocytic gap junctional communication decreases neuronal vulnerability to oxidative stress-induced disruption of ca^{2+} homeostasis and cell death. *J. Neurochem.* **70**, 958-970.

Bottenstein J. E., Sato G. H. (1979) Growth of a rat neuroblastoma cell line in serum-free supplemented medium. *Proc. natn. Acad. Sci. U.S.A.* **76**, 514-517.

Boulton T. G., Yancopoulos G. D., Gregory J. S., Slaughter C., Moomaw C., Hsu J., Cobb M. H. (1990) An insulin-stimulated protein kinase similar to yeast kinases involved in cell cycle control. *Science* **249**, 64-67.

Brissett J. L., Kumar N. M., Gilula N. B., et al. (1991) The tumor promoter 12-O-tetradecanoylphorbol-13-acetate and the ras oncogene modulate expression an dphosphorylation of gap junction proteins. *Mol. Cell Biol.* **11**, 5364-5371.

Britz-Cunningham S. H., Shah M. M., Zuppan C. W., Fletcher W. H. (1995) Mutations of the *connexin43* gap-junction gene in patients with heart malformations and defects of laterality. *N. Engl. J. Med.* **332**, 1323-1329.

Bruzzone R., White T. W., Haefliger J.-A., Gimlich R. L., Paul D. L. (1993) Connexin40, a component of gap junctions in vascular endothelium, is restricted in its ability to interact with other connexins. *Mol. Biol. Cell* **4**, 7-20.

Bruzzone R., White T. W., Scherer S. S., Fischbeck K.-H., Paul D. L. (1994) Null mutations of connexin32 in patients with X-linkec Charcot-Marie-Tooth disease. *Neuron* **13**, 1253-1260.

Bruzzone R., White T. W., Yoshizahi G., Patiño R., Paul D. L. (1995) Intercellular channels in teleosts: functional characterization of two connexins from atlantic croaker. *FEBS Lett.* **358**, 301-304.

Bruzzone R., Ressot C. (1997) Connexins, gap junctions and cell-cell signalling in the nervous system. *Eur. J. Neurosci.* **9**, 1-6.

Bruzzone R., White T. W., Paul D. L. (1996a) Connections with connexins: the molecular basis of direct intercellular signaling. *Eur. J. Biochem.* **238**, 1-27.

Bruzzone R., White T. W., Goodenough D. A. (1996b) The cellular internet on-line with connexins. *BioEssays* **18**, 709-718.

Burt J. (1987) Block of intercellular communication: interaction of intercellular H^+ and Ca^{+2}. *Am. J. Physiol.* **253**, C607-C612.

Burt J. M., Spray D. C. (1988) Single-channel events and gating behavior of the cardiac gap junction channel *Proc. Natl. Acad. Sci. U. S. A.* **85**, 3431-3434.

Butterweck A., Elfgang C., Willecke K., Traub O. (1994) Differential expression of the gap junction proteins connexin45, -43, -40, -31, and -26 in mouse skin. *Eur. J. Cell Biol.* **65**, 152-163.

Cavanagh J. F. R., Mione M. C., Pappas I. S., Parnavelas J. G. (1997) Basic fibroblast growth factor prolongs the proliferation of rat cortical progenitor cell *in vitro* without altering their cell cycle parameters. *Cerebral Cortex* **7**, 293-302.

Caveney S. (1985) The role of junctions in development. *Ann. Rev. Physiol.* **47**, 319-335.

Chalmers-Redman R. M. E., Priestly T., Kemp J. A., Fine A. (1997) In vitro propagation and inducible differentiation of multipotential progenitor cells from human fetal brain. *Neuroscience* **76**, 1121-1128.

Chang C.-C., Trosko J. E., Kang H.-L., Bombick D., Matsumura F. (1985) Potential role of the *src* gene product in inhibition of gap-junctional communication in NIH/3T3 cells. *Proc. Natl. Acad. Sci. U. S. A.* **82**, 5360-5364.

Charles A. C., Merrill J. E., Dirksen E. R., Sanderson M. J. (1991) Intercellular signaling in glial cells: calcium waves and oscillations in response to mechanical stimulation and glutamate. *Neuron* **6**, 983-992.

Choudhry R., Pitts J, D., Hodgins M. B. (1997) Changing patterns of gap junctional intercellular communication and connexin distribution in mouse epidermis and hair follicles during embryonic development. *Dev. Dyn.* **210**, 417-430.

Church J., Baimbridge K. G. (1991) Exposure to high-ph medium increases the incidence and extent of dye coupling between rat hippocampal ca1 pyramidal neurons *in vitro*. *J. Neurosci.* **11**, 3289-3295.

Cole W. C., Garfield R. E. (1986) Evidence for physiological regulation of myometrial gap junction permeability. *Am. J. Physiol.* **251**, C411-C420.

Condorelli D. F., Parenti R., Spinella F., Salinaro A. T., Belluardo N., Cardile V., Cicirata F. (1998) Cloning of a new gap junction gene (cx36) highly expressed in mammalian brain neurons. *Eur. J. Neurosci.* **10**, 1202-1208.

Conners B. W., Benardo L. S., Prince D. A. (1983) Coupling between neurons of the developing rat neocortex. *J. Neurosci* **3**, 773-782.

Cornell-Bell A. H., Finkbeiner S. M., Cooper M. S., Smith S. J. (1990) Glutamate induces calcium waves in cultured astrocytes: long-range glial signaling. *Science* **247**, 470-473.

Craig C. G. Tropepe V., Morshead C. M., Reynolds B. A., Weiss S., Van der Koop D. (1996) In vivo growth factor expansion of endogenous subependymal neural precursor cell populations in the adult mouse brain. *J. Neurosci.* **16**, 2646-2658.

Crow J. M., Atkinson M. M., Johnson R. G (1994) Micromolar levels of intracellular calcium reduce gap junctional permeability in lens cultures. *Invest. Ophtalmol. Vis. Sci.* **35**, 3332-3341.

Crow D. S., Beyer E. C., Paul D. L., Kobe S. S., Lau A. F. (1990) Phosphorylation of connexin43 gap junction protein in uninfected and Rous sarcoma virus-transformed mammalian fibroblasts. *Mol. Cell. Biol.* **10**, 1754-1763.

Dahl G., Isenberg G. (1980) Decoupling of heart muscle cells: correlation with increased cytoplasmic calcium activity and with changes of nexus ultrastructure. *J. Membr. Biol.* **53**, 63-75.

Dahl E., Winterhager E., Reuss B., Traub O., Butterweck A., Willecke K. (1996) Expression of the gap junction proteins connexin31 and connexin43 correlates with communication compartments in extraembryonic tissues and in the gastrulating mouse embryo, respectively. *J. Cell Sci.* **109**, 191-197.

Dani J. W., Chernjavsky A., Smith S. J. (1992) Neuronal activity triggers calcium waves in hippocampal astrocyte networks. *Neuron* **8**, 429-440.

Davidson J. S., Baumgarten I. M. (1988) Glycyrrhetinic acid derivatives: a novel class of inhibitors of gap-junctional intercellular communication. structure-activity relationships. *J. Pharm. Exper. Therapeutics* **256**, 1104-1107.

Davies T. C., Barr K. S., Jones D. H., Zhu D., Kidder G. M. (1996) Multiple members of the connexin gene family participate in preimplantation development of the mouse. *Dev. Genet.* **18**, 234-243.

Dean J. B., Huang R.-Q., Erlichman J. S., Southard T. L., Hellard D. T. (1997) Cell-cell coupling occurs in dorsal medullary neurons after minimizing anatomical-coupling artifacts. *Neuroscience* **80**, 1997.

Dermietzel R., Schunke D., Leibstein A. (1978) The oligodendrocytic junctional complex. *Cell Tissue Res.* **193**, 61-72.

Dermietzel R. (1998) Gap junction wiring: a 'new' principle in cell-to-cell communication in the nervous system? *Brain Res. Reviews* **26**, 176-183.

Dermietzel R., Hertzberg E. L., Kessler J. A., Spray D. C. (1991) Gap junctions between cultured astrocytes: imunocytochemical, molecular, and electrophysiological analysis. *J. Neurosci.* **11**, 1421-1432.

Dermietzel R., Spray D. C. (1993) Gap junctions in the brain: where, what type, how many and why?. *Trends Neurosci.* **16**, 186-192.

Dermietzel R., Traub O., Hwang T. K., Beyer E., Bennett M. L. V., Spray D.C., Willecke K. (1989) Differential expression of three gap junction proteins in developing and mature brain tissues. *Proc. natn. Acad. Sci. U.S.A.* **86**, 10148-10152.

Dermietzel R., Farooq M., Kessler J. A., Althaus H., Hertzberg E. L., Spray D. C. (1997) Oligodendrocytes express gap junction proteins connexin32 and connexin45. *Glia* **20**, 101-114.

Dermietzel R. (1998) Gap junction wiring: a 'new' principle in cell-to-cell communication in the nervous system. *Brain Res. Rev.* **26**, 179-183.

Deschênes S. M., Walcott J. L., Wexler T. L., Scherer S. S., Fischbeck K. H., (1997) Altered trafficking of mutant connexin32. *J. Neurosci.* **17**, 9077-9084.

DeVries S. H., Schwartz E. A. (1989) Modulation of an electrical synapse betweensolitary pairs of catfish horizontal cells by dopamine and second messengers. *J. Physiol. Lond.* **414**, 351-375.

Doyle J. P., Coleman D. R. (1993) Glial-neuron interactions and regulation of myelin formation. *Curr. Opin. Cell Biol.* **5**, 779-785.

Eghbali B., Kessler J. A., Reid L. M., Roy C., Spray D. C. (1991) Involvement of gap junctions in tumorogenesis: transfection of tumor cells with connexin 32 cDNA retards growth in vivo. *Proc. Natl. Acad. Sci U. S. A.* **88**, 10701-10705.

Ek J. F., Delmar M., Perzova R., Taffet S. M. (1994) Role of histidine 95 on pH gating of the cardiac gap junction protein connexin 43. *Circ. Res.* **74**, 1058-1064.

El Aoumari A., Fromaget C., Dupont E., Reggio H., Durbec P., Briand J.-P., Boller K., Kretman B., Gros D. (1990) Conservation of a cytoplasmic carboxy-terminal domain of connnexin43, a gap junctional protein in mammalian heart and brain. *J. Membr. Biol.* **115**, 229-240.

Elfgang C., Eckert R., Lichtenberg-Fraté H., Butterweck A., Traub O., Klein R. A., Hulser D. F., Willecke K. (1995) Specific Permeability and selective formation of gap junction channels in connexin transfected HeLa cells. *J. Cell Biol.* **129**, 805-817.

el-Sabban M. E., Pauli B. U. (1991) Cytoplasmic dye transfer between metastatic tumor cells and vascular endothelium. *J. Cell Biol.* **115**, 1375-1382.

Enomoto T., Yamasaki H. (1984) Lack of intercellular communication between chemically transformed and surrounding nontransformed BALB/c3T3 cells. *Cancer Res.* **44**, 5200-5203.

Fallon M. B., Nathanson M. H., Mennone A., Saez J. C., Burgstahler A. D., Anderson J. M. (1995) Altered expression and function of hepatocyte gap junctions after common bile duct ligation. *Am. J. Physiol.* **269**, C1186-C1194.

Flagg-Newton J. L., Dahl G., Loewenstein W. R. (1981) Cell junction and cyclic AMP. I. upregulation of junctional membrane permeability and junctional membrane particles by administration of cyclic nucleotide or phosphodiesterase inhibitor. *J. Membr. Biol.* **63**, 105-121.

Fraser S. E., Green C. R., Bode H. R., Gilula N. B. (1987) Selective disruption of gap junctional communication interferes with a patterning process in hydra. *Science* **237**, 49-55.

Frödin M., Peraldi P., Van Obberghen E. (1994) Cyclic AMP acitvates the mitogen-activated protein kinase cascade in PC12 cells. *J. Biol. Chem.* **269**, 6207-6214.

Furshpan E. J., Potter D. D. (1959) Transmission at the giant motor synapses of the crayfish. *J. Physiol.* (London) **145**, 289-325.

Gage F. H. (1998) Discussion point stem cells of the central nervous system. *Curr. Opin. Neurobiol.* **8**, 671-676.

Gahr M., Garcia-Segura L. M. (1996) Testosterone-dependent increase of gap-junctions in hvc neurons of adult female canaries. *Brain Res.* **712**, 69-73.

Gensburger C., Labourdette G., Sensenbrenner M. (1987) Brain basic fibroblast growth factor stimulates the proliferation of rat neuronal precursor cells in vitro. *FEBS Lett.* **217**, 1-5.

Giaume C., Fromaget C., el Aoumari A., Cordier J., Glowinski J., Gros D. (1991) Gap junctions in cultured astrocytes: single channel currents and characterization of channel-forming protein. *Neuron* **6**, 133-143.

Giaume C., McCarthy K. D. (1996) Control of gap-junctional communication in astrocytic networks. *Trends Neurosci.* **19**, 319-325.

Giblin L. J., Christensen B. N. (1997) Connexin43 immunoreactivity in the catfish retina. *Brain Res.* **755**, 146-150.

Goldberg G S, Moreno A P, Bechberger J F, Hearn S S, Shivers R R, MacPhee D J, Zhang Y-C, Naus C G (1996) Evidence that disruption of connexon particle arrangements in gap junction plaques is associated with inhibition of gap junctional communication by a glycyrrhetinic acid derivative. *Exp. Cell Res.* **222**, 48-53.

Goldsmith P. C., Thind K. K. (1993) Morphological basis for neural control of GnRH secretion in the monkey. In *The Neurobiology of Puberty*. (eds Plant T. M. and Lee P. A.) pp. 73-85. J. Endocrinol. Ltd., London.

Goliger J. A., Paul D. L. (1995) Wounding alters epidermal connexin expression and gap junction-mediated intercellular communication. *Mol. Biol. Cell* **6**, 1491-1501.

Goliger J. A., Paul D. L. (1994) Expression of gap junction proteins Cx26, Cx31.1, Cx37, and Cx43 in developing and mature rat epidermis. *Dev. Dyn.* **200**, 1-13.

Gomez N., Tonks N. K., Morrison C., Harmar T., Cohen P. (1990) Evidence for communication between nerve growth factor and protein tyrosine phosphorylation. *FEBS Lett.* **271**, 119-122.

Gourdie R. G., Green C. R., Severs N. J., Thompson R. P. (1992) Immunolabelling patterns of gap junction connexins in the developing and mature rat heart. *Anat. Embryol.* **185**, 363-378.

Granda B., Tabernero A., Sánchez-Abarca I. L., Medina J. M. (1998) The K-ATP channel regulates the effect of Ca2+ on gap junction permeability in cultured astrocytes. *FEBS Lett.* **427**, 41-45.

Graves J. D., Campbell J. S., Krebs E. G. (1995) Protein serine/threonine kinases of the MAPK cascade. *Ann. N.Y. Acad. Sci.*. **766**, 320-343.

Greene L. A., Tischler A. S. (1976) Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma which respond to nerve growth factor. *Proc nant. Acad. Sci. U.S.A.* **73**, 2424-2428.

Gritti A., Parati E. A., Cova L., Frolichsthal P., Galli R., Wanke E., Faravelli L., Morassutti D. J., Roisen F., Nickel D. D., Vescovi A. L. (1996) Multipotent stem cells from the adult mouse brain proliferate and self-renew in response to basic fibroblast growth factor. *J. Neurosci.* **16**, 1091-1100.

Gunning P. W., Landreth G. E., Bothwell M. A., Shooter E. M. (1981) Differential and synergistic actions of nerve growth factor and cyclic AMP in PC12 cells. *J. Cell Biol.* **89**, 240-245.

Hanson M. G., Shen S., Wiemelt A. P., McMorris F. A., Barres B. A. (1998) Cyclic AMP elevation is sufficient to promote survival of spinal motor neurons in vitro. *J. Neurosci.* **18**, 7361-7371.

Hatton G. I., Yang Q. Z., Cobbett P. (1987) Dye coupling among immunocytochemically identified neurons in the supraoptic nucleus: increased incidence in lactating rats. *Neuroscience* **21**, 923-930.

Hatton G. I. (1997) Function-related plasticity in hypothalamus. *Ann. Rev. Neurosci.* **20**, 375-97.

Heese K., Fiebich B. L., Baver J., Otten V. (1997) Nerve growth factor (NGF) expression in rat microglia is induced by adenosine A2a-receptors. *Neurosci. Lett.* **23**, 83-86.

Heidemann S. R., Joshi H. C., Schechter A., Fletcher J. R., Bothwell M. (1985) Synergistic effects of cyclic AMP and nerve growth factor on neurite outgrowth and microtubule stability of PC12 cells. *J. Cell Biol.* **100**, 916-927.

Herrmann K., Antonini A., Shatz C. J. (1994) Ultrastructural evidence for synaptic interactions between thalamocortical axons and subplate neurons. *Eur. J. Neurosci.* **6**, 1729-1742.

Hertzberg E. L., Van Eldik L. J. (1987) Interaction of calmodulin and other calcium-modulated proteins with gap junctions. *Methods Enzymol.* **139**, 445-455.

Hirschi K K, Xu C, Tsukamoto T, Sager R (1996) Gap junction genes cx26 and cx43 individually suppress the cancer phenotype of human mammary carcinoma cells and restore differentiation potential. *Cell Growth and Differentiation* **7**, 861-870.

Hosny J., Jennes L., (1998) Identification of gap junctional connexin-32 mRNAs and protein in gonadotropin-releasing hormone neurons of the female rat. *Neuroendocrinology* **67**, 101-108.

Hossain M. Z., Murphy L. S., Hertzberg E. L., Nagy J. I. (1994) Phosphorylated forms of connexin43 predominate in rat brain: demonstrated by rapid inactivation of brain metabolism. *J. Neurochem.* **62**, 2394-2403.

Huang G. Y., Cooper E. S., Waldo K., Kirby M. L., Gilula N. B., Lo C. W. (1998) Gap junction-mediated cell-cell communication modulates mouse neural crest migration. *J. Cell Biol.* **143**, 1725-1743.

Huang R.-Q., Erlichman J. S., Dean J. B. (1997) Cell-cell coupling between co_2-excited neurons in the dorsal medulla oblongata. *Neuroscience* **80**, 41-57.

Jiang J. X., White T. W., Goodenough D. A., Paul D. L. (1994) Molecular cloning and functional characterization of chick lens fiber connexin 45.6. *Mol. Cell Biol.* **5**, 363-373.

Johnston M. F., Ramón F. (1981) Electrotonic coupling in internally perfused catfish segmented axons. *J. Physiol.* (Lond) **317**, 509-518.

Jou Y. S., Matesic D., Dupont E., Lu S. C., Rupp H. L., Madhukar B. V., Oh S. Y., Trosko J. E., Chang C.-C. (1993) Restoration of gap-junctional intercellular communication in a comunication-deficient rat liver cell mutant by transfection with connexin 43 DNA. *Mol. Carcinogenesis* **8**, 234-244.

Kadle R., Zhang J. T., Nicholson B. J. (1991) Tissue-specific distribution of differentially phosphorylated forms of Cx 43. *Mol Cell Biol.* **11**, 363-369.

Kalimi G. H., Lo C. W. (1989) Gap junctional communication in the extraembryonic tissues of the gastrulating mouse embryo. *J. Cell Biol.* **109**, 3015-3026.

Kalyani A., Hobson K., Rao M. S. (1997) Neuroepithelial stem cells from the embryonic spinal cord: isolation, characterization, and clonal analysis. *Dev. Biol.* **186**, 202-223.

Kandler K. (1997) Coordination of neuronal activity by gap junctions in the developing neocortex. *Semin. Dev. Biol.* **8**, 43-51.

Kandler K., Katz L. C. (1998a) Coordination of neuronal activity in developing visual cortex by gap junction-mediated biochemical communication. *J. Neurosci.* **18**, 1419-1427.

Kandler K., Katz L. C. (1998b) Relationship between dye coupling and spontaneous activity in developing ferret visual cortex. *Dev. Neurosci.* **20**, 59-64.

Kanemitsu M. Y., Lau A. F. (1993) Epidermal growth factor stimulates the disruption of gap junctional communication and connexin43 phosphorylation independent of 12-O-tetradecanoylphorbol-13-acetate-sensitive protein kinase C: the possible involvement of mitogen-activated protein kinase. *Mol. Biol. Cell* **4**, 837-848.

Kessler J. A., Spray D. C., Sáez J. C., Bennett M. V. (1984) Determination of synaptic phenotype: insulin and cAMP independently initiate development of electrotonic coupling between cultured sympathetic neurons. *Proc. nant. Acad. Sci. U.S.A.* **81**, 6235-6239.

Kessler J. A., Spray D. C., Sáez J. C., Bennett M. V. L. (1985) In *Gap Junctions*, M. V. L. Bennett and D. C. Spray eds. (Cold Spring Harbor Press) 231-240.

Kidder G. M., Rains J., McKeon J. (1987) Gap junction assembly in the preimplantation mouse conceptus is independent of microtubules, microfilaments, cell flattening, and cytokines. *Proc. Natl. Acad. Sci U. S. A.* **84**, 3718-3722.

Kilpatrick T. J., Bartlett P. F. (1995) Cloned multipotential precursors from the mouse cerebrum require FGF-2, whereas glial restricted precursors are stimulated with either FGF-2 or EGF. *J. Neurosci.* **15**, 3653-3663.

Kirby M. L. (1993) Cellular and molecular contributions of the cardiac neural crest to cardiovascular development. *Trends Cardiovas. Med.* **3**, 18-23.

Kirby M. L., Waldo K. L. (1995) Neural crest and cardiovascular patterning, *Circ. Res.* **77**, 211-215.

Kitchens D. L., Snyder E. Y., Gottlieb D. I. (1994) FGF and EGF are mitogens for immortalized neuronal progenitors. *J. Neurobiol.* **25**, 797-807.

Kolb H., Famiglietti (1974) Rod and cone pathways in the inner plexiform layer of cat retina. *Science* **186**, 47-49.

Knobil E. (1980) The neuroendocrine control of the menstral cycle. *Rec. Prog. Horm. Res.* **36**, 53-88.

Krsmanovic L. Z., Stojilkovic S. S., Merelli F., Dufour S. M., Virmani M. A., Catt K. J. (1992) Calcium signaling and episodic secretion of gonadotrophin-releasing hormone in hypothalamic neurons. *Proc. Natl. Acad. Sci U. S. A.* **89**, 8462-8466.

Krutovskikh V., Mazzoleni G., Mironov N., Omori Y., Aguelon A.-M., Mesnil M., Berger F., Partensky C., Yamaski H. (1994) Altered homologous and heterologous gap-junctional intercellular communication in primary human liver tumors associated with aberrant protein localization but not gene mutation of connexin32. *Int. J. Cancer* **56**, 87-94.

Kuhn H. G., Winkler J., Kempermann G., Thal L. J., Gage F. H. (1997) Epidermal growth factor and fibroblast growth factor-2 have different effects on neural progenitors in the adult mouse brain. *J. Neurosci.* **17**, 5820-5829.

Kukekov V. G., Laywell E. D., Thomas L. B., Steindler D. A. (1997) A nestin-negative precursor cell from the adult mouse brain gives rise to neurons and glia. *Glia* **21**, 399-407.

Kumar N. M., Gilula N. B. (1996) The gap junction communication channel. *Cell* **84**, 381-388.

Kwak B. R., Hermans M. M. P., De Jonge H. R., Lohmann S. M., Jongsma H. J., Chanson M (1995) Differential regulation of distinct types of gap junction channels by similar phosphorylating conditions. *Mol. Biol. Cell* **6**, 1707-1719.

Laemmli N. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Laird D. W., Puranam K. L., Revel J. P. (1991) Turnover and phosphorylation dynamics of connexin43 gap junction protein in cultured cardiac myocytes. *Biochem. J.* **273**, 67-72.

Lang L. M., Beyer E. C., Schwartz A. L., Gitlin J. D. (1991) Molecular cloning of rat uterine gap junction protein and analysis of gene expression during gestation. *Am. J. Physiol.* **260**, E787-E793.

Lasater E. M. (1987) Retinal horizontal cell gap junctional conductance is modulated by dopmaine through a cAMP-dependent protein kinase. *Proc. Natl. Acad. Sci U. S. A.* **84**, 7319-7323.

Lau A. F., Kanemitsu M. Y., Kruata W. E., Danesh S., Boynton A. L. (1992) Epidermal growth factor disrupts gap-junctional communication and induces phosphorylation of connexin43 on serine. *Mol. Biol. Cell* **3**, 865-874.

Lazark A., Peracchia C. (1993) Gap junction gating sensitivity to physiological internal calcium regardless of pH in Novikoff hepatoma cells. *Biophys. J.* **65**, 2002-2012.

Le A.-C. N., Musil L. S. (1998) Normal differentiation of cultured lens cells after inhibition of gap junction-mediated intercellular communication. *Dev. Biol.* **204**, 80-96.

Lee S., Gilula N. B., Warner A. E. (1987) Gap junctional communication and compaction during preimplantation stages of mouse development. *Cell* **51**, 851-860.

Lee S. H., Kim W. T., Cornell-Bell A. H., Sontheimer H. (1994) Astrocytes exhibit regional specificity in gap-junction coupling. *Glia* **11**, 315-325.

Lee S. W., Tomasetto C., Paul D., keyomarsi R., Sager R. (1992) Transcriptional downregulation of gap-junction proteins blocks junctional communication in human mammary tumor cell lines. *J. Cell Biol.* **118**, 1213-21.

Lehman M. N., Silverman A. J. (1988) Ultrastructure of luteinizing hormone-releasing hormone (LHRH) neurons and their projections in the golden hamster. *Brain Res. Bull.* **20**, 211-221.

Li J., Hertzberg E. C., Nagy J. I. (1997) Connexin32 in oligodendrocytes and association with myelinated fibers in mouse and rat brain. *J. Comp. Neurol.* **379**, 571-579.

Li R., Gao W. Q., Mather J. P. (1996) Multiple factors control the proliferation and differentiation of rat early embryonic (day 9) neuroepithelial cells. *Endocrine* **5**, 205-217.

Lin R. C. S., Matesic D. F. (1994) Immunohistiochemical demonstration of neuron-specific enolase and microtubule-associated protein 2 in reactive astrocytes after injury in the adult forebrain. *Neuroscience* **60**, 11-16.

Liu S., Taffet S., Stoner L., Delmar M., Vallano M. L., Jalife J. (1993) A structural basis for unequal sensitivity of the major cardiac and liver gap junctions to intracellular acidification: the carboxyl tail length. *Biophys. J.* **64**, 1422-1433.

Lo C. W., Cohen M. F., Huang G.-Y., Lazatin B. O., Patel N., Sullivan R., Pauken C., Park S. M. J. (1997) Cx43 gap junction gene expression and gap junctional communication in mouse neural crest cells. *Dev. Gene.* **20**, 119-132.

Loewenstein W., R. (1981) Junctional intercellular communication: the cell-cell membrane channel. *Physiol. Rev.* **61**, 829-913.

Loewenstein W. R., Rose B. (1992) Semin. Cell Biol. 3, 89-79.

Lye S. J., Nicholson B. J., Mascarenhas M., MacKenzie L., Petrocelli T. (1993) Increased expression of connexin-43 in the rat myometrium during labor is associated with an increase in the plasma estrogen:progesterone ratio. *Endocrinology.* 132, 2380-2386.

MacVicar B., Dudek F. E. (1981) Electrotonic coupling between pyramidal cells : a direct demonstration in rat hippocampal slices. *Science* **213**, 782-784.

MacVicar B. A., Dudek F. E. (1982) Electrotonic coupling between granule cells of rat dentate gyrus: physiological and anatomical evidence. *J. Neurophysiol.* **47**, 579-592.

MacVicar B. A., Jansen H. (1985) Uncoupling of CA1 pyramidal neurons by propionate. *Brain Res.* **330**, 141-145.

Major E. O., Miller A. E., Mourrain P., Traub R. G., Widt E. D. (1985) Establishment of a line of fetal glial cells that supports virus multiplication. *Proc. nant. Acad. Sci. U.S.A.* **82**, 1275-1261.

Mark M. D., Liu Y., Wong S. T., Hinds T. R., Storm D. R. (1995) Stimulation of neurite outgrowth in PC12 cells by EGF and KCL depolarization: a Ca^{+2}-independent phenomenon. *J. Cell Biol.* **130**, 701-710.

Marshall J. C., Kelch R. P. (1986) Gonadotropin-releasing hormone: role of pulsatile secretion in the regulation of reproduction. *N. Eng. J. Med.* **315**, 1459-1468.

Matesic D. F., Hayashi T., Trosko J. E., Germak J. A. (1996) Upregulation of gap junctional intercellular communication in immortilized gonadotropin releasing hormone neurons by stimulation of the cyclic AMP pathway. *Neuroendocrinology* **64**, 286-297.

Matesic D. F., Germak J., Dupont E., Madhukar B. V. (1993) Immortalized hypothalamic LHRH neurons express a connexin 26-like protein and display functional gap junctional coupling assayed by flourescence recovery after photobleaching. *Neuroendocrinology* **58**, 485-492.

Matesic D. F., Rupp H. L., Bonney W. J., Ruch R.J., Trosko J. E. (1994) Changes in gap-junction permeability, phosphorylation, and number mediated by phorbol ester and non-phorbol-ester tumor promoters in rat liver epithelial cells. *Molecular Carcinogenesis* **10**, 226-236.

Matsumoto A., Arnold A. P., Zampighi G. A., Micevych P. E. (1988) Androgenic regulation of gap junctions between motoneurons in the rat spinal cord. *J. Neurosci.* **8**, 4177-4183.

Matsumoto A. (1997) Hormonally induced neuronal plasticity in the adult motoneurons. *Brain Res. Bulletin* **44**, 539-547.

Mazzoleni G., Camplani A., Telò P., Pozzi A., Tenganelli S., Elfgang C., Willecke K., Ragnotti G. (1996) Effect of tumor-promoting and anti-promoting chemicals on the viability and junctional coupling of human HeLa cells transfected with DNAs coding for various murine connexin proteins. *Comp. Biochem. Physiol. C Pharmocol. Toxicol. Endocrinol.* **113**, 247-256.

McBurney M. W., Rogers B. J. (1982) Isolation of male embryonal carcinoma cells and their chromosome replication patterns. *Dev. Biol.* **89**, 503-508.

McConnell S. K. (1995) Constructing the cerebral cortex: neurogenesis and fate determination. *Neuron* **15**, 761-768.

McKay R. (1997) Stem cells in the central nervous system. *Science* **276**, 66-71.

McHahon D. C. Brown D. R. (1994) Modulation of gap junction channel gating in zebrafish retinal electrical synapses. *J. Neurophysiol.* **72**, 2257-2267.

Mehta P. P., Bertram S. S., Loewenstein W. R. (1986) Growth inhibition of transformed cells correlates with their junctional communication with normal cells. *Cell* **44**, 187-196.

Mehta P., Holtz-Wagbenblatt A., Rose F., Shalloway D., Loewenstein W. (1990) Incorporation of the gene for a cell-cell channel protein into transformed cells leads to normalization of growth. J. Membr. Biol. 124, 207-225.

Mehta P. P., Loewenstein W. R. (1991) Differential regulation of communication by retinoic acid in homologous and heterologous junctions between normal and transformed cells. *J. Cell Biol.* **113**, 371-9.

Mellon P. L., Windle J. J., Goldsmith P. P., Padula C. A., Robert J. L., Weiner R. I. (1990) Immortalization of hypothalamic GnRH neurons by genetically targeted tumorgenesis. *Neuron* **5**, 1-10.

Melrose P., Gross L., Cruse J., Rush M. (1987) Isolated gonadotropin-releasing hormone neurons harvested from adult male rats secrete biologically active neuropeptide in a regular, repetitive manner. Endocrinol. 121, 993-999.

Mesnil M., Fraslin J. M., Piccoli C., Yamasake H., Guguen-Guillouzo C. (1987) Cell contact but not functional communication (dye coupling) with biliary epithelial cells is required for hepatocytes to maintain differentiated functions. *Exp. Cell Res.* **173**, 524-533.

Mesnil M., Krutovskikh V., Piccoli C., Elfgang C., Traub O., Willecke K., Yamasaki H. (1995) Negative growth control of HeLa cells by connexin genes: connexin species specificity. *Cancer Res.* **55**, 629-639.

Miller T., Dahl G., Werner R. (1988) Structure of a gap junction gene: connexin 32. *Biosci. Rep.* **8**, 455-464.

Monti-Bloch L., Abudra V., Ezyaguirre C. (1993) Electrical communication between glomus cells of the rat carotid body. *Brain Res.* **622**, 119-131.

Moorby C. D., Stoker M., Gherardi E. (1995) HGF/SF inhibits junctional communication. *Exp. Cell Res.* **219**, 657-663.

Mugnaini E. (1986) Cell junctions of astrocytes, ependyma, and related cells in the mammalian central nervous system, with emphasis, on the hypothesis of a generalized functional syncytium of supporting cells. In *Astocytes*, Vol. I. (eds Fedoroff S. and Vernadakis A.), pp 329-371. New York, Academic Press.

Musil L. S., Beyer E. C., Goodenough D. A. (1990a) Expression of the gap junction protein connexin 43 in embryonic chick lens: molecular cloning, ultrastructural localization, and post-translational phosphorylation. *J. Membrane Biol.***116**, 163-175.

Musil L. S., Cunningham B. A., Edelman G. M., Goodenough D. A., (1990b) Differential phosphorylation of the gap junction protein connexin43 in junctional communication-competent and –deficient cell lines. *J. Cell Biol.* **111**, 2077-2088.

Musil L. S., Goodenough D. A. (1991) Biochemical analysis of connexin43 intracellular transport, phosphorylation, and assembly into gap junctional plaques. *J. Cell Biol.* **115**, 1357-1374.

Nagy J. I., Yamamoto T., Shiosaka S., Dewar K., Whittaker M., Hertzberg E. L. (1988) Immunohistochemical localization of gap junction protein in rat CNS: a preliminary account. In *Modern Cell Biology Vol. 7 Gap Junctions.* E. L. Hertzberg and R. G. Johnson eds, (New York: Alan R. Liss, Inc.,) 375-389.

Nagy J. I,, Yamamoto T., Sawchuk M. A., Nance D. M., Hertzberg E. L. (1992) Quantitative immunohistochemical and biochemical correlates of connexin 43 localization in rat brain. *Glia* **5**, 1-9.

Naus C. C. G., Bechberger S. F., Paul D. L. (1991) Gap junction gene expression in human seizure disorder. *Exp. Neurol.* **111**, 198-203.

Naus C. C. G., Elisevick K., Zhu D., Belliveau D. J., Del Maestro R. F. (1992) in vivo growth of C6 glimoa cells transfected with connexin43 cDNA. *Cancer Res.* **52**, 4208-4213.

Naus C. C. G., Beechberger J. F., Zhang Y., Venance L., Yamasaki H., Juneja S. C., Kidder G. M., Giaume C. (1997) Altered gap junctional communication, intercellular signaling, and growth in cultured astrocytes deficient in connexin43. *J. Neurosci. Res.* **49**, 528-540.

Nelles E., Butzler C., Jung D., Temme A., et al. (1996) Defective propagation of signals generated by sympathetic nerve stimulation in the liver of connexin 32 deficient mice. *Proc. Natl. Acad. Sci. U. S. A.* **93**, 9565-9570.

Neuberg D. H.-H., Carenini S., Schachner M., Martini R., Suter U. (1998) Accelerated demyelination of peripheral nerves in mice deficient in connexin 32 and protein zero. *J. Neurosci. Res.* **53**, 542-550.

Nguyen T. T., Scimeca J. C., Filloux C., Peraldi P., Carpentier J. L., van Obberghen E. (1993) Co-regulation of the mitogen-activated protein kinase, extracellular signal-regulated kinase 1, and the 90-kDa ribosomal S6 kinase in PC12 cells. *J. Biol. Chem.* **268**, 9803-9810.

O'Brian J., Al-Ubaidi M. R., Ripps H. (1996) Connexin 35: a gap junctional protein expressed preferentially in the skate retina. *Mol. Biol. Cell* **7**, 233-244.

O'Donnell P., Grace A. A. (1993) Dopaminergic modulation of dye coupling between neurons in the core and shell regions of the nucleus accumbens. *J. Neurosci.* **13**, 3456-3471.

Oh S Y, Grupen C G, Murray A W (1991) Phorbol ester induces phosphorylation and down-regulation of connexin 43 in WB cells. *Biochimica et Biophysica Acta* **1094**, 243-245.

Oh S. Y., Dupont E., Madhukar B. V., Briand J.-P., Chang C.-C., Beyer E., Trosko J. E. (1993) Characterization of gap junctional communication-deficient mutants of a rat liver epithelial cell line. *Eur. J. Cell Biol.* **60**, 250-255.

Olson D. J., Christian J. L., Moon R. T. (1991) Effect of wnt-/ and related proteins on gap junctional communication in Xenopus embryos. *Science* **252**, 1773-1776.

Oyamada M., Kimura H., Oyamada Y., Miyamoto A., Ohshika H., Mori M. (1994) The expression, phosphorylation, and localization of connexin 43 and gap junctional intercellular communication during the establishment of a synchronized contraction of cultured neonatal rat cardiac myocytes. *Exp. Cell Res.* **212**, 351-358.

Paul D. L. (1995a) New functions for gap junctions. *Curr. Opin. Cell Biol.* **7**, 665-672.

Paul D. L., Yu K., Bruzzone R., Gimlich R. L., Goodenough D. A. (1995b) Expression of a dominant negative inhibitor of intercellular communication in the early Xenopus embryo causes delamination and extrusion of cells. *Development* **121**, 371-381.

Peinado A., Yuste R., Katz L. C. (1993a) Extensive dye coupling between rat neocortical neurons during the period of circuit formation. *Neuron* **10**, 103-114.

Peinado A., Yuste R., Katz L. C. (1993b) Gap junctional communication and the development of local circuits in neocortex. *Cerebral Cortex* **3**, 488-498.

Pelletier D. B., Boyton A. L. (1994) *J. Cell Physiol.* **158**,427-434.

Rabaden-Diehl C., Dahl G., Werner R. (1994) A connexin-32 mutation associated with Charcot-Marie-Tooth disease does not affect channel formation in oocytes. *FEBS Lett.* **351**, 90-94.

Perez J. Tranque P. A., Naftolin F., Garcia-Segura L. M. (1990) Gap junctions in the hypothalamic arcuate neurons of ovariectomized and estradiol-treated rats. *Neurosci. Lett.* **21**, 923-927.

Perez-Velazquez J. L., Frantseva M., Naus C. C., Bechberger J. F., Juneja S. C., Velumian A., Carlen P. L., Kidder G. M., Mills L. R. (1996) Development of astrocytes and neurons in cultured brain slices from mice lacking connexin43. *Brain Res. Dev. Brain Res.* **97**, 293-296.

Perez-Velazquez J. L., Han D., Carlen P. L. (1997) Neurtansmitter modulation of gap junctional communication in the rat hippocampus. *Eur. J. Neurosci.* **9**, 2522-2531.

Pereda A., Nairn A., Wolzson L., Faber D. S. (1994) Postsynaptic modulation of synaptic efficacy at mixed synapses on the Mauthner cell. *J. Neurosci.* **14**, 3704-3712.

Pereda A., Faber D. S. (1996) Activity-dependent short-term enhancement of intercellular coupling. *J. Neurosci.* **16**, 983-992.

Raff M. C., Miller R. H., Noble M. (1983) A glial progenitor cell that develops *in vitro* into an astrocyte or an oligodendrocyte depending on culture medium. *Nature* **303**, 390-396.

Ray J., Peterson D. A., Schinstine M., Gage F. H. (1993) Proliferation, differentiation, and long-term culture of primary hippocampal neurons. *Proc. Natl. Acad. Sci. U. S. A.* **90**, 3602-3606.

Ray J., Baird A., Gage F. H. (1997) A 10-amino acid sequence of fibroblast growth factor 2 is sufficient for its mitogenic activity on neural progenitor cells. *Proc. nant. Acad. Sci. U.S.A.* **94**, 7047-7052.

Reaume A. G., de Sousa P.A., Kilkarni S., Langille B. L., Zhu D., Davies T. C., Juneja S. C., Kidder G. M., Rossant J. (1995) Cardiac malformationin neonatal mice lacking connexin43. *Science* **267**, 1831-1834.

Revel J.-P., Brown S. S. (1976) Cell junctions in development, with particular reference to the neural tube. *Cold Spring Harbor Symp. Quant. Biol.* **40**, 443-55.

Reynolds B. A., Weiss S. (1992) Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* **255**, 1707-1710.

Reynolds B. A., Weiss S. (1996) Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. *Dev. Biol.* **175**, 1-13.

Risek B., Guthrie S., Kumar N. M., Gilula N. B. (1990) Modulation of gap junction transcript and protein expression during pregnancy in rat. *J. Cell biol.* **110**, 269-282.

Risek B., Klier F. G., Gilula N. B. (1992) Multiple gap junction genes are utilized during rat skin and hair development. *Development* **116**, 639-651.

Risek B., Klier F. G., Gilula N. B. (1994) Developmental regulation and structural organization of connexins in epidermal gap junctions. *Dev. Biol.* **164**, 183-196.

Rogers M., Berestecky J. M., Hossain M. Z., Guo H. M., Kadle R., Nicholson B. J., Bertram J. S. (1990) Retinoid-enhanced gap junctional communication is achieved by increased levels of connexin 43 mRNA and protein. *Mol. Carcinogenesis* **3**, 335-343.

Rose B., Loewenstein W. R. (1975) Permeability of cell junction depends on local cytoplasmic calcium activity. *Nature* **254**, 250-252.

Rose B., Mehta P. P., Loewenstein W. R. (1993) Gap junction protein gene suppresses tumorigenicity. *Carcinogenesis* **14**, 1073-1075.

Rosenberg E., Paris R. A., Spray D. C., Monfils B., Abreu S., Danishefsky I., Reid L. M. (1996) Correlation of expression of connexin mRNA isoforms with degree of cellular differentiation. *Cell Adhesion and Communication* **4**, 223-235.

Rosser A. E., Tyers M., ter Borg M., Dunnett S. B., Svendsen C. N. (1997) Co-expression of MAP-2 and GFAP in cells developing from rat EGF responsive precursor cells. *Dev. Brain Res.* **98**, 291-295.

Rozental R., Mehler M. F., Morales M., Andrade-Rozental A. F., Kessler J. A., Spray D. C. (1995) Differentiation of hippocampal progenitor cells in vitro: temporal expression of intercellular coupling and voltage- and ligand-gated responses. *Dev. Biol.* **16**, 350-362.

Ruch R. J., Bonney W. J., Sigler K., Guan X., Matexic D., Schafer L. D., Dupont E., Trosko J. E. (1994) Loss of gap junctions from DDT-treated rat liver. *Carcinogenesis* **15**, 301-306.

Ruch R. J., Guan X., Sigler K. (1995) Inhibition of gap junctional intercellular communication and enhancement of growth in balb/c3t3 cells treated with connexin43 antisense oligonucleotides. *Molecular Carcinogenesis* **14**, 269-274.

Rudkin B. B., Lazarovici P., Levi B. Z., Abe Y., Fujita K., Guroff G. (1989) Cell cycle-specific action of nerve growth factor in PC12 cells: differentiation without proliferation. *EMBO J.* **8**, 3319-3325

Rudnicki M. A., McBurney M. W. (1987) Cell culture methods and induction of differentiation of embryonal carcinoma cell lines. In *Teratocarcinomas and Embryonic Stem Cells. A Practical Approach.* (Robertson E. J. ed), pp 19-49. IRL Press, Oxford.

Rup D. M., Veenstra R. D., Wang H. Z., Brink P. R., Beyer E. C. (1993) Chick connexin-56, a novel lens gap junction protein. molecular cloning and functional expression. *J. Biol. Chem.* **268**, 706-712.

Sáez J. C., Spray D. C., Nairn A. C., Hertzberg E., Greengard P. (1986) cAMP increases junctional conductance and stimulates phosphorylation of the 27-kDa principal gap junction polypeptide. *Proc. nant. Acad. Sci. U.S.A.* **83**, 2473-2477.

Sáez J. C., Gregory W. A., Watanabe T., Dermietzel R., Hertzberg E. L., Reid L., Bennett M. V. L., Spray D. C. (1989) cAMP delays disappearance of gap junctions between pairs of rat hepatocytes in primary culture. *Am. J. Physiol.* **257**, C1-C11.

Sáez J. C., Spray D. C., Hertzberg E. L. (1990) Gap junctions: biochemical properties and functional regulation under physiological and toxicological conditions. *In Vitro Toxicol.* **3**, 69-86.

Sáez J. C., Berthoud V. M., Moreno A. P., Spray D. C. (1993) Gap junctions multiplicity of controls in differentiated and undifferentiated cells and possible functional implications. In *Advances in Second Messenger and Phosphoprotein Research*, S. Shenolikar and A. C. Nairn, eds. (New York: Raven Press, Ltd.) 27, 163-194.

Saitoe M., Inazawa T., Takahashi K. (1996) Neuronal expression in cleavage-arrested ascidian blastomeres requires gap junctional uncoupling from neighboring cells. *J. Physiol.* **491.3**, 825-842.

Sato K., Gratas C., Lampe J., Biernat W., Kleihues P., Yamasake H., Ohgaki H. (1997) Reduced expression of the P2 form of the gap junction protein connexin43 in malignant meningiomas. *J. Neuropath. Exper. Neurol.* **56**, 835-839.

Scemes D., Dermietzel R., Spray D. C. (1998) Calcium waves between astrocytes from cx43 knockout mice. *Glia* **24**, 65-73.

Shetty A. K., Turner D. A. (1998) In vitro survival and differentiation of neurons derived from epidermal growth factor-responsive postnatal hippocampal stem cells: inducing effects of brain-derived neurotrophic factor. *J. Neurobiol.* **35**, 395-425.

Schütte M., Chen S., Buku A., Wolosin J. M. (1998) Connexin50, a gap junction protein of macroglia in the mammalian retina and visual pathway. *Exp. Eye Res.* **66**, 605-613.

Seigel G. M., Rollins J., Mhyre T. R., Poles T., Fidele Ü., Holliday J. (1996) Immortalized cerebellar cells can be induced to display mature neuronal characteristics. *Neuroscience* **74**, 511-518.

Shatz C. J., Stryker M. P., (1988) Prenatal tetrodotoxin indusion blocks segregation of retinogeniculate afferents. *Science* **242**, 87-89.

Sherer S. S. (1996) Molecular specializations at nodes and paranodes in peripheral nerve. *Microsc. Res. Tech.* **34**, 452-461.

Simburger E., Stang A., Kremer ., Dermietzel R. (1991) Expression of connexin 43 mRNA in adult rodent brain. *Histochem. Cell Biol.* **107**, 127-137.

Snyder E. Y., Deitcher D. L., Walsh C., Arnold-Aldea S., Hartwieg E. A., Cepko C. L. (1992) Multipotent neural cell lines can engraft and participate in development of mouse cerebellum. *Cell* **68**, 33-51.

Spray D. C., White R. L., Campos de Carvalho A., et al. (1984) Gating of gap junctional conductance. *Biophys J.* **45**, 219-230.

Spray D. C. (1994) Physiological and pharmacological regulation of gap junction channels. In *Molecular Medhanisms of Epithelial Cell Junctions: From Development to Disease* (ed Citi S.), pp 195-215. Austin, Texas: R. G. Landes Co.

Spray D. C., Dermietzel R. (1996) Gap Junctions in the Nervous System: an introduction. In *Gap Junctions in the Nervous System.* (eds Spray D. C. and Dermietzel R.), pp 1-11. Austin, Texas: R.G. Landes Co.

Stagg R. B., Fletcher W. H. (1990) The hormonal regulation of contact-dependent cell-cell communication by phosphorylation. *Endocr. Rev.* **11**. 302-325.

Sullivan R., Huang G. Y., Meyer R. A., Wessels A., Linask K. K., Lo C. W. (1998) Heart malformations in transgenic mice exhibiting dominant negative inhibition of gap junctional communication in neural crest cells. *Dev. Biol.* **204**, 80-96.

Tabernero A., Giaume C., Medina J. M. (1996) Endothelin-1 regulates glucose utilization in cultured astrocytes by controlling intercellular communication through gap junctions. *Glia* **16**, 187-195.

Takeda A, Hashimoto E, Yamamura H, Shimazu T (1987) Phosphorylation of liver gap junction protein by protein kinase C. *FEBS Letters* **210**, 169-172.

Tamemoto H. T., Kadowaki K., Tobe K., Ueki T., Izumi Y., Chatani M., Kohno M., Kasuga Y., Yazaki Y., Akanuma Y. (1992) Biphasic activation of two mitogen-activated kinases during the cell cycle in mammalian cells. *J. Biol. Chem.* **267**, 20293-20297.

Tornatore C., Baker-Cairns B., Yadid G., Hamilton R., Meyers K., Atwood W., Cummins A., Tanner V., Major E. (1996) Expression of tyrosine hydroxylase in an immortalized human fetal astrocyte cell line; in vitro characterization and engraftment into the rodent striatum. *Cell Transplantation* **5**, 145-163.

Traverse S., Gomez N., Paterson H., Marshall C., Cohen P. (1992) Sustained activation of the mitogen-activated protein (MAP) kinase cascade may be required for differentiation of PC12 cells. *Biochem. J.* **288**, 351-355.

Traub O., Look J., Paul D., Willecke K. (1987) Cyclic adenosine monophosphate in cultured mouse hepatocytes. *Eur. J. Cell Biol.* **4**, 48-54.

Traub O., Look J., Dermietzel R., et al. (1989) Comparative characterization of the 21-kD and 26 kD gap junction proteins in murine liver and cultured hepatocytes. *J. Cell Biol.* **108**, 1039-1051.

Trosko J. E., Madhukar B. V., Chang C.-C. (1993) Endogenous and exogenous modulation of gap junctional intercellular communication: toxicological and pharmacological implications. *Life Sciences* **53**, 1-19.

Trosko J. E., Ruch R.J. (1998) Cell-cell communication in carcinogenesis. *Frontiers in Bioscience* **3**, 208-236.

Turin L., Warner A. E. (1977) Carbon dioxide reversibly abolishes ionic communication between cells of early amphibian embryo. *Nature* **270**, 56-57.

Vaney D. I. (1994) Patterns of neuronal coupling in the retina. *Progr. Ret. Eye Res.* **13**, 301-353.

Vaney D. I., Nelson C. J., Pow D.V. (1998) Neurotransmitter coupling through gap junctions in the retina. *J. Neurosci.* **18**, 10594-10602.

van Kempen M. J., Formaget C., Gros D., Moorman A. F., Lamers W. H. (1991) Spatial distribution of connexin 43, the major cardiac gap junction protein, in the developing and adult rat heart. *Circ. Res.* **68**, 1638-51.

Verity A. N., Wyatt T. L., Lee W., Hajos B., Baecker P. A., Eglen R. M., Johnson R. M. (1999) Differential regulation of glial cell line-derived neurotrophic factor (GDNF) expression in human neuroblastoma and glioblastoma cell lines. *J. Neurosci. Res.* **55**, 187-197.

Wade M. H., Trosko J. E., Schlindler M. (1986) A fluorescence photobleaching assay of gap junction-mediated communication between human cells. *Science* **232**, 525-528.

Wang Q., Zheng J. Q. (1998) cAMP-mediated regulation of neurotrophin-induced collapse of nerve growth cones. *J. Neurosci.* **18**, 4973-4984.

Wang X., Li L., Peracchia L. L., Peracchia C. (1996) Chimeric evidence for a role of the connexin cytoplasmic loop in gap junction channel gating. *Pflügers Arch.* **431**, 844-852.

Wang Y., Rose B. (1995) Clustering of Cx43 cell-to-cell channels into gap junction plaques: regulation by cAMP and microfilaments. *J. Cell Sci.* **108**, 3501-3508.

Warner A. E., Guthrie S. C., Gilula N. B. (1984) Antibodies to gap junctional protein selectively disrupt junctional communication in the early amphibian embryo. *Nature* **311**, 127-131.

Weiner E. C., Loewenstein W. R. (1983) Correction of cell-cell communication defect by introduction of a protein kinase into mutant cells. ***Nature*** **305**, 433-435.

Weiss S., Dunne C., Hewson J., Wohl C., Wheatley M., Peterson A. C., Reynolds B. R. (1996) Multipotent CNS stem cells are present in the adult mammalian spinal cord and ventricular neuroaxis. *J. Neurosci.* **16**, 7599-7609.

Weliky M., Katz L. C. (1997) Disruption of orientation tuning in visual cortex by artificially correlated neuronal activity. *Nature* **386**, 680-685.

Welsh J. P., Lang E. J., Suglhara I., Llinas R. (1995) Dynamic organization of motor control within the olivocerebellar system. *Nature* **374**, 453-457.

Werner R., Levine E., Rabadan-Diehl C., et al. (1991) Gating properties of connexin32 cell-cell channels and their mutants expressed in Xenopus oocytes. *Proc. R. Soc. Lond.[Biol.]* **243**, 5-11.

Wetsel W. C., Valenca M. M., Merchenthaler I., Liposts Z., Lopez F. J., Weiner R I., Mellon P. L., Negro-Vilar A. (1992) Intrinsic pulsatile secretory activity of immortalized luteinizing hormone-releasing hormone-secreting neurons. *Proc. Natl. Acad. Sci. U. S. A.* **89**, 4149-4153.

White T. W., Bruzzone R., Wolfram S., Paul D. L., Goodenough D. A. (1994) Selective interactions among the multiple connexin proteins expressed in the vertebrate lens the second extracellular domain is a determinant of compatibility between connexins. *J. Cell Biol.* **125**, 879-892.

White T.W., Paul D. L., Goodenough D.A., Bruzzone R. (1995) Functional analysis of selective interactions among rodent connexins. *Mol. Biol. Cell* **6**, 459-470.

White R. L., Doellar J. E., Verselis V. K., et al. (1990) Gap junctional conductance between pairs of ventricular myocytes is modulated synergistically by H+ and Ca+. *J. Gen. Physiol.* **95**, 1061-1075.

Wilgenbus K. K., Kirkpatrick C. J., Knuechel R., Willecke K., Traub O. (1992) Expression of Cx26, Cx32, and Cx43 gap junction proteins in normal and neoplastic human tissues. *Int. J. Cancer* **51**, 522-529.

Willecke K., Hennemann H., Dahl E., Jungbluth S., Heynkes R. (1991) The diversity of connexin genes encoding gap junctional proteins. *Eur. J. Cell Biol.* **56**, 1-7.

Williams B. P., Read J., Price J. (1991) The generation of neurons and oligodendrocytes from a common precursor cell. *Neuron* **7**, 685-693.

Xie H., Laird D W., Chang T. H., Hu V. W. (1997) A mitosis-specific phosphorylation of the gap junction protein connexin43 in human vascular cells: biochemical characterization and localization. *J. Cell Biol.* **137**, 203-210.

Yamamoto T., Ochalski A., Hertzberg E. L., Nagy J. I. (1990) On the organization of astrocytic gap junctions in rat brain as suggested by lm and em immunohistochemistry of connexin43 expression. *J. Comp. Neurol.* **302**, 853-883.

Yamamoto T., Kardami E., Nagy J. I. (1991) Basic fibroblast growth factor in rat brain: localization to glial gap junctions correlates with connexin 43 distribution. *Brain Res.* **554**, 336-343.

Yamasaki H. (1990) Gap junctional intercellular communication and gap junctional intercellular communication and carcinogenesis. *Carcinogenesis* **11**, 1051-1058.

Yuste R., Peinado A., Katz L. C. (1992) Neuronal domains in developing neocortex. *Science* **257**, 665-669.

Yuste R., Nelson D. A., Rubin W. W., Katz L. C. (1995) Neuronal domains in developing neocortex: mechanisms of coactivation. *Neuron* **14**, 7-17.

Zhu D., Kidder G. M., Caveney S., Naus C. C. G. (1992) Growth retardation in glioma cells cocultured with cells overexpressing a gap junction protein. *Proc. Natl. Acad. Sci. U. S. A.* **89**, 10218-21.